Xylaria sp. The Candle Snuff Fungus from West Java

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ABSTRACT

Candle snuff fungus belongs to Xylaria group. Generally, Xylaria has a form like stick or candle or elongated fruit of shapes. Xylaria is classified into Ascomycota within Xylariaceae. This study found one species of candle-shaped mushroom in IPB Unversity. This study aimed to identified and characterized the specimen using molecular and morphological data. The specimen was collected and preservedinto FAA solution and deposited into Herbarium Bogoriense as BO 24426. Molecular analyses using Large Subunit as a region for amplification showed that the BO 24426 was classified into Xylaria sp. This species closes to Xylaria consociata. The stromata were erected, unbranched, and tapered to the apex. The texture was rigid and hard. Ascus bore 8 ascospores. The ascospores were fusiform or bean-shaped and smooth. The morphological observations supported molecular identification of BO 24426 as Xylaria sp. Other genes were needed to ensure the exact species of Xylaria

Introduction

Mushroom is a macro-fungus that belongs to Ascomycota and Basidiomycota (Brundrett et al., 1996). Some mushrooms show the unique characters as the unusual shapes. In Indonesia, many unique shapes of mushrooms are found as a specific name of genus, such as puffball for *Calvatia* (Hermawan & Putra, 2018, 2021), Cannonball for *Sphaerobolus* (Hermawan & Maulana, 2020), goblet for *Trichaleurina* (Hermawan, Amelya, et al., 2020), fan for *Telephora* (Hermawan, Imaningsih, et al., 2020), coral for *Ramaria* (Hermawan, Imaningsih, et al., 2020), trumpet for *Cantharellus* (Hermawan, Imaningsih, et al., 2020), microphone-like for *Lysurus* (Hermawan et al., 2021), and parasol for *Chlorophyllum* (Hermawan, Imaningsih, et al., 2020). Others unique mushroom is *Xylaria*. The *Xylaria* is famous with name of the dead men's finger or the candle snuff fungus.

Xylaria contains 819 name records (Index Fungorum, 2021). *Xylaria* is classified into Xylariales within Pezizomycotina (Mycobank, 2021). The traits of this genus are known as saprobic and also endophytic fungi. *Xylaria* as an endophyte lives inside the plant tissue without causing any disease symptoms for the plant (Petrini, 1991). A special trait of *Xylaria* also emerges from nest of termites (Rogers et al., 2005). This study found the *Xylria* stromata on the ground (without substrate). It indicates the possibility of the trait as symbiont with insect.

Traditional classification and identification of fungi has relied upon microscopic features, colony characteristics on media and also biochemical reactions (Sutton & Cundell, 2004). Sometimes the traditional method

cannot ensure the name of species, especially for *Xylaria*. The *Xylaria* are difficult to identify and classify using only the stromata characteristics (Whalley, 1996). Some researchers in *Xylaria* study using the molecular comprehensive for identifying the species (Ju et al., 2009; Ma et al., 2013; Okane et al., 2008). In Indonesia, study of *Xylaria* is usually conducted using only morphological character (Kristin et al., 2020). The molecular study is important to make sure the name of species and will be supported using morphology. The internal transcribed spacer (ITS) region is a good region to identify the fungi (Brandt et al., 2005; White et al., 1990). But, other region such as Large Subunit is also needed to make stronger analyses in fungi. For Xylariaceae, the sequences that popular to be analyzed are Large Subunit region (Okane et al., 2008), some ITS sequences are rarely available in GenBank. Therefore, this study used molecular study for Large Subunit sequences.

Materials and Methods

Mushrooms Sampling Site

The stromata were collected in August 2019 and located in the Arboretum of IPB University. The fungus was found as a *Xylaria* stroma based on the morphology. The stroma was collected, documented, and observed the morphological characters. The observation was conducted in the mycology laboratory of Biology Department, Mathematics and Natural Sciences, IPB University, Indonesia. The apothecium was preserved in FAA (Seshagirirao et al., 2016) and deposited into Herbarium Bogoriense Indonesia.

