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Research Article

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Complementary Molecular Data for Two Species from Herbarium Bogoriense: *Sphaerobolus stellatus* and *Trichaleurina javanica*

Data Molekuler Pendukung untuk Dua Jenis Spesimen Herbarium Bogoriense: *Sphaerobolus stellatus* dan *Trichaleurina javanica*

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ABSTRACT

The micro fungus *Sphaerobolus stellatus* (BO 24422) and the macro fungus *Trichaleurina javanica* (BO 24420) were found in the IPB University Landscape Arboretum. Current identification used morphological characteristics and molecular studies with Large Subunit DNA markers, respectively. According to their identification, the database of fungal identification is commonly from Internal Transcribed Spacer (ITS) marker for molecular study. However, these two specimens do not yet have an ITS sequence. This research aims to study the molecular data of two specimens using ITS sequences. The research method used molecular identification using an internal transcribed spacer (ITS) primer: ITS 4 as a reverse and ITS 5 as a forward. Two phylogenetic trees were generated by Randomized Axelarated Maximum Likelihood (RAxML) Black box. Bootstrap (BS) values $\geq$ 90% were displayed on the phylogenetic tree branches. The results showed that the bootstrap value for *S. stellatus* BO 24422 was 96% classified as *S. stellatus*. The fungus *T. javanica* BO 24420 shows a BS value with a percentage of 93% classified as *T. javanica*. The ITS is a general marker and very useful for molecular identification.

INTRODUCTION

Fungi Kingdom includes true fungi and fungus-like organisms (Lucking *et al*., 2020). The structural characteristics that indicate a fruiting body are called mushrooms. Some of the mushroom can be easily identified based on their specific features or characteristics of fruiting bodies. However, other fungi do not necessarily have specific features or characteristics of fruiting bodies to distinguish them. The main difficulty is to observe and find the specific characters that define the boundaries of their species or genus.

Two mushrooms, micro-mushroom specimen BO 24422 and macro-mushroom specimen BO 24420 were found in Landscape Arboretum of IPB University. The identification used morphological characteristics (Hermawan & Maulana, 2020) and molecular study of Large Subunit DNA marker (Hermawan *et al*., 2020), respectively. According to their identification, the database of fungal identification is commonly from Internal Transcribed Spacer (ITS) marker for molecular study (Nilsson *et al*., 2015). But both specimens, *S. stellatus* BO 24422 and *T. javanica* BO 24420, have not the ITS sequences yet.

Sphaerobolus, commonly called *artillery fungus* due to a unique single gleba, contained basidiocarp and it produces basidiospores in an enclosed structure. This genus is included in Gasteromycetes along with bird’s nest fungi, puffballs, stinkhorn, and also the earth stars mushroom (Geml *et al.*, 2005). Systematics of *Sphaerobolus* was documented with each species well separated on the phylogram and well known to be distributed in America and the Netherlands (Geml *et al.*, 2005). However, in Asia, the data on *S. stellatus* is still lacking. The documented data of *S. stellatus* was reported in the Philippines (The Global Fungal Red List

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Initiative, 2015), and morphological identification data was from Indonesia (Hermawan & Maulana, 2020). The molecular evidence is still needed as supporting data, so this report is included the complementary molecular data of *S. stellatus* specimen BO 24422. The ITS marker is very expected to be conducted. Baetsen-young *et al.* (2015), have identified the species of *Sphaerobolus* from turfgrass of nine golf courses using the ITS marker.

The *T. javanica* specimen has an adult Apothecium that is goblet shaped, hairy and dark grayish cyan in color, the top is concave with a soft orange color, while the immature Apothecium is less cylindrical in shape with wrinkles at the top (Hermawan *et al.*, 2020). In previous study by *Hermawan et al*. (2020), The specimen which found in west java have identified using Large Subunit (LSU). The phylogenetic tree showed that the species *T. javanica* with 81% bootstrap value. While another possibility of good bootstrap value may be gotten if the identification using the ITS marker.

According to the current molecular identification in fungi, ITS markers are very useful for making fungal taxonomic databases well and clearly identified. Therefore, this study provided the ITS sequences of two mushrooms for *S. stellatus* BO 24422 and *T. javanica* BO 24420. Perhaps these ITS sequences might be useful for making clear the position of these specimens towards other specimens in the world.

MATERIAL AND METHOD

Sample Description

Two samples as S*phaerobolus stellatus* BO 24422 and *Trichaleurina javanica* BO24420. Both samples had been already deposited into Herbarium Bogoriense Indonesia. *S. stellatus* BO 24422 was identified by morphological identification (Hermawan & Maulana 2020), whereas *T. javanica* BO 24420 was identified by molecular study using Large Subunit (LSU) marker (Hermawan *et al*. 2020). The part of samples was put into Cetyl Trimethyl Ammonium Bromide (CTBA) solution.

