The diversity of endophytic fungi on *Annuia* mutant plantation (*Artemisia annua* L.) based on ITS rDNA marker

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**Abstract.** *Artemisia annua* is a plant that produces artemisinin which is an antimalarial compound. The production of artemisinin compounds is influenced by the interaction of endophytic molds with their host plants. This study was conducted to identify endophytic molds found in plant tissues of *A. annua* which previously had been treated by mutations with Ethyl methanesulfonate (EMS) to increase artemisinin production levels. Identification was carried out by using BLAST analysis, while the phylogenetic tree analysis used the minimum evolution (ME) method on MEGA 7 and the maximum parsimony (MP) method on PAUP 4.0b10. Phylogenetic analysis used noncoding sequences of the *Internal Transcribe Spacer* (ITS) rDNA region. Twelve endophytic molds (8 isolates from leaves, 2 isolates from stems, 1 isolate from petioles, and 1 isolate from flowers) in the Biogen Culture collection. This study identified to the species level phylogenetically. The endophytic molds identified were dominated by the *Sordariomycetes* group. It consist of 4 species, such as *Clonostachys rosea*, *Fusarium oxysporum*, *Microascus gracillis*, and *Scopulariopsis brevicaulis*, and another species belonging to the *Eurotiomycetes* group, is *Aspergillus sydowii*. A total of 4 families were obtained, such as *Nectriaceae*, *Bionectriaceae*, *Microascaceae*, and *Aspergillaceae*. All acquired classes belong to the *Ascomycota* division.

**Keywords:** *Artemisia annua*, Endophytic fungi, Internal Transcribe Spacer, Phylogenetic tree.

1. **Introduction**

*Artemisia annua* (L.) is known as a medicine plant that produce *artemisinin* compounds. *Artumsin* is a bioactive compound that functions as an anti-malarial [1]. *Artemisinin* compounds have function as antimicrobial, anti-inflammatory, and anti-oxidant [2]. The content of *artemisinin* in *A. annua* plants is low, that range from 0.1 to 2%, that is necessary to make efforts to increase the levels of artemisinin production in *A. annua* plants [3]. Previous studies [4] have made efforts to increase artemisinin levels by giving mutation treatment to wild type *A. annua* plants that used 1% Ethyl methanesulfonate (EMS) and succeeded in increasing the size of glandular trichomes on leaves and increasing *artemisinin* levels.

The content of artemisinin in *A. annua* plantation is influenced by the interaction between *endophytic fungi* and their host plants quantitatively and qualitatively [5, 6]. Research on the diversity of endophytic fungi found in *A. annua* plants, described that *Pseudonocardia* sp. and *Colletotrichum* sp. can stimulate artemisinin production on leaves [7]. It’s potentials and interactions is the basis for the isolation and
Identification of endophytic fungi which are found in plant tissues of *A. annua*. Isolation and identification are the first steps to obtain endophytic fungi that have the potential to produce artemisinin.

Identification of endophytic fungi can be done by morphological and molecular identification methods. The use of morphological identification can be used, but it can only identify the fungi to the genus level but endophytic fungi that do not have a spore stage (*mycelia sterilia*), so they can not be identified. In recent decades, the molecular identification of endophytic fungi that use the ITS rDNA barcode has been used very popular to identify endophytic fungi until the species level. The molecular identification has high accuracy even for fungi that do not have a spore stage (*mycelia sterilia*) due to the diversity and specificity of the ITS rDNA region sequence [8].

Based on the molecular identification of ITS rDNA [9, 10], it was reported that the type of endophytic fungi in *A. annua* are *Cladosporium* sp., *Edenia* sp., *Cephalosporium* sp., *Acremonium persicum*, *Mucor* sp., *Leptoshaeria* sp., *Gleospoioides* sp., *Culvularia pallescens*, *Chochiliobolus lunatus*, and *Hypocylon* sp.

This study used 12 endophytic fungi isolates from *A. annua* mutant plantation that was taken from the Biogen Culture Collection (Biogen CC). It was from *A. annua* plants that has been treated by EMS mutation. Twelve endophytic fungi isolates have been identified morphologically such as macroscopic and microscopic[11]. However, in the identification process, some endophytic fungi, some of reproductive organ structures or (*mycelia sterilia*) was not obtained. Therefore, molecular identification of endophytic fungi is necessary to provide more accurate information about endophytic fungi species in *A. annua* based on DNA specificity sequence.