Morphological Observation

The morphological data of stromata were observed and documented to confirm the genus as *Xylaria*. The data would be used to support the molecular analyses. The observation was conducted using an Olympus stereo and binocular microscope cs22LED. The features of macro-and micro-morphology such as asci and ascospores. The observations were about the shape, size, and ornamentation, then compared with the other publication of the species within *Xylariaceae*.

Molecular Identification

The stromata were identified using molecular analyses. The genomic DNA was extracted using the protocol as in (Hermawan, Amelya, et al., 2020). The genomic DNA quality and quantity were verified using a nanodrop spectrophotometer. The amplification was used Large Subunit (LSU) as forward LR0R (5'-GTA CCC GCT GAA CTT AAG C-3') and reverse LR5 (5'-ATC CTG AGG GAA ACT TC-3') primers. PCR amplification was performed in 40 µL total reaction containing 12 µL ddH2O, 2 µL of 10 pmol of each primer, 20 µL PCR mix from 2X Kappa Fast 2G, and 4 µL 100 ng template DNA. Amplification used a Thermoline PCR. The PCR condition was set as follows: initial denaturation at 94 ℃ for 2 minutes, followed by 30 cycles of denaturation at 94 ℃ for 45 seconds, annealing at 56 ℃ for 1 minute, and extension at 72 ℃ for 1 minute. Then final extension was set at 72 ℃ for 10 minutes. The amplicons were estimated on 1 % agarose gels and visualized by the Gel DocTM XR system. PCR products were sent to the 1st Base Malaysia for sequencing.

Phylogenetic Analyses

The sequence was deposited in GenBank. This sequence, *Xylaria* species in (Okane et al., 2008), some *Xylaria* species from GenBank, and *Vamsapriya bambusicola* (outgroup) were constructed into phylogenetic tree (Table 1). Sequences were aligned using Clustal X Ver. 2.1 software and saved as PHYLIP format files. All sequences were aligned using 600 base pairs of the ITS region. The phylogenetic tree of Randomized Axelerated Maximum Likelihood (RAxML) Black Box was generated on CIPRES (Stamatakis, 2014). Bootstrap analyses with 1000 replicates assessed the phylogenetic tree. Bootstrap (BS) $\geq$ 50 was shown on the branch.

Table 1. Collection code, species, GenBank accession numbers of Internal Transcribed Spacer used in this study.

Species	Collection code	GenBank acc. no
		LSU
Xylaria allantoidea	BCC 1340	AB376730
Xylaria anisopleura	BCC 17352	AB376732
Xylaria apiculata	BCC 1136	AB376700
Xylaria arbuscula	BCC 1156	AB376703
Xylaria arbuscula	RHP 21	MT215561
Xylaria arbuscula	CBS 126416	MH875561
Xylaria arbuscula	DHK-10	MN376820
Xylaria aristata	BCC 1229	AB376716
Xylaria aristata	BCC 1260	AB376722
Xylaria badia	BCC 1171	AB376705
Xylaria badia	BCC 1190	AB376711
Xylaria bambusicola	BCC 22739	AB376809
Xylaria bambusicola	BCC 23628	AB376821
Xylaria bambusicola	BCC 23659	AB376825
Xylaria bambusicola	MFLUCC 11-0606	KU863148
Xylaria bambusicola	BCC 23627	AB376820
Xylaria coccophora	BCC 1085	AB376688
Xylaria consociata	BCC 18196	AB376733
Xylaria cubensis	BCC 1321	AB376729
Xylaria cubensis	BCC 1144	AB376701
Xylaria cubensis	BCC 1303	AB376725
Xylaria cubensis	BCC 11027	AB376683
Xylaria cubensis	BCC 1219	AB376715
Xylaria curta	BCC 1007	AB376681
Xylaria curta	BCC 1151	AB376702
Xylaria enteroleuca	CBS 128357	MH876349
Xylaria escharoidea	BCC 23279	AB376818
Xylaria escharoidea	BCC 23634	AB376882
Xylaria feejeensis	BCC 1115	AB376696
Xylaria grammica	IHI A82	MK408621
Xylaria grammica	BCC 1002	AB376679
Xylaria grammica	BCC 1170	AB376704
Xylaria grammica	5084	JQ862608
Xylaria hypoerythra	BCC 22968	AB376812
Xylaria hypoxylon	AFTOL-ID 51	AY544648
Xylaria hypoxylon	CBS 126417	MH875562
Xylaria hypoxylon	DM1153	MT773340
Xylaria hypoxylon	DSM 108379	MK577428
Xylaria juruensis	BCC 1232	AB376717
Xylaria juruensis	BCC 1086	AB376689
Xylaria juruensis	BCC 1083	AB376687
Xylaria juruensis	BCC 1263	AB376723
Xylaria juruensis	BCC 1234	AB376719
Xylaria juruensis	BCC 1233	AB376718
Xylaria laevis	BCC 1182	AB376709
Xylaria longipes	19GCAS018	MW077324
Xylaria longipes	CBS 148.73	MH872351
Xylaria longipes	CBS 347.37	MH867427
Xylaria longipes	DSM 107183	MK408619

