Second Record of *Phaeoclavulina aeruginea* (P. Zhang) Worldwide

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Abstract- *Phaeoclavulina* was previously placed in the genus *Ramaria* subgenus *Echinoramaria. Phaeoclavulina* has a broad distribution from temperate to tropical regions worldwide. One of its species, *P. aeruginea,* is acknowledged to be an interesting coral fungus with blue to blue-green apical branches as the distinctive character. To date, there has been no information on the occurrence of *P. aeruginea* in Indonesia. In 2023, the Indonesian mushroom hunter community obtained some unusual specimens of coral fungi that resembling *P. cyanocephala* and *P. aeruginea* during a mushroom hunting in West Kalimantan. The goal of this study was to ensure the taxonomical position of our coral fungi specimens based on morphological evidence. The fresh specimens were subjected to morphological and molecular investigation. The basidiomata were described based on the macro- and micromorphological features. Molecular analysis using ITS 1/2 sequences was conducted to construct a phylogenetic tree. We confirmed our specimen as *Phaeoclavulina aeruginea* (P. Zhang) FIPIA-DEP44 was easily distinguished from other species of *Phaeoclavulina* by the greenish blue apical color on the branches of fruiting bodies, having 2 sterigmata on basidia, and produced spiky spores with nodes (echinulates) on its surface. However, *P. aeruginea* is difficult to distinguish morphologically from *P. cyanocephala*. The BLAST comparison of our specimen revealed a 96% similarity to *P. aeruginea* from China as the top hits. In addition, the phylogenetic tree (Maximum Likelihood) placed our specimens within the *P. aeruginea* clade (BS value 100%). The current study reveals the information on distribution of *P. aeruginea* in Indonesia after previously reported from China.

Index Terms- Coral fungus, taxonomy, new record, west Kalimantan

I. Introduction

The genus *Phaeoclavulina*, typified by *P. macrospora*, was originally described by Brinkmann (1897) to accommodate the coral-like mushroom (ramarioid) which produced two sterigmata of basidia and have spiny spores. However, Corner (1970) placed the species having these features in genus *Ramaria* subgenus *Echinoramaria*. In addition, Petersen (1981) rejected the *Phaeoclavulina* as a new genus. Recently, *Phaeoclavulina* was resurrected once more as a valid genus, based on morphological and molecular evidence (Giachini, 2004; Giachini *et al*., 2010; Giachini and Castellano, 2011). This genus contains ramarioid, unipileate, merismatoid, sometimes lignicolous basidiomata with spiny basidiospores (Giachini and Castellano, 2011). Macroscopically, *Phaeoclavulina* can be recognized by the funnel-shaped or fan-shaped pileus with ranges of branch surfaces color white, brown-green, blue to green, pale to sordid olivaceous, violet, brown-yellow or red cinnamon, gray, brick red, or pale to dark orange yellow (Giachini and Castellano, 2011). Microscopically, the basidiospores are acute spines (echinulate), verrucose, or reticulate (Giachini and Castellano, 2011).

Phaeoclavulina are clustered in the Gomphales, Gomphaceae (Giachini *et al*., 2010; Hosaka *et al.*, 2006; Hibbett *et al*., 2007). *Phaeoclavulina* has a cosmopolitan distribution around the globe, especially in the tropics and subtropics (González-Ávila *et al*., 2013). Giachini & Castellano (2011) acknowledged 41 species in *Phaeoclavulina.* To date, the Index fungorum (2025) recorded 65 taxa of *Phaeoclavulina.* Of those species, *P. aeruginea* and *P. cyanocephala* are considered unique due to the bluish green appearance on the tips of basidiomata. *P. aeruginea* was just recently reported as new species from China (Liu *et al*., 2022). Meanwhile, *P. cyanocephala* was previously reported on the ground in the forest of Africa, the Americas, and tropical Asia (Corner, 1950; Petersen, 1981). Recently, Maneevun *et al*. (2012) reported the distribution of this species from Thailand, and Hosaka (2018) from Japan. The GBIF (2025) records 235 occurrence of *P. cyanocephala* worldwide, no data for *P. aeruginea,* and no data for both species from Indonesia.

