

## Study of *Lentinus squarrosulus* from West Java on The Basis of Molecular and Morphological Data

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### ABSTRACT

*Lentinus* is a unique genus within Polyporales, because of the lamellate basidiocarp. In Indonesia, *Lentinus* is commonly studied about their potential, rarely for their taxonomy. BO 24427 specimen was found in West Java, Indonesia, situated in the Landscape Arboretum of IPB University. The specimen was identified using molecular study and supported by some morphological data of the fresh fruiting bodies. Molecular identification used ITS 4 and 5 regions. Phylogenetic tree was constructed using MEGA Version X software. The morphology was based on macroscopic and microscopic observation. The result of molecular analyses showed that BO 24427 specimen was identified as *Lentinus squarrosulus* with 99% bootstrap value and classified into section Rigididi. The morphological data of the fresh fruiting body supported the molecular identification. The important morphological data that were classified into *Lentinus squarrosulus* were scabrous cap and size of basidiospores. This study was the first record to publish the finding of *Lentinus squarrosulus* in Indonesia.

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### Introduction

By naming Polyporales, it is an order which generally has pores characteristic (Hawksworth *et al.*, 1996). Nevertheless, this order is heterogeneous of basidiocarp types (Binder *et al.*, 2005). The basidiocarp types are bracket-shaped (*Fomitopsis* P. Karst.; *Trametes* Fr.), resupinate to effused-reflexed (*Ceriporiopsis* Domański; *Phlebia* Fr., *Antrodia* (Fr.) Donk), stipitate (*Polyporus* P. Micheli) and hymenophore configuration such as poroid (*Perenniporia* Murrill), lamellate (*Lentinus* Fr.), hydnyaceous

(*Steccherinum* Gray) and smooth (*Podoscypha* Pat.) (Binder *et al.*, 2013). Some of those genera have the characteristics that are opposite by naming Polyporales, i.e. *Lentinus* with lamellate basidiocarp. In the previous study, *Lentinus* has been placed in the agaric family as Tricholomataceae because of a lamellate hymenophore and white spore print (Miller 1973). Newly, Studies in Pegler (1975), Corner (1981), and Singer (1986) put *Lentinus* within Polyporales. It was supported by molecular data by Hibbett & Vilgalys (1993). Lamellate basidiocarp in

Polyporales has two genera, i.e. *Lentinus* and *Panus* (Hibbett *et al.*, 1993).

Hibbett *et al.*, (2007) and Nilsson *et al.*, (2014) suggested studying fungi using molecular analyses that are a very powerful tool to identify them until species. Schoch *et al.*, (2012) recommended the Internal Transcribed Spacer (ITS) area had been formally proposed as the main marker in the identification of fungi molecularly. It was agreed with Seelan *et al.*, (2015) that study about *Lentinus* using molecular study. The best single gene that could clade the species within *Lentinus* was ITS.

To date, *Lentinus* has 40 species (Kirk *et al.*, 2008), excluding the synonyms, on the basis of 146 species records in Index Fungorum (2020). Then, many reports mentioned the wide distribution in Asia, including Indonesia (Sulistiany & Sudirman, 2015), Thailand (Somchai, 2012), Malaysia (Bolhassan *et al.*, 2012), India (Manimohan *et al.*, 2004), and China (Nazura *et al.*, 2010). In India, *Lentinus* is becoming one of the potential mushrooms for edible mushrooms containing many vitamins and good nutrition (Gulati *et al.*, 2011), antimicrobial (Sudirman, 2010), and antioxidant (Omar *et al.*, 2011). One of the potential *Lentinus* is *L. squarrosulus* (Mont.) Singer (Omar *et al.*, 2011). Recently in this study, *L. squarrosulus* was found growing in West Java, Indonesia. Compared with many studies done on *Lentinus* in Indonesia, none has been done on its taxonomy.

## Materials and Methods

### Mushrooms Sampling Site

The sampling was conducted on 20 January 2020 and located in Arboretum Landscape of IPB University. The mushroom was collected, documented, and observed the morphological characters, such as size, color, shape, ornamentation, and spores. The

observation was conducted in the mycology laboratory of Biology Department, Mathematics and Natural Sciences, IPB University using Olympus stereo and binocular microscope cs22LED. The sample was preserved in FAA (Kottapalli *et al.*, 2016) and deposited into Herbarium Bogoriense Indonesia.