Table 1. Continue

Species	Collection code	GenBank acc. no
		LSU
Xylaria mellissii	BCC 1005	AB376680
Xylaria mellissii	BCC 1186	AB376710
Xylaria multiplex	BCC 1177	AB376707
Xylaria multiplex	BCC 1036	AB376684
Xylaria obovata	BCC 1053	AB376685
Xylaria obovata	BCC 18718	AB376737
Xylaria obovata	BCC 1100	AB376690
Xylaria papulis	BCC 22966	AB376811
Xylaria phyllocharis	BCC 1352	AB376731
Xylaria phyllocharis	BCC 1065	AB376686
Xylaria piperiformis	BCC 22987	AB376814
Xylaria polymorpha	CBS 162.22	MH866242
Xylaria psidii	BCC 1199	AB376713
Xylaria psidii	BCC 1127	AB376698
Xylaria schweinitzii	BCC 1013	AB376682
Xylaria schweinitzii	BCC 1001	AB376678
Xylaria sicula	CBS 401.58	MH869355
Xylaria sp.	BO 24426	MW433688
Xylaria sp.	BCC 1181	AB376708
Xylaria sp.	BCC 1133	AB376699
Xylaria sp.	BCC 1288	AB376724
Xylaria tuberoides	BCC 18361	AB376736
Xylaria xylarioides	CBS 127883	MH876177
Xylaria xylarioides	CBS 128018	MH877980
Vamsapriya bambusicola	MFLUCC 11-0577	KM462836

Results and Discussion

Specimen

Two stromata were found that grew on the ground without substrate (Figure 1a). Sexual morphology: Stromata look like greyish candle snuff with whitish powder covered stromata from upper (Figure 1a-b). The stromata were erected, unbranched, and 6.5-9.1 cm of height. The stromata were tapered to the apex of stromata. The texture was rigid and hard. Asci were appeared inside the stromata. Ascus contains 8 ascospores (Figure 1c). The ascospore has oil globular inside, 1 or 2 oil globules appeared in the corner of ascospore, fusiform or bean-shaped and smooth (free of ornament). The size was 12.2–13.2 x 5.0–5.9 µm (Figure 1d). Specimen examined: Landscape Arboretum of IPB University, BO 24426, Rudy Hermawan. GenBank Submission: LSU: MW433688.

Xylaria has the unique morphology as a candle, spoon and finger (Kirk et al., 2008). Then, Fournier et al. (2018) described the updated morphology of *Xylaria* species as in pulvinate to depressed-spherical shape. *Xylaria* BO 24426 has the morphology with candle snuff with whitish powder covering the stromata. The whitish powder does not contain spore or other sexual structure. The spore as ascospore is inside the stromata part.

Rogers and Samuels (1986) described *Xylaria* species has dark carbonaceous stromata and pigmented ascospores as dark color with a germ slit. *Xylaria* BO 24426 showed the dark ascospores. The ascospores was produced inside the ascus with 8 spores. Germ slit character is really hard to be observed. The observation should use the advanced microscope with the high magnification of lens. the observation of BO 24426 has not successful observed the germ slit characteristic.