Genomic DNA Extraction

The CTAB suspension containing samples were mashed and destructed using sterile pestle. The genomic DNA was extracted using the protocol from Hermawan *et al.* (2020). The molecular identification using internal transcribed spacer (ITS) primer: ITS 4 as a reverse and ITS 5 as a forward. Polymerase Chain Reaction (PCR) was performed with 40 µL total reaction volume. It was containing 20 µL of PCR mix from 2× KappaFast 2G, 2 µL (10 pmol) of each primer, 4 µL (100 ng) of template DNA, and 12 µL of ddH_2O. The DNA was amplified follows initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and last for the final extension was 72 °C for 10 min. The PCR products were sequenced by First Base Malaysia company. Then, the sequences were deposited into GenBank NCBI website (https://www.ncbi.nlm.nih.gov).

Phylogenetic Tree Analyses

Two phylogenetic trees were generated by Randomized Axelarated Maximum Likelihood (RAxML) Black box (Stamatakis, 2014). The first phylogenetic tree was *Sphaerobolus* species phylogenetic tree. The phylogenetic tree was constructed using *Sphaerobolus* species from Geml *et al*. (2005). The second phylogenetic tree was *Trichaleurina* species phylogenetic tree. The phylogenetic tree was constructed using *Trichaleurina* species from Carbone *et al*. (2013). *Geastrum pseudostriatus* voucher Type BP22110 was used as an outgroup in both phylogenetic trees. All sequences that were used in these phylogenetic tree constructions were listed in Table 1. The bootstrap (BS) method as 1000 replicates was used in these phylogenetic tree constructions. Bootstrap (BS) values ≥ 90% were displayed on the phylogenetic tree branches.

Table 1. List of sequences used in this study.

Species	Strain / voucher	ITS acc. number	Reference
Geastrum pseudostriatus	Type BP22110	KC581997	Jeppson *et al*., 2013
Sphaerobolus ingoldii	SS42	AY654738	Geml *et al*., 2005
Sphaerobolus ingoldii	T800	AY654737	Geml *et al*., 2005
Sphaerobolus iowensis	SS9	AY487956	Geml *et al*., 2005
Sphaerobolus iowensis	SS4	AY487953	Geml *et al*., 2005
Sphaerobolus iowensis	ATCC 52850	AY487958	Geml *et al*., 2005
Sphaerobolus stellatus	SS12	AY487959	Geml *et al*., 2005
Sphaerobolus stellatus	BO 24422	OP218935	This study
Sphaerobolus stellatus	ATCC 18339	AY487957	Geml *et al*., 2005
Trichaleurina javanica	TUR-A 198585	KF418256	Carbone *et al*., 2013
Trichaleurina javanica	TNM F11334	KF418252	Carbone *et al*., 2013
Trichaleurina javanica	TNM F8917	KF418251	Carbone *et al*., 2013
Trichaleurina javanica	BO 24420	OP218936	This study
Trichaleurina tenuispora	TNM F10376	KF418244	Carbone *et al*., 2013
Trichaleurina tenuispora	TNM F4705	KF418243	Carbone *et al*., 2013
Trichaleurina tenuispora	TNM F17898	KF418245	Carbone *et al*., 2013

Result and Discussion

The ITS sequences of *S. stellatus* BO 24422 and *T. javanica* BO 24420 were successfully gotten with a good quality. Then the sequences have been already submitted into GenBank NCBI as OP218935 and OP218936, respectively. The sequences were used for the molecular identification of *S. stellatus* BO 24422 (Fig. 1) and *T. javanica* BO 24420 (Fig. 2). The bootstrap value for *S. stellatus* BO 24422 on Fig. 1 showed the good value as 96% classified as *S. stellatus*. *T. javanica* BO 24420 on Fig. 2 had a better BS value with 93% compared with the LSU phylogenetic (Hermawan *et al*., 2020) with 81%.

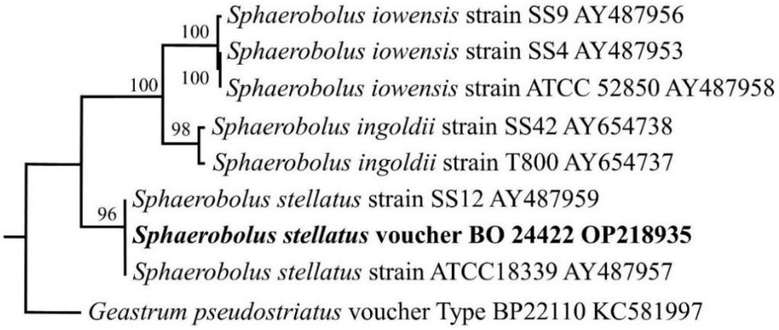

Figure 1. Sphaerobolus phylogenetic tree. Our species is in bold letters. Bootstrap values >90% is presented on the branches.