The species of *Phaeoclavulina* are less known and poorly studied in Indonesia. Due to the wide range of available niches, we believe that there are still many unrecorded and undescribed species of coral fungus in Indonesia. In addition, no previous information of *P. aeruginea* and *P. cyanocephala* in Indonesia are available. Hence, to increase the data of *Phaeoclavulina* in Indonesia, more field sampling should be done. In 2023, during the fungus foray carried by the Indonesian mushroom hunter community (KPJI) in West Kalimantan, some basidiomata of coral fungi resembling *P. cyanocephala* or *P. aeruginea* was obtained. The current study was aimed at ensuring the taxonomical identity of our specimens based on morphological evidence.

II. MATERIALS AND METHODS

The specimens were obtained in November 2023 at Twi Mentibar, Selakau, Sambas Regency, West Kalimantan, Indonesia. The specimens were photographed in situ and in the laboratory. The habit, habitat, location, bedrock and elevation and surrounding vegetation were noted. Subsequent investigation was done in laboratory of the Integrated Laboratory of Bioproducts (iLaB), National Research and Innovation Agency (BRIN), Bogor, Indonesia and Mycology laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Indonesia. Some fruiting bodies were deposited at the Herbarium Bandungense Indonesia with the collection number FIPIA-DEP44.

Macro- morphological characters were investigated using fresh basidiomata for fruiting bodies texture, size, shape, color, ornamentation on surface, stalk, and branches. Micro-morphological features observation was performed using a digital bright field microscope Olympus BX-63, Japan. The microscopic examination includes hyphal system, clamp connection, pileipelis, context (trama), basidia, basidiospores, and cystidia. The specimens were identified following Giachini (2004), Giachini and Castellano (2011), and Maneevun *et al*. (2012). The nomenclature and currently accepted name were following Index Fungorum (http://www.indexfungorum.org/) and Mycobank (https://www.mycobank.org/). The basidia and basidiospores were also subjected to scanning electron microscopy (SEM) investigation. Specimen were prepared following Goldstein et al. (1992). The lamellae were cut into small pieces (5x5 mm), pre-fixed in 2.5% glutaraldehyde of caccodylate buffer pH 8.4 at 27˚C for 2 days, pre-fixed in 2 % tannic acid for 6 hr and washed with 4 cacodylate buffer change. The samples were dehydrated in 50-100% ethanol series, infiltrated with t-butanol for 10 minutes twice, and then freeze dried. Freeze dried samples were mounted on an aluminum stub with double-sided carbon tape and coated with gold. The sample then observed using a JSM IT 200 SEM system (JEOL, Tokyo, Japan).

Fresh basidiomata were used to isolate DNA. DNA was extracted using a Qiagen Dneasy Plant Mini Kit according to the manufacturer's instructions. A Thermo Scientific Arktik Thermal Cycler was used for DNA amplification (ThermoFisher-Scientific). The amplification reaction was carried out using the pairs of primers ITS 1F/ITS 4B (Gardes and Bruns, 1993). The PCR amplification was performed in 50 µL total reaction containing 9 µL ddH2O, 1.5 µL of 10 pmol of each primer, 25 µL PCR mix from 2× PCR buffer for KOD FX Neo (Toyobo), and 2 µL 100 ng template DNA. The PCR condition was set as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 45 seconds, and extension at 72°C for 1.5 minute. The final extension was set at 72°C for 8 minutes. PCR product was analyzed using 1.5% agarose gel electrophoresis run with TAE buffer (40 mm Tris-acetate, pH 8.0 1 mM EDTA), stained with FloroSafe DNA stain and visualized by the Gel Doc EZ Gel Documentation System (Biorad). PCR products were sent to the 1st Base Malaysia for sequencing.

