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The first record of *Nectriopsis rexiana* Carried in *Sphaerobolus stellatus* Mycelium

Rudy Hermawan [1*], Indra Maulana [1], Yuyun Nishaul Khairillah [1]

[1] Department of Biology, IPB University, Jl. Raya Dramaga, Babakan, Bogor Regency, West Java, Indonesia 16680.
* Correspondence: hermawan_rudy@apps.ipb.ac.id

Abstract

Background: Fungicolous fungi are micro-fungi that inhabit or attach to the other fungus. These fungi can penetrate the host tissue area. Fungicolous fungi are naturally living on the host on the field. *Sphaerobolus stellatus* basidioma was found on the rotten wood with other species such as *Ceratiomyxa arbuscula*, *Lentinus squarrosulus*, *Stemonitis pallida*, etc. Many possibilities are other fungal species which not appear the basidioma live on the substrate or, moreover, on the *Sphaerobolus stellatus* basidioma. This study aimed to analyze the fungicolous fungi on *Sphaerobolus stellatus* mycelium using molecular analysis. **Methods:** The mycelium (SSmycelium sample) on the field was picked and put into a CTAB solution. Then, the mycelium was processed for molecular identification. The genomic DNA was then amplified using Internal Transcribed Spacer primers. The sequences were assembled and analyzed for the homology test in BLAST NCBI. Then, it was continued to phylogenetic tree reconstruction for three types of phylogenetic trees. They were Neighbor Joining by MEGA, Maximum Likelihood by MEGA, and Randomized Axelarated Maximum Likelihood. **Results:** The best phylogenetic tree was from the Randomized Axelarated Maximum Likelihood phylogenetic tree. The SSmycelium was identified as Nectriopsis rexiana with a 75 % BS value. The species did not have a morphological description, only the DNA source and ITS sequence. **Conclusions:** *Nectiropsis rexiana* was a parasitic and fungicolous fungus on other fungi. This study was the first record that Nectriopsis rexiana was found on Sphaerobolus stellatus.

Keywords: Fungicolous fungi, MEGA software; Parasitic fungi; RaxML; Rotten wood.

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Introduction

Fungi have lots of roles that include interactions with other microbes or hosts. Fungicolous fungi is an interaction between a fungus with another fungus that has various symbiotic relationships from mutualistic, commensal, parasitic, or saprotrophic or with other organisms such as plants (Yao et al., 2019). Fungicolous fungi can colonize other fungi, such as mushrooms. The outer area of the mushroom fruiting body is not free from the presence of other microorganisms. Even in edible mushrooms, the presence of microbes is an essential factor for the successful formation of fruit bodies besides environmental, for example, in the cultivation of Morels (Longley et al., 2019). The interaction between the mushroom and fungicolous fungi is widely happening on wild or undomesticated mushrooms. The fungicolous fungus Fusarium exquisite was recently isolated from the Lysurus periphragmoides fruiting body (Hermawan & Maulana, 2022).

Sphaerobolus stellatus had been found at IPB University, Bogor, Indonesia. The fungus was characterized as a Basidiomycota group that produced many mycelial nets on the substrate (Hermawan & Maulana, 2020). On the rotten wood, *S. stellatus* did not penetrate the substrate; other fungi also appeared on the same substrate spot, such as Lentinus

sajor-caju (Hermawan & Sari, 2021), *Ceratiomyxa arbuscula* (Hermawan & Amalia, 2022), and *Stemonitis pallida*. The possibility for other microorganisms to penetrate or be harbored together with the Sphaerobolus mycelial nets could happen in nature. *S. stellatus* mycelia were collected directly from the spot and continued to do molecular identification using the Internal Transcribed Spacer region. This region was beneficial for identification in general fungal taxonomy (Schoch et al., 2012). As our molecular identification result, this study got the species name *Nectriopsis rexiana*. The species did not match with our sample as *S. stellatus*. According to that result, we raised the information about the species as *N. rexiana* carried in *S.s stellatus* mycelial nets in nature. The herbarium did not designate our Nectriopsis as a specimen. This species was selected based accidentally on *S. stellatus* molecular identification. Molecular identification is very convenient (Gherbawy & Voight, 2016), especially for some fungi, which are only mycelium without any specific form. In this study, we aimed to analyze the fungicolous fungi that possibly attach or are carried by *N. rexiana* mycelium using molecular analysis without any isolate in a culture medium. This study reports new information on the fungicolous fungus topic.