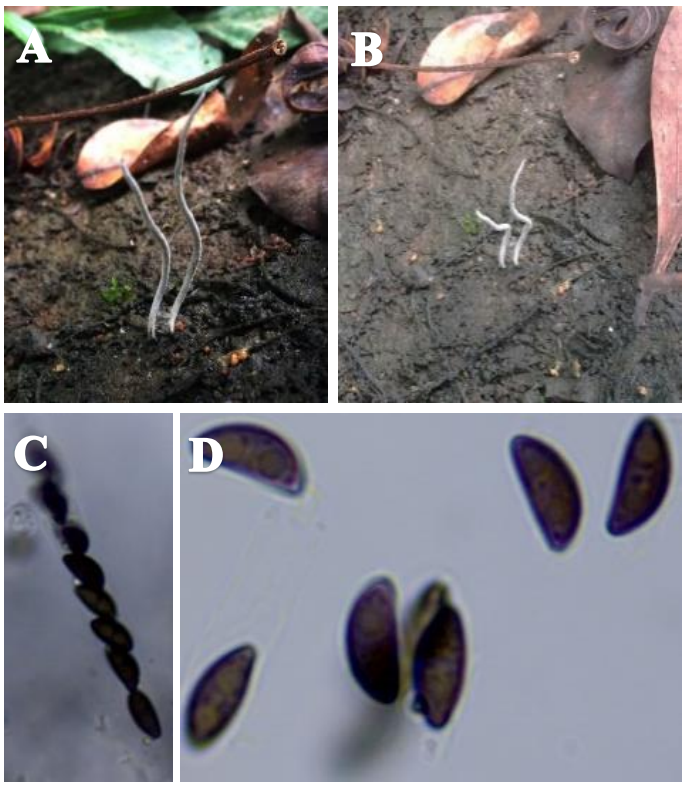

Figure 1. Morphology of *Xylaria* BO 24426 (A; B) Stromata on substrate; (C) ascus; (D) ascospores. Scale bars: (A) 10 cm; and (C; D) 10 µm.

The ascospore shape of BO 24426 has fusiform or bean-shaped. Other species of *Xylaria* have many shapes such as ellipsoid slightly inequilateral with broadly to narrowly rounded ends (as *X. berteroi*), ellipsoid-inequilateral to sub-oblong with broadly rounded ends (as *X. alboareolata*) etc. (Fournier et al., 2018). Based on our BO 24426, the ascospores also have the oil globules inside the ascospores.

Molecular work

The phylogenetic tree showed that the specimen BO 24426 was classified as *Xylaria* sp. with 41% bootstrap value (Figure 2) making other clades from *Xylaria consociata*. Okane et al. (2008) mentioned the *X. consociata* as a saprobic *Xylaria*. Whereas the *Xylaria* sp. BO 24426 is not saprobic *Xylaria*. According to Figurure 2, BO 24426 has a different line length with the *Xylaria consociata*. This line was indicated that the evolutionary based on their sequences is different. It can be showed that *Xylaria* BO 24426 is not *Xylaria consociata*. In Okane et al. (2008) also mentioned some of *Xylaria* as *Xylaria* sp. The region of gene amplification for molecular identification within *Xylaria* species sometimes can't show the strong identification. As a single gene or region in this study, BO 24426 only can be identifying until the Genus name

Based on this phylogenetic tree of *Xylaria* BO 24426, the species can be assumed as a new species or unidentified. The BO 24426 is assumed as a new species because the BO 24426 makes other clade among other species. Or it can also be assumed as unidentified species. The other gene regions are needed to complete the differences among the species and make the perfect phylogenetic tree. This additional work will ensure the BO 24426 as a new species or not.

The other functional genes for identification are Internal Transcribed Spacer, Actin, Small Sub Unit, etc. Schoch et al. (2012) mentioned the ITS region is the most useful for identification and also as a general gene for identification among fungal species. The existence of ITS as a general gene in *Xylaria* is not really helpful to be found in NCBI or GenBank. Okane et al. (2008) had used the LSU as additional gene for making the phylogenetic tree among *Xylaria* species. The phylogenetic was enough clearly to distinguish the species among *Xylaria* species.

