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New records of the diversity of *Scleroderma* spp. from Papua, Indonesia

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Abstract. *Sufaati S, Suharno, Agustini V, Suwannasai N. 2023. New records of the diversity of* Scleroderma *spp. from Papua, Indonesia. Biodiversitas 24: 4269-4276.* Papuan forest is one of the highest diversity tropical rainforests in the world. However, in some areas it is undergoing in process of land degradation. Reforestation using local plant inoculated with native mycorrhiza may have better results since they will be more adaptable. Mycorrhiza helps in plant nutrient uptake and ameliorates heavy metals in mining areas. Genus *Scleroderma* is a group of ectomycorrhiza that can be found at early stage of succession. However, to date there has been little study on its taxonomy. This study was conducted to construct the database on the diversity of *Scleroderma* spp. in Papua, Indonesia. Samples were collected from several areas in the provinces of Papua and West Papua from 2003 to 2022. Morphological characters and its habitat were observed. The results show, that 23 isolates had been documented. Molecular identification using ITS sequences was used to confirm the *Scleroderma* to the species level. The results found at least 3 species of *Scleroderma*, namely: *Scleroderma suthepense*, *S. xanthochroum*, and *S. sinnamariense* were identified based on morphological and molecular analysis, while *S. citrinum* was identified morphologically. This finding provides new data on the distribution of Sclerodermataceae in Papua. The results of this preliminary study are important for selecting native *Scleroderma* spp. for inoculation programs in degraded land.

Keywords: DNA, ectomycorrhiza, fungi, ITS, phylogenetic tree

INTRODUCTION

Forests in Papua, Indonesia, have very high biodiversity, including fungi, which associated with plants known as mycorrhizae (Agustini et al. 2009; Suharno et al. 2022). In several areas in Papua, forest ecosystems are experiencing a process of land degradation due to mining (Suharno et al. 2017), shifting cultivation, fires, and the conversion of forest into plantations (oil palm) (Kadir et al. 2020). This condition is a concern of the government to maintain and protect forests as an important part of human life. Restoration of degraded land using local plants inoculated with native mycorrhiza can give better results, because they are more adaptable to environmental changes. Meanwhile, there is still little research on mycorrhizae in Papua, some of which are related to arbuscular mycorrhizae (Suharno et al. 2022), orchid mycorrhizae (Agustini et al. 2009), and early collections of ectomycorrhizae (Nugroho et al. 2010).

Ectomycorrhiza (ECM) is a symbiotic association of fungi with the feeder roots of higher plants in which both the partners are mutually benefited and indeed the association appears to be significant for the existence of both the partners. The ecological role of ectomycorrhiza is to help plants absorb nutrients and water and increase plant resistance to disease (Zuo et al. 2022; Kebert et al. 2022). ECM presents an alternative to strengthen the sustainability of plantation forests and reduce dependence on chemical fertilizers from non-renewable sources (Wu et al. 2023). Generally, ectomycorrhizal fungi are used as biological fertilizers for food crops, plantations, forestry and even rehabilitation of degraded lands (Kałucka and Jagodziński 2016; Weidlich et al. 2020; Sánchez-Ledesma et al. 2022). The ectomycorrhizal fungi include three divisions namely: Ascomycota, Basidiomycota and Zygomycota, there are >250 genera and an estimated 20,000 species (Janowski and Leski 2023). Amongst them is the *Scleroderma* group which is distributed worldwide in temperate and tropical regions, and forms ectomycorrhizae with various woody plants including members of the families Caesalpiniaceae, Dipterocarpaceae, Fagaceae, Myrtaceae, Phyllanthaceae, and Pinaceae (Hibbett et al. 2007; Mrak et al. 2017; Leonardi et al. 2018). In Index Fungorum data (online), it is found about 199 of *Scleroderma* listed (https://www.indexfungorum.org/names/Names.asp, accessed on 4 August 2023). *Scleroderma* species have also been used to increase the growth of tree seedlings, both in nurseries and in the field (Chen 2006; Bechem and Alexander 2012; Sánchez-Ledesma et al. 2022). One type of ectomycorrhizae that is often used as a biological fertilizer in pine, eucalyptus and Dipterocarpaceae nurseries is *Scleroderma* spp.