Method

Sample Collection

The sample was *Sphaerobolus stellatus* mycelium from Hermawan & Maulana (2020). The mycelium was collected in the Landscape Arboretum of IPB University in January 2020. The mycelium (Figure 1) was collected from the fresh *S. stellatus* on the field and labeled SSmycelium. The mycelium was put into sterile CTAB (N-cetyl-N, N, N, trimethyl ammonium bromide) sol in the centrifuged tube. The composition of Cfollowedwing the composition from Hermawan et al. (2020). The transferring of mycelium into the tube was conducted in semi-aseptic conditions.

Figure 1. *S. stellatus* mycelia as Ssmycelium

DNA Extraction

The mycelium suspension was resuspended gently using the pipetting technique. The mechanical pipetting process would destroy the mycelium. Then, the DNA of the mycelium was extracted using the protocol from Hermawan et al. (2020). The genomic DNA was measured the quality using Nanodrop Spectrophotometer. The DNA concentration was prepared at 100 ng/µl.

DNA Amplification

The genomic DNA was amplified using a PCR machine. The Internal Transcribed Spacer region was used to identify this sample. A pairing of ITS (White et al., 1990), as ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') was used in this study. PCR amplification was performed in 30 µL as a total reaction. The PCR mixture contained a 15 µL PCR mix of 2X Kappa Fast 2G, 1.5 µL of 10 pmol of each ITS primer, three µL 100 ng template DNA, anined 9 µL ddH2O (Hermawan, 2020). A

Thermoline PCR machine was used and set as follows: initial denaturation at 94 °C for 2 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute. The last final extension was set at 72 °C for 10 minutes. The amplicon was checked using the electrophoresis method on TEB buffer 1x and agarose 1.5%. The electrophoresis product was visualized using Gel DocTM XR System. Amplicon as PCR product was sent to the 1st Base Malaysia company for sequencing process using Sanger dideoxy method.

BLAST Analysis

The sequences from sample SSmycelium were assembled using ChromasPro Software. The sequence was submitted and deposited into GenBank NCBI to get GenBank Accession Number. Then, the sequence was done for BLAST analysis in NCBI GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LIK_LOC=blasthome). The TYPE option was chosen. The highly similar sequences (megablast) option was selected for this BLAST analysis. As a result, the genus would have appeared on BLAST analysis. The genus Nectriopsis was the sample genus identification. All Nectriopsis species in the GenBank were downloaded.

Phylogenetic Tree Analysis

The sequences of all Nectriopsis species, our sequence, and one sequence Neopestalotiospsis zimbabwana (Hermawan et al., 2021), were aligned using clustal X software. Then, the phylogenetic tree was prepared using three methods. The first and second would use MEGA X software as the Maximum likelihood method and the Neighbor-Joining Method. The third phylogenetic tree used CIPRES Website for the RAxML method (Stamatakis, 2014). The phylogenetic tree reconstruction from the RAxML method followed the protocol from Hermawan et al. (2020). All phylogenetic trees used 1000 replicates as the Bootstrap method to assess the phylogenetic tree. The phylogenetic trees from MEGA software used the best-fit model for the Maximum likelihood and Neighbor-Joining assessment. The phylogenetic trees were edited as a final work using Treegraph Ver. 2 software. The bootstrap value as (BS)≥70 was shown on the branch.

Table 1. The Species and Sequences Which be used in this Study

Species	Isolate or Specimen Code	GenBank Accession Number
		ITS
Nectriopsis exigua	G.J.S.98-32	HM484865
Nectriopsis fuliginicola	CBS 399.82	KU382174
Nectriopsis fuliginicola	CBS 400.82	KU382175
Nectriopsis lindauiana	CBS 897.70	KU484865
Nectriopsis rexiana	CBS 542.92	KU382178
Nectriopsis rexiana	CBS 112780	KU382179
Nectriopsis rexiana	SSmycelium	OM568949
Nectriopsis rexiana	CBS 248.70	KU382177
Nectriopsis rexiana	CBS 305.70a	KU382181
Nectriopsis rexiana	CBS 840.70	KU382180
Nectriopsis violacea	CBS 440.65	KU382184
Nectriopsis violacea	CBS 849.70	KU382185
Nectriopsis violacea	CBS 278.80	KU382183
Nectriopsis violacea	CBS 914.70	KU382182
Neopestalotiopsis zimbabwana	X1	MW422813

Result and Discussion

The *Sphaerobolus stellatus* has many sequences for 32 data of the Internal Transcribed Spacer (ITS) region as molecular data in NCBI (NCBI, 2022). Hermawan & Maulana (2020) identified the *Sphaerobolus stellatus* using a morphological study. Then, it was continued to extract the DNA and amplify the ITS region. But unfortunately, the

sequence was analyzed as other species. The species as *Nectriopsis rexiana* was identified in the molecular identification. We assumed that the species *Nectriopsis rexiana* was attached/harbored on the *Sphaerobolus stellatus* mycelium. Furthermore, the DNA concentration of the *Nectriopsis rexiana* was higher than the *Sphaerobolus stellatus*. Those conditions made the *Nectriopsis rexiana* detected accidentally during the amplification step.