The molecular approach was carried out by using sequences in the Internal Transcribe Spacer (ITS) rDNA region. The ITS regional sequences that have identified more than 90,000 on fungi representing 17,000 taxa fungal have been published. The use of ITS regional sequences was highly recommended [12]. Molecular identification by using DNA sequences was more efficient in terms of time and accuracy, especially for indistinguishable endophytic fungi morphologically [13].

This study used phylogenetic analysis by the minimum evolution method in Molecular Evolutionary Genetics Analysis (MEGA) and the maximum parsimony method in Phylogenetic Analysis Using Parsimony (PAUP) to determine the relationship among endophytic fungi species. The aim of this research is to obtain the diversity of endophytic fungi from the *A. annua* mutant plant of the BB Biogen culture collection by molecular identification. The research can provide an inventory and database of endophytic fungi from *A. annua* mutant plants from Indonesia.

2. Materials and Methods

2.1 Materials

The researchers used 12 endophytic fungi isolates that has been isolated by Shabrina, Radiastuti, Susilowati (2019) from the leaves, stalks, stems, and flowers of *Artemisia annua* mutant plant. The culture was obtained from the BB Biogen culture collection.

| Sample code | Isolate source |
|-------------|----------------|
| M1A1D2      | leaf           |
| M1A2D2      | leaf           |
| M1C1D2      | leaf           |
| M2A1D3U     | leaf           |
| M3A2D3U     | leaf           |
| M10C2D2U    | leaf           |
| M12D1T1U    | stalk          |
| M14C1B1     | stem           |
| M15B1BG1    | flower         |
| M17C1B2     | stem           |
| M18A1D2U    | leaf           |
2.2. Reculture of endophytic fungi
Reculture of endophytic fungi isolates was carried out by using the cork borer method. The pure endophytic fungi isolates were transferred to new PDA medium and incubated for 5-7 days at room temperature.

2.3. DNA extraction
DNA extraction used mycelium molds that was grown on PDB medium for 5-7 days in a 100 rpm rotary shaker at room temperature. Mycelium was taken and was put into sterile plastic then crushed by using a sterile pestle. DNA isolation was carried out by using the PhytopureTM Kit (Amersham) with protocol by manufacture.

The sample was added with chloroform as much as 500 μL that had been store in -20°C degree. DNA extraction resin suspension was added as much as 100 μL and inverted for 10 minutes, then it was centrifuged at 1300 xg for 10 minutes. The centrifuged sample will form 3 phases. The top sample phase of the microtube was transferred into a new microtube, then isopropanol was added as much as the volume of the transferred sample. The sample was centrifuged at 4000 xg for 5 minutes until a DNA pellet was formed.

The mycelia was added with reagent 1 as much as 600 μL and stirred by using a spatula. Furthermore, 200 L of reagent 2 was added and inverted until homogeneous. Mycelium solution was incubated for 10 minutes in a water bath at 65°C and incubated in the freezer for 20 minutes.

The pellets of DNA were washed and added with 100 μL of 70% cold ethanol, then centrifuged at 4000 xg for 10 minutes. The supernatant was discarded and the pellet was dried for 1 hour until the ethanol was evaporated. The DNA pellet was resuspended by adding 50 μL of nuclease-free water.

2.4. DNA amplification
DNA amplification was carried out by using the Polymerase Chain reaction (PCR) method in 25 μL of the reaction mixture. The reaction mixture consisted of 12.5 μL of go taq green master mix, 8.5 μL of nuclease free water, and 1 μL of ITS4 and ITS5 primer. Then 2 μL of DNA template was added. The settings of the PCR machine follows 90 seconds at 95°C for initial denaturation, followed by 35 cycles that consist of 30 seconds at 95°C for denaturation, 30 seconds at 55°C for annealing, 90 seconds at 72°C for extension and 5 minutes at 72°C for the final extension. The final stage storage temperature is 15°C [42].