Scleroderma spp. is very wide in almost all parts of the world (Mrak et al. 2017; Clasen et al. 2018). Taxonomically, the genus *Scleroderma* is included in the family Sclerodermataceae. Taxonomic studies of *Scleroderma* in temperate climates have been carried out since 1801 (Phosri et al. 2009), and the research developed rapidly until now in America, Europe, and Asia (Watling 2006; Nouhra et al. 2012; Rusevska et al. 2014). In Indonesia, research on ectomycorrhizae especially the *Scleroderma* spp. includes inventory, inoculum production, and growth response compatibility testing (Nugroho et al.

2010; Sufaati 2014; Helbert et al. 2019; Putra 2020). However, its taxonomic studies have not been widely elucidated yet. So far, most of the identification of ectomycorrhizae has been carried out based on the morphological characteristics of the fruit bodies. Since there are several morphological characteristics of ectomycorrhizae that are similar between one species and another, some difficulties occurred in the identification process. With the advancement of molecular identification techniques that are developing very rapidly, modern taxonomy does not only rely on morphological characteristics, but is also supported by molecular data.

Based on previous research, *Scleroderma* spp. was found in Papua (Nugroho et al. 2010). However, until now its taxonomic status has not been confirmed yet precisely because the identification was only based on morphological characters. Thus, it is necessary to correctly identify the *Scleroderma* from Papua using molecular techniques such as those with the internally transcribed spacer (ITS) region rDNA sequence of *Scleroderma*. Based on the development of molecular techniques in fungi identification, it is necessary to use this method to determine the species with certainty. This technique will provide more precise information than just looking at the morphological characters. Thus, the doubts and uncertainties in determining the type of *Scleroderma* can be minimized. In present study, several *Scleroderma* species were collected and identified as new records in Papua.

MATERIALS AND METHODS

Samples collection

The samples were collected for isolation of *Scleroderma* spp. from the provinces of Papua and West Papua. The morphological observation was carried out in Mycology Laboratory, Department of Biology, Cenderawasih University, Jayapura, Papua, Indonesia. The research began with the collection of ectomycorrhizae from various areas which were conducted from 2003 to 2022. The location of survey and collection included some districts in Papua i.e., Jayapura City, Jayapura District, Mamberamo Raya District, and Manokwari District in West Papua (Figure 1).

This research was conducted through several steps. Firstly, habitat was recorded on elevation above sea level (asl), geographic position coordinates and associated plants. Then, *Scleroderma* was collected by taking the entire part of the fruiting bodies in various sizes that grow on the soil surface or in the litter of forest floors. Lastly, the samples were characterized and identified both morphologically and molecularly.

Morphological characterization of *Scleroderma*

The morphological character such as the shape, size, color, present of stalk, surface and peridium as well as other characteristics of the fruiting body was observed (Sims et al. 1995; Watling 2006). About 15 spores from each type of fruit body were taken, then viewed under a microscope at a magnification of 200-1000x. The characteristics of the spores i.e., size, shape and spores ornamentations were observed, and the spores were photographed using Scanning Electron Microscope (SEM) (Hitachi TM3000) at the Zoology Laboratory, Indonesian Institute of Sciences. The identification is done based on various reference identification key (Nouhra et al. 2012; Sims et al. 1995; Watling 2006).