Nectriopsis rexiana, a mesophilic fungus from myxomycetes was reported to produce Monacolin K (Wagner et al., 1998). Genus of *Nectriopsis* belongs to Bionectriaceae with *Clonostachys* spp. (Zare & Gams, 2016) and other relative families Clavicipitaceae, Cordvcipiticeae. Hypocreaceae, Nectriaceae, Niessliaceae, and Ophiocordvcipitaceae in Hypocreales (Hirooka et al., 2010). *Nectriopsis rexiana* was described as a fungicolous fungus of myxomycetes such as *Lycogala epidendron*, *Physarum nutans*, *Fuligo septica*, *Lycogala flavofuscum* (Zare & Gams, 2016). In addition, the other species of *Nectriopsis* were reported to act as Lichenicolous fungus like *N. cladoniicola*, *N. parmeliae*, and *N. rubifaciens* (Cole & Hawksworth, 2001). The behavior information of *Nectriopsis rexiana* as a fungicolous fungus that inhabited *Sphaerobolus stellatus* is still lacking.

This study only resulted from the sequences of *N. rexiana* from *Sphaerobolus stellatus* mycelium. There was no isolate or other morphological observation. Then, the sequences were assembled and conducted the BLAST analysis in GenBank NCBI. The result showed in table 1. The genus was identified as *Nectriopsis*. Four species showed as Genus *Nectriopsis*, and one species as Genus *Verticillium*. The four best results showed as *Nectriopsis*. We adopted these results for SSmycelium identification as Genus Nectriopsis. The *Verticillium rexianum* CABI: IMI320287 might be another possibility. The GenBank description of the *Verticillium rexianum* CABI: IMI320287 with JQ647443 accession number was described as a publication by Cannon et al. (2012). But, the *Verticillium rexianum* CABI: IMI320287 was not mentioned in the publication. According to Chaverri et al. (2011), on their study said that *Verticillium rexianum* was an asexual stage name of *Nectriopsis exigua*.

Blast's analysis results could not confirm the name of the species. An analysis of the phylogenetic tree is required to ensure the name of the species. According to Hall (2013), too many different ways of analyzing the phylogenetic tree Many ways to explore the phylogenetic tree. This study used three methods for phylogenetic analyses, such as Neighbor-Joining by MEGA (Figure 2), Maximum Likelihood by MEGA (Figure 3), and Randomized Axelarated Maximum Likelihood (Figure 4). All phylogenetic trees showed the exact structure of SSmycelium as *Nectriopsis rexiana*.

The phylogenetic trees were built using three types of analyses, such as Neighbor-Joining by MEGA (Figure 2), Maximum Likelihood by MEGA (Figure 3), and Randomized Axelarated Maximum Likelihood (Figure 4). Figure 3 classified Ssmycelium, including *N. rexiana,* with an 81% BS value. But the *N. exigua* was too closed with the *N. rexiana* clade. From this phylogenetic tree, the branching among *N. rexiana* and *N. exigua* was too short of separating them as different species. Since the analysis used a single gene, this phylogenetic tree formed an unclearly phylogenetic tree structure. Then, the other phylogenetic tree in figure 4 showed that the *N. rexiana* clade looked better than in figure 3. The maximum likelihood was prevalent for the phylogenetic tree in this era. Figure 3 showed that SSmycelium was classified as *N. rexiana* with a 69% BS value. This value was lower than the NJ analysis. But the value of 69% was strong enough to identify the species on the clade branching. The third phylogenetic tree analysis is RAxML, as shown in figure 4. The best structure is in figures 2 and 3. A BS value of 75% classifies SSmycelium in the clade *N. rexiana*.

Nectriopsis rexiana was identified as *Nectriopsis exigua* previously. Many species in *Nectriopsis* were named *N. exigua* before the molecular study was comprehensively studied. Zare & Gams (2016) wholly mentioned and attached the *Nectriopsis* species to their phylogenetic tree. There were one species as *N. exigua*, but there was a note or sign that it was not sure as the correct species of N*. exigua*. Currently, many *Nectriopsis exigua* were changed into *N. rexiana*.