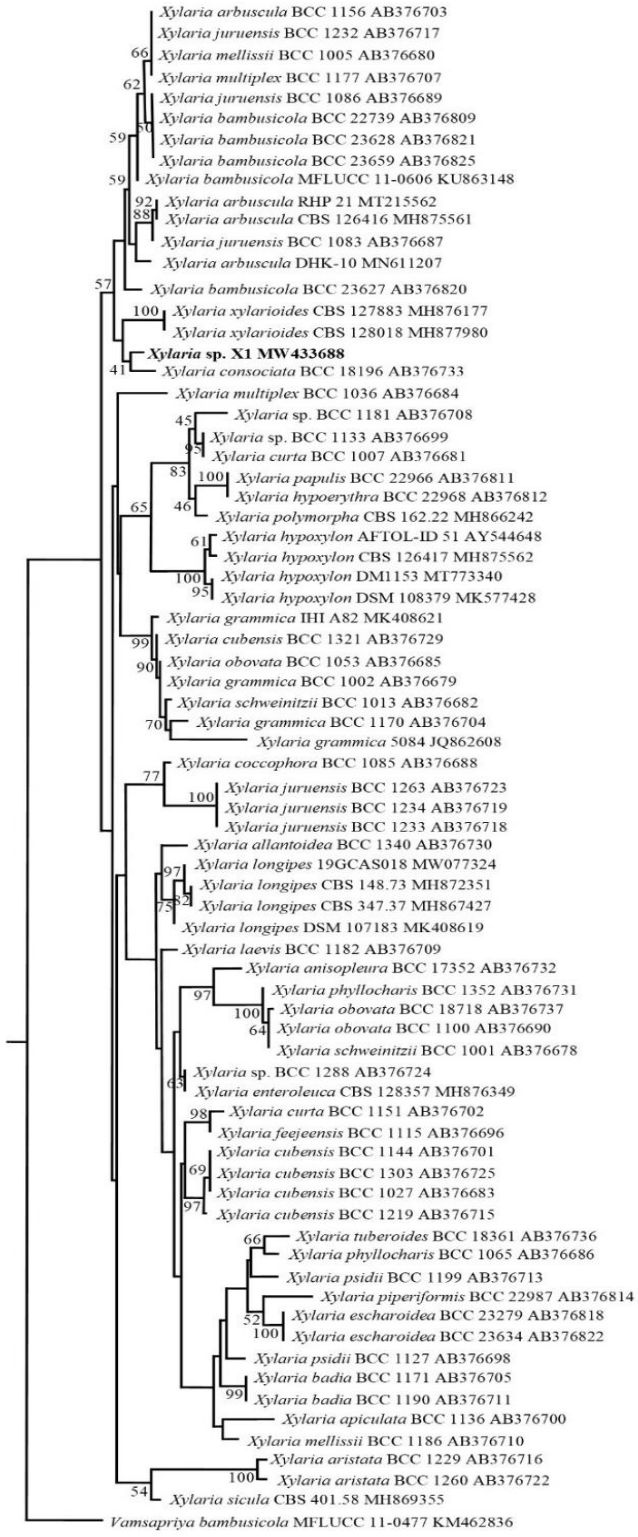

Figure 2. *Xylaria* BO 24426 phylogenetic tree based on the LR0R/LR5 region using RAxML. Bootstrap (BS)≥50 was shown on the branch. The *Xylaria* BO 24426 must be in bold.

Conclusion

Xylaria BO 24426 has been identified as *Xylaria* sp. with 41% BS value and closed to *X. consociata*. The stromata look like greyish candle snuff with whitish powder covering stromata. As a single gene identification using LSU region is not enough and strong to identify BO 24426 into a species among *Xylaria*.

Moreover, BO 24426 has potential as a new species, because the clade of them showed the branch length that separated with other species.

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