### Identification of Mushrooms

The species was identified using molecular analyses. The genomic DNA was extracted manually using CTAB method (Hermawan *et al.*, 2020<sup>a</sup>). Sterile part of the fruiting body was mashed up until being a paste, 500 µL CTAB-buffer (cetyl-trimethyl ammonium bromide) was added into fruiting body paste and incubated in 65 °C for 30 min. Then, 500 µL of chloroform isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 min. The supernatant was collected, 500 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 rpm for 10 min. The supernatant was collected, and 50 µL 2M NaOAc (Sodium Hypo-Acetate) and 500 µL absolute ethanol were added. This mixture was stored in -20 °C overnight, then centrifuged at 15,000 rpm for 30 min. The supernatant was removed. 300 µL of 70 % ethanol was added and centrifuged at 10,000 rpm for 5 min. The supernatant was removed and the DNA sample was in the pellet. The pellet was air-dried using a Speed Vacuum on 30 °C for 30 min. The pellet was resuspended in 50 µL of Tris-EDTA buffer and 10 µL 1 mg/mL of RNase. Then, it was incubated at 37 °C for 10 min. RNase was then deactivated by incubating the mixture at 70 °C for 10 min. DNA quality and quantity were verified using a nanodrop spectrophotometer.

After that, the DNA quality and quantity were verified using a nanodrop spectrophotometer. The amplification used Internal Transcribed Spacer (ITS) as

forward ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and reverse ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers. PCR amplification was performed in 30 µL total reaction containing 15 µL PCR mix from 2X Kappa Fast 2G, 1,5 µL of 10 pmol of each primer, and 3 µL 100 ng template DNA, and 9 µL ddH<sub>2</sub>O. Amplification used a Thermoline PCR. The PCR condition was 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. Then the final extension was set at 72 °C for 10 minutes. The amplicons were estimated on 1 % agarose gels and visualized by the Gel Doc™ XR system. PCR products

were sent to the 1st Base Malaysia for sequencing.

The Sequence was deposited in GenBank. This sequence, 28 reference sequences of updated *Lentinus* species (Seelan *et al.*, 2015), and *Polyporus arcularius* (outgroup) were used for phylogenetic tree reconstruction (Table 1). Sequences were aligned and built the phylogenetic tree using MEGA Ver. X software. The phylogenetic tree was built by bootstrap analyses with 1000 replicates. Maximum likelihood was chosen for phylogenetic tree construction with the best method based on MEGA selection. Phylogenetic tree was built by bootstrap analyses with 1000 replicates. Bootstrap (BS) ≥ 60 was shown on the branch.

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

Collection code (Voucher/Cultures)	Species	GenBank acc. no ITS
BO 24427	<i>Lentinus squarrosulus</i>	MT815466
DED07668	<i>Lentinus badius</i>	KP283480
PU00436	<i>Lentinus badius</i>	KP283481
JSKT5858	<i>Lentinus badius</i>	KP283479
JS0094	<i>Lentinus badius</i>	KP283478
TFB11754	<i>Lentinus bertieri</i>	GU207302
TFB11756	<i>Lentinus bertieri</i>	GU207301
DSH9243C	<i>Lentinus crinitus</i>	KP283495
KM141387	<i>Lentinus polychrous</i>	KP283487
AH00024	<i>Lentinus polychrous</i>	KP283485
JS00054	<i>Lentinus polychrous</i>	KP283486
SNP24989	<i>Lentinus sajor-caju</i>	KP283493
JS0056	<i>Lentinus sajor-caju</i>	KP283494
FRI62056	<i>Lentinus sajor-caju</i>	KP283492
TFB11164	<i>Lentinus scleropus</i>	GU207310
FRIM4180	<i>Lentinus squarrosulus</i>	KP283483
CUI6513	<i>Lentinus squarrosulus</i>	KP283482
BORHF0009	<i>Lentinus squarrosulus</i>	KP283484
NG9	<i>Lentinus striatulus</i>	KX580188
Tage Roland M0135	<i>Lentinus striatulus</i>	GU207311
TENN19955	<i>Lentinus suavissimus</i>	KP283504
ADD7	<i>Lentinus suavissimus</i>	KP283501
LE0791625	<i>Lentinus suavissimus</i>	KP283500
DSH2011 (AL57)	<i>Lentinus suavissimus</i>	KP283496
TFB6206	<i>Lentinus swartzii</i>	GU207276
CCCT 17.106	<i>Lentinus swartzii</i>	MN192922
DSH92D787	<i>Lentinus tigrinus</i>	KP283488
ATCC 9406	<i>Lentinus tigrinus</i>	GU207269
ATCC 28757	<i>Lentinus tigrinus</i>	GU207271
DSH92-132	<i>Polyporus arcularius</i>	KP283522

### Morphological Observation

The species identification was confirmed by morphological data. All characters of the fruiting body were observed and documented. The data were important such as size, shape and ornamentation of the fruiting body and basidiospores. The data will be compared with reference of species within *Lentinus* using a literature study. Describing fresh specimens used a description of the fruiting body by Brundrett *et al.* (1996).