N. rexiana was primarily a parasite fungus (Zare & Gams, 2016). In this study, *N. rexiana* SSmycelium was found on the Sphaerobolus stellatus mycelium collected on the field. This is the first report that *Sphaerobolus stellatus* harbored the *N. rexiana*. Based on Zare & Gams (2016), many *N. rexiana* were isolated and identified as fungicolous and as a parasite on other fungi, such as *N. rexiana* CBS 248.70 from Lycogala epidendron, *N. rexiana*, CBS 305.70A from Physarium, *nutans*, *N. rexiana* CBS 840.70 from *Fuligo septica*, *N. rexiana* CBS 542.92 from *Trametes* sp., and *N. rexiana* CBS 112780 from *Lycogala epidendron*. Many possibilities for *N. rexiana* to inhabit or attach the *Sphaerobolus stellatus* mycelium on the field. In nature, the *Nectriopsis* can form an asexual stage as *Verticillium*. The spores as conidia can be flown up by the air or distributed by water drops from rain or other abiotic interactions. According to figure 1, we assumed that the black spot on the *Sphaerobolus stellatus* mycelium was *N. rexiana* colonies.

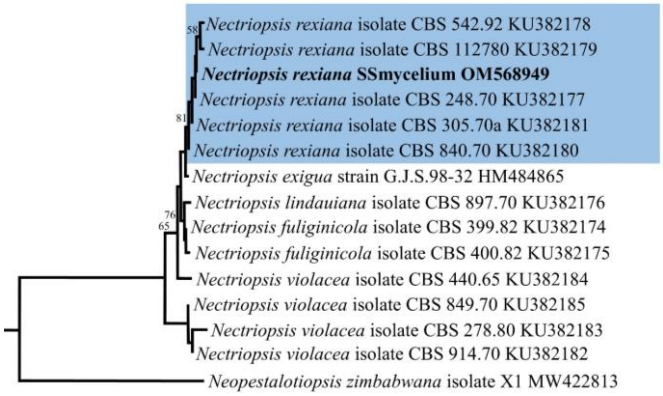

Figure 2. *SSmycelium* phylogenetic tree generated from ITS 4/5 using Neighbor-Joining on MEGA X.

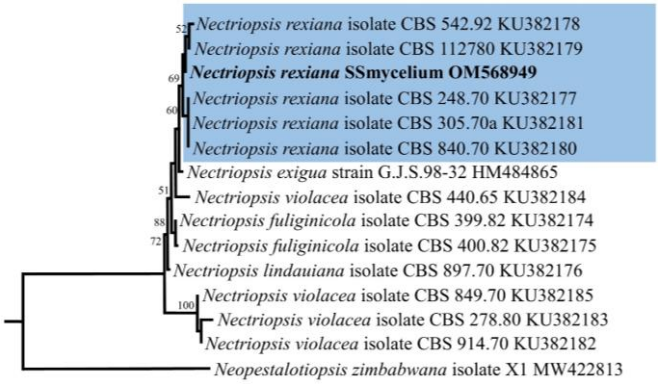

Figure 3. SSmycelium phylogenetic tree generated from ITS 4/5 using Maximum Likelihood on MEGA X.

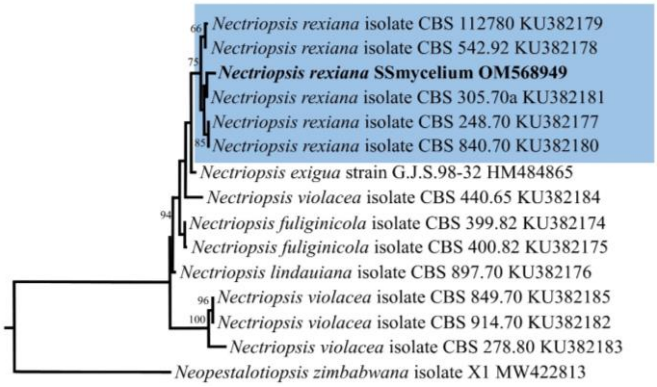

Figure 4. SSmycelium phylogenetic tree generated from ITS 4/5 using Maximum Likelihood on RAxML Black Box.

Conclusions

Sphaerobolus stellatus harbors another fungus on the mycelium in the field. The fungicolous fungus identification uses molecular study. Three phylogenetic trees are built as Neighbor-Joining by MEGA, Maximum Likelihood by MEGA, and Randomized Accelerated Maximum Likelihood by RAxML Black Box. The SSmycelium sample is identified as *Nectriopsis rexiana*. The best phylogenetic tree for single gene analyses as ITS region in SSmycelium is a phylogenetic tree constructed with Randomized Accelerated Maximum Likelihood by RAxML Black Box.

Declaration statement

The authors reported no potential conflict of interest.

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