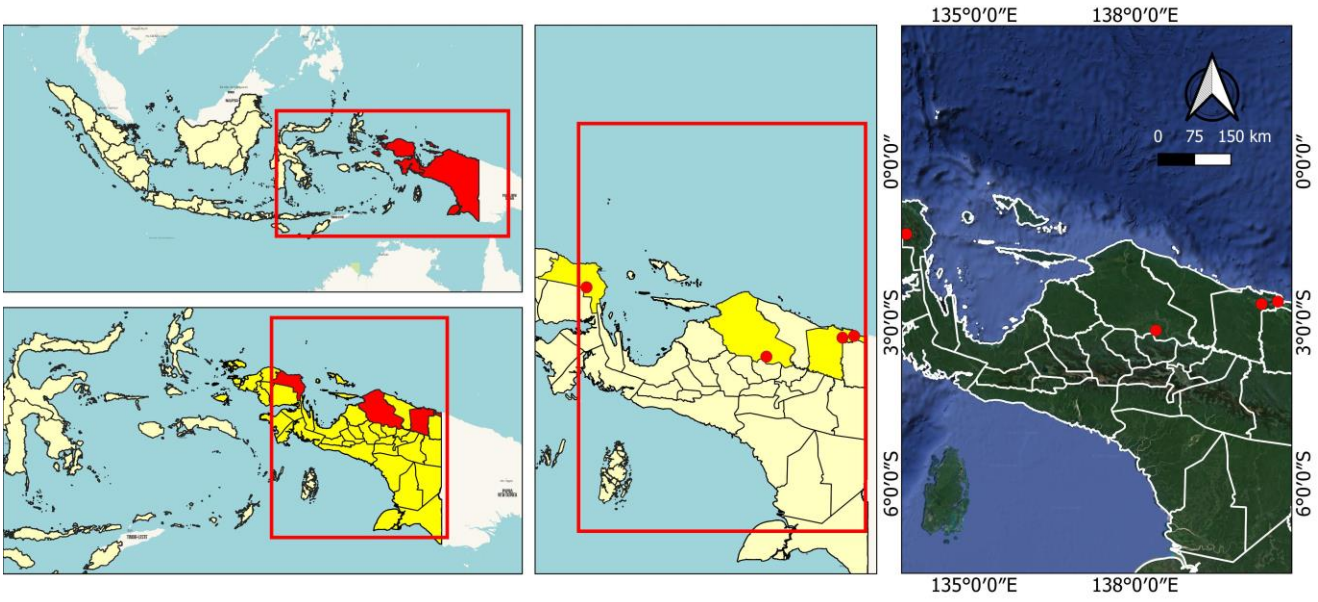

Figure 1. Location of sampling areas in Papua and West Papua Provinces, Indonesia

Molecular identification

Molecular identification was done by isolating the DNA sample. DNA was taken from the spores contained in the fruiting body of the fungi. Isolation and amplification with polymerase chain reaction (PCR) were carried out at the Biotechnology Laboratory, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand. The spores were crushed with liquid nitrogen and isolated using the cetyltrimethylammonium bromide (CTAB) method (Kumla et al. 2013). The primers used in this analysis were ITS1 and ITS4B (Gardes and Bruns 1993). Aliquots of 12.5 µL diluted DNA were combined with equal volumes of PCR mix containing buffer, nucleotide triphosphate, and Taq polymerase. The final concentrations of the components were: 200 µM each of dATP, dCTP, dGTP and dTTP, 50-mM KCl, 10-mM Tris HCl (pH 8.3), 0.1-mg/mL gelatin, 0.5 units of Taq DNA polymerase per 25-µL reaction (Boehringer Mannheim) and 1 µM each of the forward and reverse primers used. The temperature cycle of each initial denaturation step was 94°C for 85 sec followed by 35 cycles of amplification, denaturation, annealing and extension. The temperatures and times for these steps in the first 13 cycles are 95ºC for 35 sec, 55°C for 55 sec, and 72°C for 45 sec. Cycles 14-26 and 27-35 use the same parameters except that the extension step is extended to 2 and 3 min, in two sets of cycles, respectively. After the 35 cycles were completed, the samples were incubated for an additional 10 min at 72°C.