2.5. Electrophoresis and sequencing
As many as 3 μL amplification products was put into 1% (w/v) agarose and were immersed in 1x TAE buffer in the chamber. 2 μL DNA marker 100 bp was added into the agarose. The agarose that contains the sample and DNA marker was electrophoresed at 220 volts for 25 minutes and the agarose are visualized by using UV light on Gel Doc XR. Sequencing reactions were carried out by using the Cycle Sequencing Ready Reaction Kit. The DNA sequencing was carried out in Malaysia's First Base (1ˢᵗ Base) Laboratory.

2.6 Data analysis
2.6.1 BLAST Analysis and Sequence Alignment
The electropherogram was analyzed by using the BioEdit ver. 7.2.5 [43]. The DNA sequences were analyzed for the similarity of each base sequence to the gene data in Gen Bank at the National Center for Biotechnology Information (NCBI) by using Basic Local Alignment Search Tools (BLAST). 99-100% level of similarity indicates that the endophytic fungi obtained are the same species. The sequences data set were aligned by using MUSCLE on MEGA 7 software [39].

2.6.2. Phylogenetic Analysis
Phylogenetic analysis was performed by using the Maximum Parsimony (MP) method on PAUP 4.0.b10 [40] and the Minimum Evolution (ME) method on the MEGA 7 software [39]. The heuristic method
used Tree Bisection Reconstruction (TBR), by adding of 1000 algorithm random sequences to obtain an optimal tree. Tree Length (TL), Consistency Index (CI), Retention Index (RI), Related Consistency Index (RC), and Homoplasy Index (HI) were calculated. Internal branch strength of phylogenetic tree in MP analysis was tested by Bootstrap (BS) analysis with 1000 replications. Values > or = BS 50% are displayed in the phylogenetic tree. Homogeneity partition was tested by 1000 replications.

3. Results and Discussion
3.1 Similarity Analysis by BLAST (Basic Local Alignment Search Tool)
Endophytic fungi that were isolated from several parts of the A. annua plant were 8 isolates such as: from leaves (M1A1D2, M1A2D2, M1C1D2, M2A1D3U, M3A2D3U, M10C2D2U, M18A1D2U, and M19B1D3), two isolates from stems (M14C1B2 and M14C1B2), one isolate from the stalk (M12D1T1U) and one isolates from the flower (M15B1BG1). Molecular identification of twelve endophytic fungi isolated from the A. annua mutant plantation was identified as 5 different species based on BLAST analysis. The most abundant endophytic fungi that found from the A. annua mutant plantation was Fusarium oxysporum.

Table 2. Similarity Sequences of Endophytic fungi from the Artemisia annua Mutant to Gen bank by using BLAST.

| Isolate code | BLAST sequence          | Genbank Accession | Query Cover | Max Ident | Max Score |
|--------------|-------------------------|-------------------|-------------|-----------|-----------|
| M2A1D3U      | Aspergillus sydowii     | MT312735          | 100%        | 99.82%    | 992       |
| M10C2D2U     | Aspergillus sydowii     | MT312735          | 100%        | 99.82%    | 996       |
| M1C1D2       | Clonostachys rosea      | KR183785          | 100%        | 100%      | 961       |
| M1A2D2       | Fusarium oxysporum      | MT530243          | 100%        | 100%      | 968       |
| M3A2D3U      | Fusarium oxysporum      | MT530243          | 100%        | 100%      | 948       |
| M12D1T1U     | Fusarium oxysporum      | MT530243          | 100%        | 100%      | 952       |
| M14C1B1      | Fusarium oxysporum      | MT530243          | 100%        | 100%      | 965       |
| M17C1B2      | Fusarium oxysporum      | MT530243          | 100%        | 100%      | 968       |
| M1A1D2       | Microascus gracilis     | KP132759          | 100%        | 100%      | 1086      |
| M15B1BG1     | Scopulariopsis brevicaulis | MN88599       | 100%        | 99.84%    | 1116      |
| M18A1D2U     | Scopulariopsis brevicaulis | KP132743   | 100%        | 99.50%    | 1086      |
| M19B1D3      | Scopulariopsis brevicaulis | MN88599    | 100%        | 99.02%    | 1094      |

Based on the BLAST results, the percentage of query coverage for all samples is 100% (Table 2). The query coverage value that reaches 100% indicates the total length of the sample sequence is the same as the species sequence contained in the Gen bank database. The BLAST results are supported by good electropherogram results that an average final base length is 554.7 bp. This shows that the reference sequence contained in the NCBI covers the entire analyzed sample sequence.