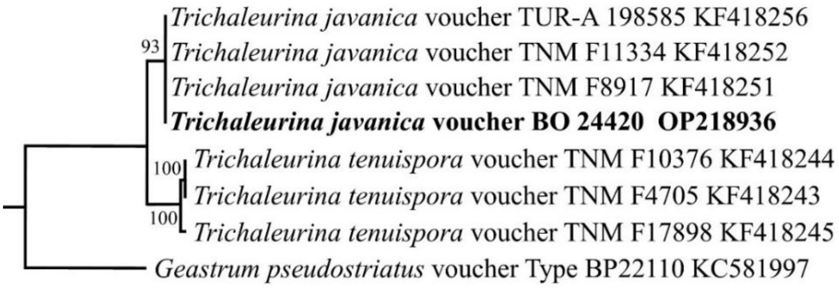

Figure 2. *Trichaleurina* phylogenetic tree. Our species is in bold letters. Bootstrap values >90% is presented on the branches.

Morphological and molecular data *on S. stellatus* from the tropical region of Asia are still needed to be observed. Currently in the world, there are only very few studies that identify micro-fungi, one of which is *Sphaerobollus* (Geml *et al*., 2005). The first record of *S. stellatus* in Asia specifically Indonesia was documented morphologically by Hermawan and Maulana (2020). The specimen (BO 24422) is further observed in the molecular method then it is identified through phylogenetic analysis. The first DNA sequence from the extracted mycelium was not *S. stellatus*. It was *Nectriopsis rexiana* that was carried in *S. stellatus* mycelium. It was assumed that *N. rexiana* (Hermawan *et al*., 2022) has a role as a fungicolous fungus or is associated with *S. stellatus* although this information still needs more evidence to be collected. The *S. stellatus* BO 24422 lives openly in nature and is in direct contact with the substrate. This is what causes these micro-fungi to have symbiosis or a fungicolous fungus when they are in the external environment. The second trial of the DNA extraction process gave the right targeted sequence. The specimen (*S. stellatus*) position is strongly separated from the other two species (Fig.1). The branch of *S. stellatus* BO 24422 shows a 96% bootstraps value which is having a strong similarity with *S. stellatus* ATCC18339 and strain SS12. The genetic distance of ITS sequences of each species is qualitatively seen to be far enough between *S. stellatus* towards *S. ingoldii* and *S. iowensis*. The Fig. 1 also showed that our specimen was identified as *Sphaerobolus stellatus* and have a close correlation with *Sphaerobolus iowensis*. This report is similar to Geml *et al*., (2005) that have constructed the phylogenetic analyses using LSU and ITS of *Sphaerobolus* and found that *Sphaerobolus stellatus* related to *Sphaerobolus iowensis*. The second specimen as *T. javanica* showed a good BS value in the *T. javanica* clade. In the GenBank NCBI, *Trichaleurina* species are only available for two species as *T. javanica* and *T. tenuispora*. The marker that was very popular to be used in *Trichaleurina* was ITS marker (Carbone *et al*., 2013). This is proofed by Fig. 2 in this study that showed the best BS value for *T. javanica* BO 24420 molecular identification. Therefore, the ITS marker can be a good identification marker for *Trichaleurina* species. Schoch *et al*. (2012) stated that the ITS is a general marker and very useful for molecular identification. On the other hand, not all mushroom will show a good phylogenetic tree with the ITS marker as a molecular marker. But some other markers also have an important role for making a good phylogenetic tree.

In many cases, species identification using DNA sequences can be representing the only means for high-precision species identification (Kang et al., 2010). Not only that, the delimitation of the species into other infra species also can be decided clearly. However, molecular identification in mycology is not devoid of complications. Mostly, the good samples are also important to be collected as the DNA source. In Indonesia, many researchers still use a morphological approach to identify fungi. Therefore, a fungal identification approach using a molecular approach is very important to ensure that the identification results are very correct and clear.

CONCLUSION

The *S. stellatus* BO 24422 and *T. javanica* BO 24420 have been identified successfully using ITS. *S. stellatus* BO 24422 has the phylogenetic tree with boostrap value 96% and *T. javanica* BO 24420 with boostrap value 93%. The phylogenetic tree also showed that *Sphaerobolus stellatus* has a close correlation with *Sphaerobolus iowensis*. There are only two species of *Trichaleurina*, namely *T. javanica* and *T. tenuispora*.

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