The ITS gene identification was performed by the BLASTn on GenBank databases. The sequencing results were identified for similarities to the GenBank database via BLASTn. The phylogenetic tree was constructed using the MEGA version 7.0 program (Kumar et al. 2016).

RESULTS AND DISCUSSION

Diversity of fungi

Samples collections were carried out at different times from 2003 to 2022. The results showed that a number of samples of the *Scleroderma* spp. fungus were found in Papua and West Papua Province, Indonesia. *Scleroderma* samples were found in several locations in various areas including Jayapura City (11 locations), Jayapura District (5 locations), Mamberamo Raya District (1 location), and Manokwari District (3 locations). There were 23 collections of fruiting bodies of *Scleroderma* spp. stored in the Mycology Laboratory, Department of Biology, Cenderawasih University, Jayapura, Papua.

Those *Scleroderma* spp. grow under several host plants (rhizosphere) such as *Gnetum gnemon*, *Gliricidia sepium*, *Calophyllum inophyllum*, *Salaca edulis*, *Cocos nucifera*, and *Pinus merkusii* (Figures 1, 2, and 3). However, some host plants were remains unknown (Table 1). The *Gnetum gnemon* (Familia: Gnetaceae), *Pinus merkusii* (Familia: Pinaceae) and dipterocarps are grouped as ectomycorrhizal host plants (Sims et al. 1995; Brearley 2011; Bechem and Alexander 2012). While other plants such as *Gliricidia sepium*, *Calophyllum inophyllum*, *Salaca edulis*, and *Cocos nucifera* were uncommon as their host plants. Therefore, this case needs further investigation.

Based on morphological characters, the *Scleroderma* found during the surveys consists of several species. Among them are *Scleroderma citrinum*, *Scleroderma* spp., and *S. sinnamariense*. The results of the observations showed that the samples from all collections had similarities. Some samples have a limited distribution such as *S. citrinum*, which was only found in Jayapura District. On the other hand, the samples identified as *S. sinnamariense* were found in several districts.

Figure 2. A-B. *Scleroderma* Art1 (S.McArt1) found under the *Pinus merkusii* at the Mc.Arthur monument, Sentani, Jayapura, Indonesia. C-D. *Scleroderma* associated with *Salaca edulis* in Warmare, Manokwari, E-F. *Scleroderma* dar (S.dar) associated with *Gnetum gnemon* found in Waena, Jayapura, and G-H. *Scleroderma* associated with *Nephelium lappaceum* in Warmare, Manokwari