The maximum identity value indicates the similarity percentage of the nucleotide sequence samples to the species sequence samples in the Gen bank. The value of max identity in this study is quite varied, ranging from 99.02 to 100%. Endophytic molds that have a max identity value of 100% are M1A1D2 samples with the microascus gracilis (KP132759), M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, M17C1B2, with the Fusarium oxysporum (MT530243), and M1C1D2 with clonostachys rosea (KR183785). Mean while the BLAST results that values range from 99%, are M2A1D3U and M10C2D2U with Aspergillus sydowii (MT312735), M15B1BG1, with Scopulariopsis brevicaulis (MN88599), M18A1D2U, with Scopulariopsis brevicaulis (KP132743), and M19B1D3 with Scopulariopsis brevicaulis (MN88599). The max identity value of 99-100% indicates that the sequence is the same as the sequence of the species that emerged from the BLAST analysis.

The process of matching the sample sequence with the database was carried out by calculating the penalty gap for scoring alignment on the sequence by looking at the match/mismatch with a value of 2/-
3 [44]. M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, M17C1B2 samples were identified as (MT530243) *Fusarium oxysporum* but it has different scores, such as 968, 948, 952, 965, and 968 (Table 2). The isolate code of M2A1D3U with a score of 992 and M10C2D2U with a score of 996 as (MT312735) *Aspergillus sydowii* and M15B1BG with a score of 1116 as (MN88599) *Scopulariopsis brevicaulis*. A higher score indicates that the sequence has a higher degree of similarity compared to other sequences, while DNA with a score of less than 50 is declared to have no similarity at all among the sequences [45].

BLAST analysis is used to ensure the amplified DNA sequence is in the right region, such as the Internal Transcribe Spacer (ITS) rDNA fragment. In addition to functioning as a data set search tool for phylogenetic analysis, However BLAST analysis cannot indicate a taxonomic classification automatically for a particular sequence so supported by phylogenetic analysis is needed[46].

3.2 Classification of endophytic fungi based on BLAST analysis

Based on the classification, 5 species that were identified by using ITS rDNA regional sequences were from the *Sordariomycetes* and *Eurotiomycetes* classes, and consisted of three ordos, such as *Microascales*, *Hypocreales*, and *Eurotiales*, and four families, such as *Microascaceae*, *Nectriaceae*, *Bionectriaceae*, and *Aspergillaceae*. The majority of endophytic fungi identified by BLAST analysis were from the *Sordariomycetes* class (*Microascus gracillis*, *Scopulariopsis brevicaulis*, *Fusarium oxysporum*, and *Clonostachys rosea*) and other species were from the *Eurotiomycetes* class (*Aspergillus sydowii*).

Table 3. Taxonomy and nucleotide length of ITS regions endophytic fungi.

| Group  | Taxonomy              | Species                  | average of nucleotide length (bp) |
|--------|-----------------------|--------------------------|----------------------------------|
| Class  | *Sordariomycetes*     | *Microascus gracillis*   | 588                              |
| Ordo   | *Microascales*         | *Scopulariopsis brevicaulis* | 606,3                           |
| Family | *Microascaceae*        |                          |                                  |
| Class  | *Sordariomycetes*     | *Fusarium oxysporum*     | 519,6                            |
| Ordo   | *Hypocreales*          |                          |                                  |
| Family | *Nectriaceae*          |                          |                                  |
| Class  | *Sordariomycetes*     | *Clonostachys rosea*     | 520                              |
| Ordo   | *Hypocreales*          |                          |                                  |
| Family | *Bionectriaceae*       |                          |                                  |
| Class  | *Eurotiomycetes*       | *Aspergillus sydowii*    | 540                              |
| Ordo   | *Eurotiales*           |                          |                                  |
| Family | *Aspergillaceae*       |                          |                                  |
| average|                       |                          | 554,7                            |