### Results and Discussion

#### Specimen

The fruiting body of *Lentinus* was deposited into Herbarium Bogoriense with code BO 24427. It was collected by Rudy Hermawan on a rotten wood substrate. All of the phases of the fruiting body were collected and preserved in the FAA. The fruiting body clearly had a stem and a cap. The cap was lamellate type. The fruiting body was white milk color. They were gregarious in their environment.

*Lentinus* is classified into Polyporales. The different hymenophore type within Polyporales makes *Lentinus unique* and as the exception in Polyporales which has lamellate type of its cap. Within *Lentinus*, there are many species that have been mentioned in other publications. Despite Index Fungorum (2020) mentioned 146 species, only 11 species were confirmed that were available for the sequences in GenBank (Seelan *et al.*, 2015). They are *L. badius*, *L. bertieri*, *L. crinitus*, *L. polychrous*, *L. sajor-caju*, *L. scleropus*, *L. squarrosulus*, *L. striatulus*, *L. suavissimus*, *L. swartzii*, and *L. tigrinus*. Hibbet *et al.*, (1993) mentioned and described in their study that there are two ways *Lentinus* gills can develop, i.e. descending growth and

radiate growth. The hymenophores within *Lentinus* can be divided into two types (Seelan *et al.*, 2015), i.e. lamellae and subporoid lamellae.

#### Phylogenetic tree

*Lentinus* BO 24427 specimen was identified as *Lentinus squarrosulus* by 99 % Bootstrap (BS) value (Fig 1). The length of ITS 4 and 5 regions of this sequence was amplified for 634 base pairs of nitrogen bases. *Lentinus squarrosulus* was first reported to grow in Indonesia. This species had been found and reported in Borneo, Malaysia, Thailand, Papua New Guinea, and South Africa (Seelan *et al.*, 2015). The *Lentinus squarrosulus* was within the section *Rigidi*.

The phylogeny (Figure 1) showed that the BO 24427 specimen was *L. squarrosulus* and classified within section *Rigidi*. According to Seelan *et al.*, (2015) and Pegler (1983), there were three species inside section *Rigidi*, i.e. *L. polychrous*, *L. squarrosulus*, and *L. sajor-caju*. Three of them are different from each other, such as only *L. sajor-caju* has annulus, *L. squarrosulus* has a slender stem, *L. polychrous* has a thick stem (Seelan *et al.*, 2015; Corner, 1981; Pegler, 1983). Some studies also reported the comparison of *L. squarrosulus* and *L. tigrinus* that make them for similar characters of the fruiting body (Dulay *et al.*, 2012; Bolhassan *et al.*, 2012; Pegler, 1983). Between *L. tigrinus* and *L. squarrosulus* are confused about making a difference. A similar surface type of them is the scabrous cap surface. But, the phylogenetic of them is distinguished by separated clades. Based on phylogeny (figure 1), between *L. squarrosulus* and *L. tigrinus* is certainly placed as a sister clade.