Table 1. *Scleroderma* collection from Papua and West Papua Provinces, Indonesia

Code of sample	Taxon name	Site collection	Position (coordinates)	Host plant (rhizosphere)	Date collection
S.Yong1	*Scleroderma citrinum*	Yongsu de soyo, Jayapura	S: 02º.25'99,4", E: 140º.29'14,7"	unknown host	Jun 2003
S.Horti	*Scleroderma* sp.1	Hortikultura Waena, Kota Jayapura	S: 02º.35'23,7", E: 140º.38'26,6"	*Gnetum gnemon*	Jul 2003
S.Yong2	*Scleroderma* sp.2	Yongsu de soyo, Jayapura	S: 02º.25'99,4", E: 140º.29'14,7"	*Gnetum gnemon*	Jun 2003
S.Kuc1	*Scleroderma sinnamariense*	Uncen Forest, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	*Calophyllum inophyllum*?	May 2004
S.Kuc2	*Scleroderma sinnamariense*	Uncen Forest, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	unknown host	5 Mar 2004
S.Kuc3	*Scleroderma sinnamariense*	Uncen Forest, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	unknown host	5 Apr 2004
S.Kem1	*Scleroderma* sp.3	Kemiri River, Jayapura	S: 02º.32'43,4", E: 140º.29'16,0"	unkown host	25 Sep 2005
S.Kem2	*Scleroderma* sp.4	Kemiri River, Jayapura	S: 02º.32'43,4", E: 140º.29'16,0"	unknown host	25 Sep 2005
S.War1	*Scleroderma* sp.5	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Gliricidia sepium*	28 Dec 2007
S.War2	*Scleroderma* sp.5	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Gnetum gnemon*	1 Jan 2007 8 May 2020
S.Kuc3	*Scleroderma sinnamariense*	Uncen Forest, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	unknown host	13 May 2008
S.War3	*Scleroderma* sp.6	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Nephelium lappaceum*	4 Jul 2008 13 Jun 2022
S.War4	*Scleroderma* sp.7	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Cocos nucifera*	4 Jul 2008
S.Kuc4	*Scleroderma* Ar1	Uncen Forest, Kota Jayapura	S: 02º.34'56,3", E: 140º.39'01,8"	*Gnetum gnemon*	14 Sep 2008
S.Ent1	*Scleroderma* Ar2	Kali Entrop, Kota Jayapura	S: 02º.34'56,3", E: 140º.39'01,8"	unknown host	14 Sep 2008
S.Ent2	*Scleroderma* Ar3	Kali Entrop, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	unknown host	14 Sep 2008
S.Kuc5	*Scleroderma* Ar4	Uncen Forest, Kota Jayapura	S: 02º.33'47,9", E: 140º.39'33,0"	unknown host	14 Sep 2008
S.Kw	*Scleroderma* Ar5	Kamp Wolker River, Kota Jayapura	S: 02º.34'02,7", E: 140º.38'45,2"	unknown host	11 Oct 2008
S.War5	*Scleroderma* Mkw1	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Salaca edulis*	8 Jun 2009 17 May 2020 13 Jun 2022
S.War6	*Scleroderma* Mkw2	Warmare, Manokwari	S: 00º.58'19,1", E: 133º.56'36,3"	*Gnetum gnemon*	8 Jun 2009 7 May 2021 13 Jun 2022
S.Mc.Art1	*Scleroderma* Mc.Art1	Mc.Arthur Sentani, Jayapura	S: 02º.33'48,1", E: 140º.32'36,9"	*Pinus merkusii*	24 Apr 2016
S.Mc.Dar1	*Scleroderma* Dar	Maralex, Waena, Kota Jayapura	S: 02º.35'36,9", E: 140º.38'21,7"	*Pinus merkusii*	26 Jun 2016
S.Mamra	*Scleroderma sinnamariense*	Kali Dorman, Mamberamo Raya	S: 03º.15'17,0", E: 138º.35'12,5"	*Gnetum gnemon*	28 Nov 2018

Table 2. The blast results of *Scleroderma* spp. from Papua and West Papua Provinces, Indonesia

No. of collection	Sample code	Taxon name	ITS size (bp)	BLAST results	Accession no.	% identity
11	S.kuc3	*Scleroderma sinnamariense*	586	*Scleroderma sinnamariense*	AB908177	97
18	S.kw	*Scleroderma* Ar5	643	*Scleroderma xanthochroum* isolate AWW254	EU718126	98
21	S. Mc.Art1	*Scleroderma* Mc.Art	636	*Scleroderma suthepense* SDBR-CMU55-SC2	NR132871	99
22	S.Dar 1	*Scleroderma* Dar	643	*Scleroderma xanthochroum* isolate AWW254	EU718126	97

The fruiting bodies of *Scleroderma* spp. from Papua are vary in terms of shape, size, and color of the gleba (Figure 2). Sample No. 21 (Table 1) identified molecularly as *S. suthepense* has a spore diameter of between 7-10 µm (Figure 3). According to Kumla (2013), the average diameter of *S. suthepense* spores is around 8-13 µm. Samples No. 18 and 22 (Table 1) showed 8-12 µm spore sizes. Molecularly, this sample was identified as *S. xanthochroum* and has a high degree of similarity with the accession number EU718126. Based on a literature search, only a few information were found related to this species, one of them was related to the morphological characteristics (Sims et al. 2004). Another species, *S. sinnamariense* (Sample No. 11), has a spore's spine with a size >1 µm. According to Sims et al. (1995) spore's spine should be <1 µm.