Table 3 shows the average length of the ITS rDNA region from each species in the range of 520-606 bp. Average of *Fusarium oxysporum* species which isolate code is M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, and M17C1B2 is 519.6 bp. *Clonostachys rosea* species with isolate code is M1C1D2 has 520 bp sequence length. *Microascus gracillis* species, which sequence length is 588 bp, the species *Scopulariopsis brevicaulis* species which isolate codes M15B1BG1, M18A1D2U, and M19B1D3 has an average sequence length is 606.3 bp, and *Aspergillus sydowii* species has 540 bp sequence length. The results sequence length sequence average for each isolate is in accordance with the results of the sequence length that using ITS primers. Short sequences in the ITS rDNA region have high variability with nucleotide lengths. It ranges from 500 to 600 bp which can be used to determine species in the fungal kingdom. The size of the nucleotide sequence length can give the best results in ITS sequence.
3.3 Phylogenetic Tree of Mutant Plant Endophytic Mold Artemisia annua

The success of the sequence alignment on MEGA 7 is shown by the phylogram in Figure 1. All datasets of the same species are already at the end of the same branch with good bootstrap values. According to [52] a bootstrap value of >85% indicates a strong phylogram branch so that it has a high level of confidence. Based on the phylogram (Figure 2), the endophytic mold isolates of mutant A. annua are classified into 4 families, such as Nectriaceae, Bionectriaceae, Microascales, and Aspergillaceae which were classified into 2 classes, such as Sordariomycetes and Eurotiomycetes. These results are the same as the results of the identification based on the BLAST analysis of the NCBI.

Clade 1 is a Eurotiomycetes which includes M2A1D3U and M10C2D2U isolates. Both isolates formed a monophyletic group with G7strain Aspergillus sydowii, DTO245H7strain A. sydowii, A. DTO245H6 strain A. sydowii, DTO245C9 strain A. sydowii, and A. sydowii with CBS 593 65 type strain with a bootstrap value is 86% and formed taxa sister with CBS 129 55 strain A. sydowii. These results indicate that isolates M2A1D3U and M10C2D2U are identified as A. sydowii.

Clade 2 is a Sordariomycetes, that consist of Fusarium oxysporum, Clonostachys rosea, Microascus gracilis, and Scopulariopsis brevicaulis species. M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, and M17C1B2 isolates formed a monophyletic group with Fusarium oxysporum strain HDXG0, F. oxysporum strain CBS F143, F. oxysporum strain G15, F. oxysporum strain GFR32, and F. raposyssporum strain SFR967 with bootstrap value is 99%. This indicates that M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, and M17C1B2 isolates can be identified as F. oxysporum based on ITS rDNA.

The other Sordariomycetes group is from M1C1D2 isolates that formed a monophyletic group with Clonostachys rosea strain CP2 and C. rosea f catenulata strain ATCC 52622 with 67% of a low bootstrap value. In addition, M1C1D2 also forms taxa sister with C. rosea strain MR44 and a bootstrap value is 98% and C. rosea f catenulata strain CBS15427T. The bootstrap value is 100%. Referring to Nilsson et al. (2008), it can support the identification of identical M1C1D2 isolates to the C. rosea species, but differences in the genetic code of the ITS1 or ITS2 regions cause species variations.

The M1A1D2 isolate formed a monophyletic group with Microascus cirrosus strain ATCC MYA 4885, Scopulariopsis gracilis strain CBS 369 70 type is FMR 12224, Microasco gracilis strain BMU02787, M. gracilis strain BMU02786, and M. gracilis strain 12205 FMR with bootstrap value is 99 %. Microascus gracilis is the latest name from Scopulariopsis gracilis [53]. This indicates that the M1A1D2 isolate can be named M. gracilis.

M18A1D2U and M19B1D3 isolates formed one clade with Scopulariopsis brevicaulis strain CBS 115540 in a low bootstrap value (57%). This indicated that the isolate is identified as S. brevicaulis. Meanwhile, M15B1BG1 isolate formed a monophyletic group with S. brevicaulis strain WNF 16, S.
brevicaulis' voucher JUF0049, *S. brevicaulis* strain FMR 12216, and *S. brevicaulis* strain WNF 23 in a bootstrap value is 61%, so it can be said that M15B1BG1 isolate is a species of *S. brevicaulis*.