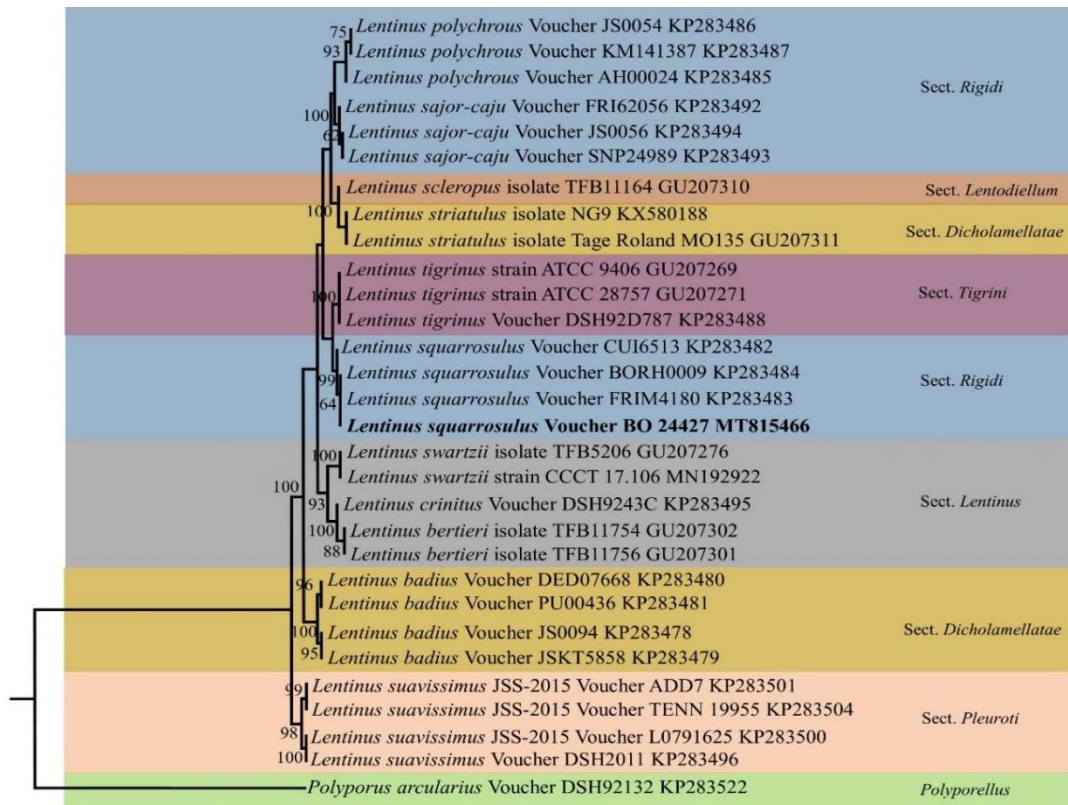


Figure 1. *Lentinus* phylogenetic tree based on the ITS4/ITS5 region using MEGA X with Maximum Likelihood using Kimura 2 Parameter + Gamma + Invariant Site. Bootstrap (BS)  $\geq 60$  was shown on the branch

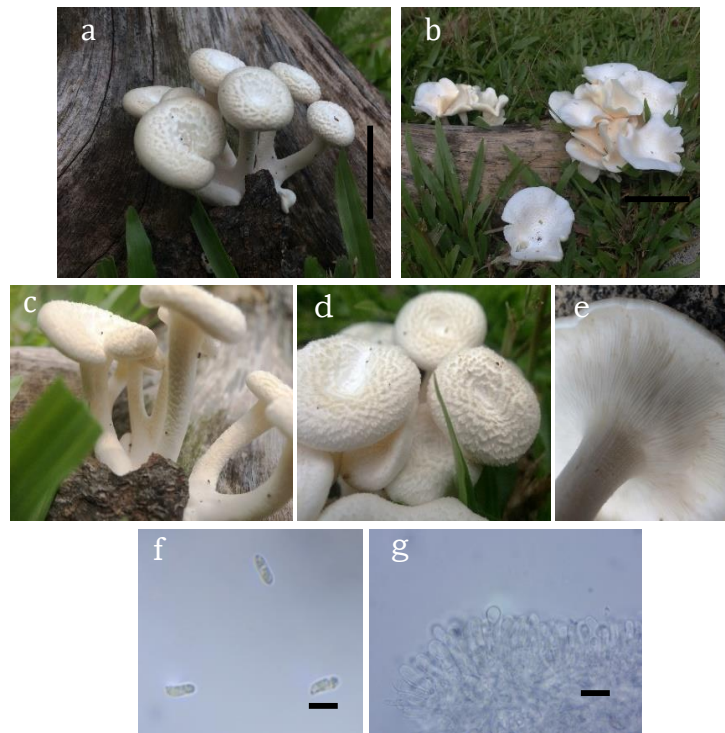


Figure 2. *Lentinus squarrosulus* BO 24427. (a) young fruiting body; (b) mature fruiting body; (c) the stem; (d) the cap; (e) the lamellae; (f) basidiospores; (g) the hymenial hyphae. Scale bars: (a-b) 5 cm; (f) 5  $\mu\text{m}$ ; (g) 10  $\mu\text{m}$