Beside the morphological characters, the size of spores was also measured and the spore ornamentation of several samples was observed under the scanning electron microscope (Table 2; Figure 3). Spore size and spore ornamentation are important character for the identification of *Scleroderma* (Sims et al. 1995). The spores morphotypes and diameter of the samples indicated

that S.Kuc2, S.Kuc3, S.Kuc4, S.Ent are probably *Scleroderma sinnamariense*. *Scleroderma sinnamariense* has thin peridium when dry (<1 µm). The peridium colored lemon-yellow to chrome-yellow with dark brown scales on surfaces, frequently with second thinner, inner orange peridium. Spores reticulate or sub reticulate are small (5-8 µm) with spines less than 1 µm long. The spores were distinctly ornamented (Sims et al. 1995).

Basidiome S.Yong1 has scales and cracks peridium, dark brown/black gleba and no stipe. According to Sims et al. (1995), *Scleroderma citrinum* has a thick peridium (1-2 µm) when dry, covered with irregular scales and cracks that are arranged in a rosette near the top of the basidiome. The basidiome colors are often pale or dull. Stipe-like base are lacking, and spores are >8 µm with <1 µm spines. Spores often have a well-defined reticulum.

Molecular identification

Based on the results of molecular identification from four samples with different morphological characteristics, three species of *Scleroderma* were identified. These species are *Scleroderma suthepense*, *S. xanthochroum*, and *S. sinnamariense* with the percentage of identity was between 97-99 (Table 2).

***Scleroderma* phylogenetic tree**

The results of the phylogenetic tree arrangement show that the samples from the *Scleroderma* collection belong to two different clades. The first clade shows one sample (No. 21) identified as *Scleroderma suthepense* and has a kinship with *S. citrinum*, *S. meridionale*, *S. septentrionale*, *S. laeve*, *S. michiganense*, and *S. bovista*. The second clade shows the *S. sinnamariense*, *S. areolatum*, *S. xanthochroum*, *S. polyrhizum*, and *S. bermudense* groups. Sample No.11 was identified and has a high degree of similarity with *S. sinnamariense*. According to (Kumla et al. 2013), *S. suthepense* is one of the newest species found in Thailand. Previously, according to Chandrasikul et al. (2011) there were about 10 types of *Scleroderma* in Thailand, namely: *Scleroderma areolatum*, *S. bovista*, *S. cepa*, *S. citrinum*, *S. dictyosporum*, *S. flavidum*, *S. lycoperdoides*, *S. polyrhizum*, *S. sinnamariense*, and *S. verrucosum*. *Scleroderma suthepense* is also found in China (Zhang et al. 2020). Furthermore, it is known that only 1 species of *Scleroderma* (*S. xanthochroum*) has been found in Malaysia (Baseia et al. 2016). The discovery of this type in Papua is new information, thus adding information about the distribution of *S. xanthochroum* in the Asian region. These samples were found in two different locations in Jayapura City, Papua, Indonesia.