![Figure 1](image.png)

**Figure 1.** Phylogenetic tree endophytic fungi of *A. annua* mutant plant with Minimum evolution (ME) analysis based on ITS rDNA CC by MEGA 7 software.

The endophytic fungi of *A. annua* was also analyzed by using the PAUP software ver 4.0b10 (Figure 2) and obtained a total of 805 characters that consist of 446 constant characters, 121 non-informative characters, and 238 parsimony informative characters. The constant and highly informative characters of parsimony were used to reconstruct the phylogenetic tree. Phylogenetic analysis in this study used *Botrytis deweyae* strain CBS 134650 type as an outgroup from the *Helotiales*ordo. The results of the evaluation of phylogenetic analysis based on the maximum parsimony method have Consistency Index (CI) value = 0.887, Retention Index (RI) value = 0.978, Related Consistency Index (RC) value = 0.868 and Homoplasy Index (HI) = 0.112 and Tree length = 534. The phylogenetic reconstruction of the phylogenetic tree in Figure 2 shows that the endophytic fungi of the mutant plant *A. annua* were classified into 2 classes, such as *Sordariomycetes* and *Eurotiomycetes*. This result of PAUP program is the same as the phylogenetic tree analysis that uses MEGA 7 software.

Clade 1 is a *Sordariomycetes* group that consist of *Microascus gracilis*, *Scopulariopsis brevicaulis*, *Fusarium oxysporum*, and *Clonostachys rosea* species. The M1A1D2 isolate formed a monophyletic group with *S. gracilis* FMR 12205 strain, *Microascus gracilis* BMU02786 strain, *M. gracilis* strain
BMU02787, *S. gracilis* strain FMR 12224, *M. cirrosus* strain ATCC MYA 4885, and *S. gracilis* type CBS 369 70 strain with a bootstrap value is 59%. This indicates that the M1A1D2 isolate was identified as *M. gracilis* because one clade with the type strain has been formed, namely *S. gracilis* CBS 3966 70 strain type.

**Figure 2.** Phylogenetic tree of endophytic fungi of *Artemisia annua* mutant plant based on ITS rDNA using PAUP software ver 4.010b.
The M18A1D2U and M19B1D3 isolates formed a monophyletic group with 115540 CBS strain Scopulariopsis brevicaulis with a 57% bootstrap value. This indicates that the M18A1D2U and M19B1D3 isolates are closely related to the identified S. brevicaulis. Meanwhile, M15B1BG1 isolate formed a monophyletic group with S. brevicaulis Voucher J UF0049, S. brevicaulis strain WNF 16 and S. brevicaulis strain 12216 FMR with 55% bootstrap value. This indicates that M15B1BG1 isolate can be named as S. brevicaulis. The M18A1D2U, M19B1D3, and M15B1BG1 isolates are a paraphyletic group.

The other Sordariomycetes group came from M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, and M17C1B2 isolates that forming a monophyletic group with Fusarium oxysporum strain SF967, F. oxysporum strain GFR32, F. oxysporum strain G15, F. oxysporum strain HDXG0 and F. oxysporum CBS F143, strain with a 42% of bootstrap value. The M1A2D2, M3A2D3U, M12D1T1U, M14C1B1, and M17C1B2 isolates can be identified as F. oxysporum. The taxa sister of M1A2D2, M3A2D3U M12D1T1U, M14C1B1, and M17C1B2 are M1C1D2 isolates. The isolates form a monophyletic group with Clonostachys rosea strain CP2, C. rosea isolate 182, and C. rosea f. catenulata strain ATCC 52622 with a 45% bootstrap value. This indicates that the M1C1D2 isolate is identified as C. rosea.

Clade 2 is a Eurotiomycetes that includes isolates M2A1D3U and M10C2D2U. Both form taxa sister with Aspergillus sydowii strain DTO245C9, A. sydowii strain G7, A. sydowii strain DTO245H7, A. sydowii strain DTO245H6, A. sydowii strain CBS 129 55, and A. sydowii type strain CBS 593 65 with 52% bootstrap value. Both come from the same ancestor so that it can be said they have a fairly close relationship with A. sydowii, so it can be said those the isolates are A. sydowii.