### Morphology of *L. squarrosulus* BO 24427

*Lentinus squarrosulus* BO 24427 was Basidiomycotina and within the Polyporales. The morphology of the young fruiting body (Fig 1a) has a circular cap incurved or enrolled inside. Then, the mature fruiting body (Fig 1b) has a wider cap than the young. Mushroom cap shapes are central depression (young fruiting body) or infundibuliform (mature fruiting body). Mushroom caps are ornamented and the entire cap surface and margin. The stem is erected from bases (Fig 1c). The young fruiting body has a pattern of universal veil remnant (scabrous) on the cap surface (Fig 1d). But, in mature fruit bodies, it may be absent or remain as scales on the cap surface. Mushroom gills are adnexed (young fruiting body) or decurrent (mature fruiting body) of attachment to the stem, crowded of gill spacing (Fig 1e), and smooth (young fruiting body) or wavy (mature fruiting body) of gill margins. Mushroom stems are equal in stem shape, bulbous of stem base, free of the membranous annulus, and fragmented bands on the stem surface. The cap is 1,5-3,7 cm (young fruiting body) and 5,3-6,9 cm (mature fruiting body) in diam. The stem is 2,4-6,1 cm in height and 0,7-1,3 in diam. The basidiospores are cylindrical, smooth, and  $6,6-8,9 \times 2,5-3,3 \mu\text{m}$  (Fig 1f). The hymenial hyphae is woven and well-development (Fig 1g).

*Lentinus squarrosulus* has scabrous on the cap surface. This morphology is the main character to identify the *Lentinus* fruiting body to *L. squarrosulus*. Sometimes the morphology makes a difference to identify them specifically. Therefore, molecular identification is more needed to make them sure of species. The ITS is strong enough and valuable to identify within *Lentinus*. Our phylogeny is a little similar to Seelan *et*

*al.*, (2015) which used the multigene for the phylogeny.

The morphology among young and mature fruiting bodies has a little different type of the cap. The young have exactly the circle shape of the cap and are enrolled in cap margin, whereas the mature has a widely circular shape than the young fruiting body and are not enrolled in cap margin. The mature has a decurrent of cap and wavy of the margin. Despite the difference in cap shape, the scabrous cap appears between the young and mature fruiting bodies. Hermawan *et al.*, (2020<sup>b</sup>) also found this pattern growth among the young and mature fruiting bodies. In Hermawan *et al.*, (2020<sup>b</sup>), the *Chlorophyllum* mushroom had a different type of cap among young and mature fruiting bodies. This normally happens when the development of the fruiting body is still growing at the time. Basidiospores of *L. squarrosulus* BO 24427 are cylindrical and free of ornamentation. It agrees with *Lentinus' description* in Pegler & Young (1983). This shape was almost finely cylindrical, sometimes with the small appendage or tube extension on the basis of basidiospore, namely sterigma (Hawksworth *et al.*, 1996). Basidia were showing a well-developed sub hymenial layer and finely loose woven trama.

*Lentinus squarrosulus* are popular as an edible mushroom in central Africa (Watling, 1993), likewise *L. sajor-caju* and *L. strigosus* (Chin, 1981). *Lentinus* has known as white root fungi (Sulistiany & Sudirman, 2015). Therefore, *Lentinus* can be saprobic fungi at all time phases. *Lentinus squarrosulus* BO 24427 had been found on rotten wood. This was the first record for publishing *L. squarrosulus* in Indonesia. *Lentinus* is not easy to culture into a medium. But some researchers had been successful to culture and domesticate them. De leon *et al.*, (2013) had been successful in domesticating *L. squarrosulus* in

Philippine. Current research, Omar *et al.*, (2011), studied the potential of *L. squarrosulus*. The water extract of the mycelium is successful as an antioxidant ingredient in some functional food and nutraceuticals.

### Conclusion

*Lentinus* BO 24427 specimen was identified as *L. squarrosulus* using ITS region with 99% bootstrap value on the phylogenetic tree. The *Lentinus squarrosulus* was within the section *Rigidi*. The morphology of *L. squarrosulus* BO 24427 central depression (young fruiting body) or infundibuliform (mature fruiting body), ornamented as a scabrous cap, and free of the annulus on the stem. The basidiospores are cylindrical, smooth, and  $6,6-8,9 \times 2,5-3,3 \mu\text{m}$ . This study was the first record of finding *L. squarrosulus* in Indonesia.

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