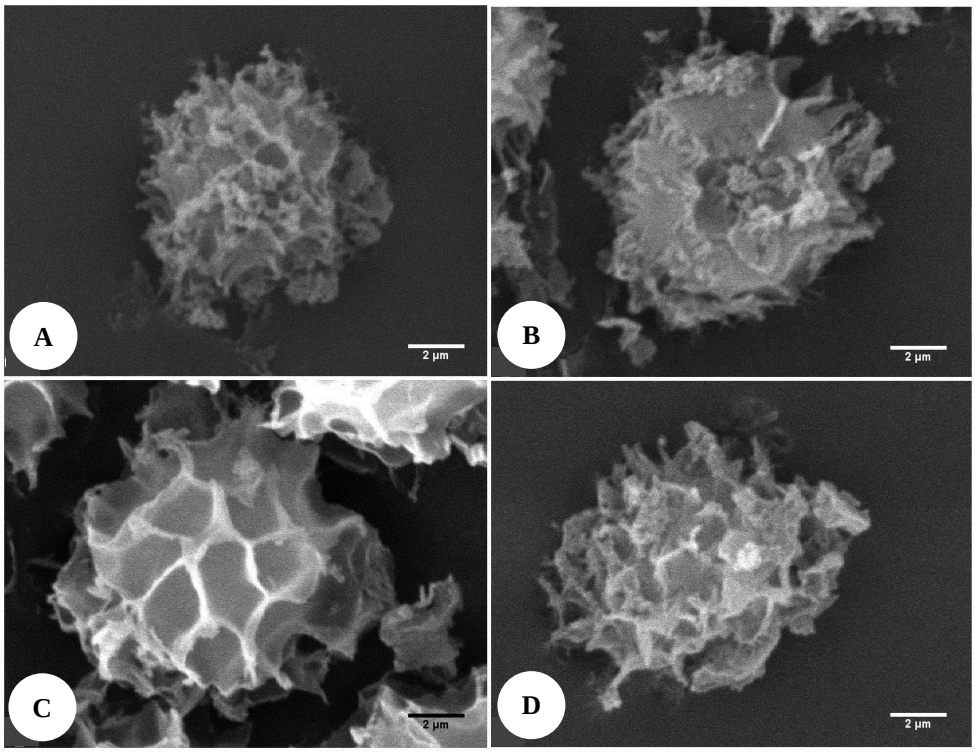

Figure 3. Spores of *Scleroderma* spp. from Papua photographed by SEM. A. *Scleroderma sinnamariense* (S. Kuc.3), B. *Scleroderma* Ar5 (S. kw), C. *Scleroderma* Mc.Art1 (S.Mc.Art1), and D. *Scleroderma* Dar (S.Dar1).