The phylogenetic analysis produced by PAUP ver 4.010b software has a low bootstrap value, that range from 42 to 59%. The number of constant character and identified informative parsimony in the analyzed nucleotides will affect the bootstrap value. The more constant character and informative parsimony, the more chances of homplasy occurring in the phylogenetic tree reconstruction process so that the bootstrap value become low [54].

A bootstrap value that range from 50 to 69% indicates a weak phylogram branch, while a bootstrap value that less than 50% indicates a weak branch [52]. The phylogenetic results of phylogenetic analysis that using MP in PAUP have a low level of confidence. According to [55], although the bootstrap value in a phylogram is low, topology phylogenetic analysis has a higher level of confidence than grouping organisms based on morphological characters. This study has used the ITS rDNA marker that a conservative area in the fungal kingdom with good similarity and query covers values in BLAST analysis. Phylogenetic analysis on PAUP ver 4.0b10 produced 5687 phylogenetic trees with best score “ length tree”=534. Tree length is the total number of substituting tree branches.

Based on the phylogram, both analysis either MEGA 7 software that using the minimum evolution (ME) method or PAUP ver 4.010b that using the maximum parsimony (MP) method resulted in the same grouping of organisms (Figures 2). Based on that case, the phylogenetic analysis using ME on the MEGA software with a good bootstrap value can support phylogenetic analysis that using MP in PAUP with a low bootstrap value. Endophytic fungi species that identified from both analytical software are divided into 2 classes, namely Sordariomycetes (Fusarium oxysporum, Clonostachys rosea, Microascus gracilis, and Scopulariopsis brevicaulis) and Eurotiomycetes (Aspergillus sydowii). The five species that identified by using ITS rDNA sequences in both software are related evolutionarily. Microascus gracilis and Scopulariopsis brevicaulis are descendants of the same ancestor. Based on taxonomy, both species come from the same ordo and family, so they obviously have similar sequence characters. Fusarium oxysporum and Clonostachys rosea are descendants of the same ancestor. Taxonomically, both species belong to the same ordo but from different families. Meanwhile, A. sydowii has formed a different class even though it is a descendant of the same ancestor with Fusarium oxysporum and Clonostachys rosea. This case illustrates that there has been a process of adaptation and selection, thus giving rise to new characters that cause the three species to be separated taxonomically. Based on the phylogram, the five species came from the same primitive ancestor.
The identified species in this study are also found in other endophytic fungi diversity studies. *S. bravicaulis* was found on the stems of soybean plants [56]. The endophytic mold *M. gracilis* was also found on leaves, stems, and roots of maize plants [57]. *F. oxysporum* was found in *Centella asiatica* Bengkulu accession [51]. *C. rosea* was found on leaves, stems, and roots of maize plants [57]. *F. oxysporum* was found in *Centella asiatica* Bengkulu accession [51]. *C. rosea* is found in plants as a bio control agent. Some researches [58] indicates that *C. rosea* that is found in strawberries were able to act as a bio control botrytis (plant pest) better than fungicides. *A. sydowii* is also found in the *hemidesmus indicus* plant (Indian sarsaparilla [59]. *S. bravaicanus* plant as a medicinal plant produces the artemisinin bioactive substance and is used as an antimalarial. Based on research [7], *Colletotrichum* is an endophytic fungus found in anuma plants and has special interaction to stimulate artemisinin production. However, in this study, endophytic fungi from the genus Colletotrichum were not identified.

4. Conclusion
Phylogenetic analysis of twelve endophytic fungi isolates of mutant *A. annua* from the Biogen CC collection could be identified entirely up to the species level using ITS rDNA sequences. The identified species belong to the classes *Sordariomycetes* and *Eurotiomycetes*. The species identified were dominated by the *Sordariomycetes* class, namely *Fusarium oxysporum*, *Clonostachys rosea*, *Microascus gracilis*, and *Scopulariopsis brevicaulis*. Meanwhile, from the *Eurotiomycetes* class, *Aspergillus sydowii* was identified. All classes belong to the *Ascomycota* division.

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