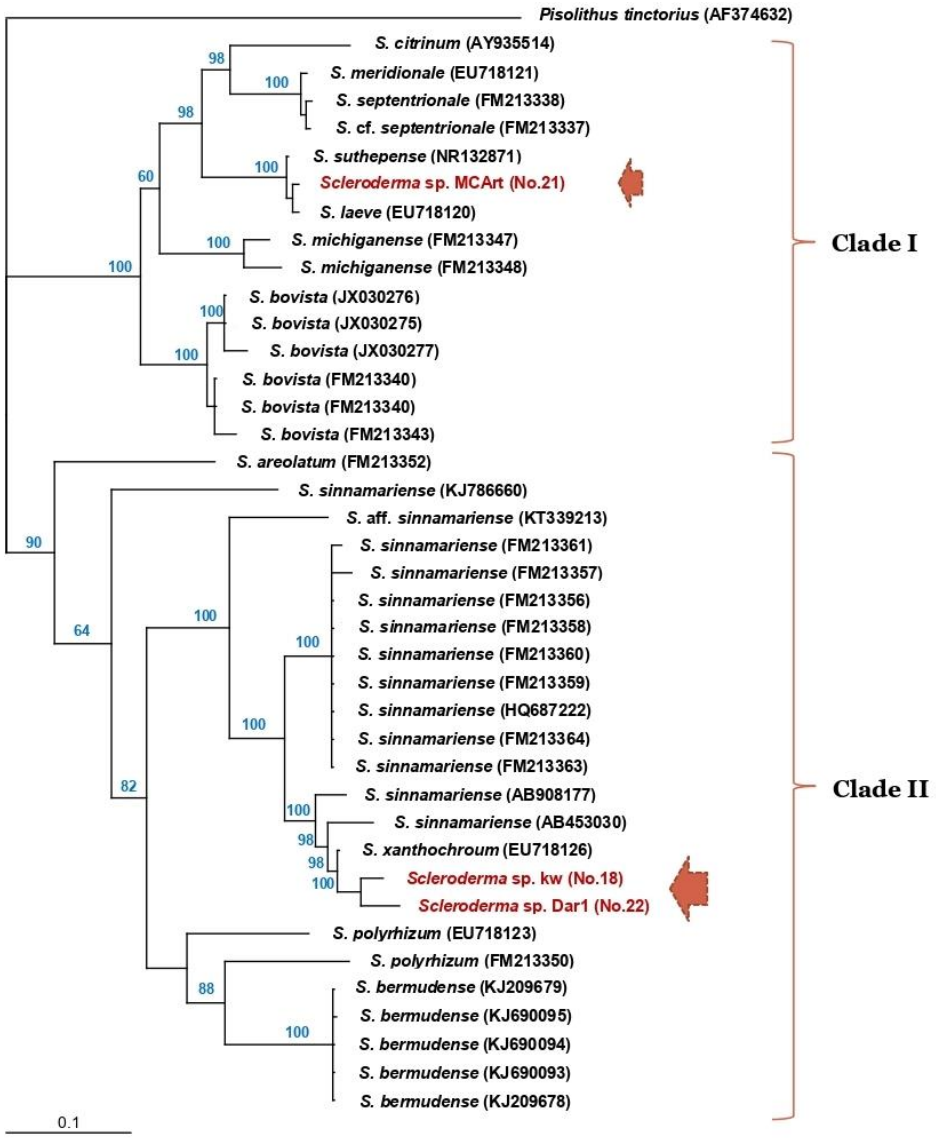

Figure 4. Molecular phylogenetic analysis with Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2344.4252) is shown. The percentage of trees where related taxa are clustered together is shown next to the branches. The initial tree(s) for the heuristic search was obtained automatically by applying the Neighbor-Join and BioNJ algorithms to the pairwise distance matrices estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with branch length measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences. All positions containing gaps and missing data were removed. There were a total of 469 positions in the final dataset. Evolutionary analyses were performed on MEGA7 (Kumar et al. 2016)

The species of *S. sinnamariense* has been identified molecularly from samples collected in Jayapura City. This species has a wide distribution in Papua province, because these samples are morphologically similar to several samples collected from other areas such as Jayapura district, Mamberamo Raya, and Manokwari District. *Scleroderma sinnamariense* is a type of *Scleroderma* that has a wide distribution in the world (Baseia et al. 2016; Guzmán et al. 2013). On the other hand, *S. xanthochroum* has a limited distribution in the Asian region (Baseia et al. 2016). The rest of the samples have not been identified yet, because it is difficult to distinguish only based on the morphology of the spores. Therefore, further identification using molecular technique has to be done for confirmation to the species level (Jacobson et al. 1993; Junghuns et al. 1998). The ITS region of rDNA sequence is the important sequence for identification and phylogenetic analysis of fungi (Schoch et al. 2014; White et al. 1990), such as rusts and mycorrhizal fungi (Gardes and Bruns 1993), endophytic fungi and orchid mycorrhizal fungi (Agustini et al. 2016; Sufaati et al. 2016; Tian et al. 2022), as well as ectomycorrhizal fungi (Rusevska et al. 2014; Henrion et al. 1992; Phosri et al. 2009; Montagner et al. 2015; Helbert et

al. 2019; Zhang et al. 2020; Raut et al. 2020; Almeida et al. 2021).

In conclusion, there are 23 collections of fruiting bodies of *Scleroderma* spp. from Papua which were associated with several host plants such as *Gnetum gnemon*, *Gliricidia sepium*, *Calophyllum inophyllum*, and *Pinus merkusii*. However, there are some unknown host plants. Characterization and identification of several samples are difficult to be done based solely on morphological data. Among them, four species were identified based on DNA sequences as *Scleroderma sinnamariense*, *S. suthepense*, and *S. xanthochroum*. Further work such as molecular identification using ITS ribosomal DNA sequences has to be carried out to complement the existing data. With molecular techniques, the possibility of discovering new species in Papua is very possible.

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