The sequences were assembled using ChromasPro software. The final aligned sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/) to obtain the accession number. The sequence was subjected to Basic Local Alignment Search Tool (BLAST) in NCBI to compare the homology with previous data. Sequencing results were analyzed with taxonomic matches based on the BLASTN result with the highest sequence similarity (http://blast.ncbi.nlm.gov/Blast.cgi). In addition, the sequences from selected BLAST results of this study (bold), thirty sequences from Gene Bank, were used to reconstruct the phylogenetic tree. *Ramaria admiratia and R. calvodistalis* were used as the outgroup Hughes *et al.* 2014 (Table 1). The phylogenic tree was constructed with the maximum likelihood (ML) method using MEGA X software (Kumar *et al*., 2018). The Bootstraps 1000 replication (BS) was used to test the strength of the internal branches of the phylogenetic trees. Other parameters used in the ML analysis were selected according to the default standard in MEGA X software. Bootstrap values of 60% or higher were shown.

III. RESULTS

Taxonomy

Phaeoclavulina aeruginea P. Zhang, in Liu, Yan, Deng, Qin & Zhang, Phytotaxa 561(1): 31 (2022) Figure (1-4)

Ten basidiomata were arbitrarily picked out for investigation. Basidiomata terrestrial, 5-14 cm tall, 2-8 cm wide, ramarioid, gregarious, and resembling the coral shape. Stipe single, strong and thick (1-2 cm), 2-5 cm tall, light brown to reddish brown, dark purple to dark brown when bruised, moderately tapering downward with white to cream mycelia and the rhizomorph can be found at the end of the base. Stipe extensively branches, 4-5 times, cylindrical, smaller near the terminal, brick to reddish brown. Dichotomously at apical, tips subacute and blue to greenish blue, white on top and blunt (upper side) of tips, up to 1.7 mm thick in the apex. The surface is slightly smooth, with yellowish brown powder. The inner flesh is firm, dry, whitish, yellowish, to yellowish brown. Hyphae of subhymenial trama parallel, slightly inflated, up to 8.5 µm broad, hyaline, thin-walled, clamp connection present. Basidia clavate, 40-50 µm × 4-6 µm, stout, persistent sterigmata up to 6 µm in length. Basidiospores ellipsoid with prominent apiculus, 8-10 µm × 4-5 µm, rust-colored, in mass, with echinulate spines, 1-2 µm long.

Habitat: Growing on soil near tree roots.

Specimens examined: Twi Mentibar, Selakau, Sambas Regency, West Kalimantan, Indonesia (1°05'57.5"N 109°00'57.8"E), on ground near *Barringtonia* sp, *Salacca* sp, *Daemonorops longipes*, *Dicranopteris linearis, Nephenthes* spp., 107 m a.s.l, relative humidity 99%, Temperature 26 °C, 2023, collected by Alvian, FIPIA-DEP44.

Molecular Analyses

The final aligned sequence was submitted to GenBank with the registration number ITSPQ824460. The BLAST results displayed that specimen FIPIA-DEP44 posed similarity to *Phaeoclavulina pseudozippelii* as the top hit. The ITS 1/2 gene-based phylogenetic tree (Figure 5) grouped specimen FIPIA-DEP44 together with *Phaeoclavulina aeruginea* specimen from China in the same clade with a high bootstrap value (100%). *Phaeoclavulina aeruginea* FIPIA-DEP44 clade is a sister clade to *P. pseudozippelii* from Thailand. In addition, *P. cyanocephala* (specimen from Guyana) is closely related to *P. aeruginea* FIPIA-DEP44 clade.

IV. DISCUSSION

The coral fungus genus *Phaeoclavulina* has proved to be monophyletic, grouped in the Gomphales, Gomphaceae (Giachini *et al*., 2010; Hosaka *et al.*, 2006; Hibbett *et al*., 2007), with broad color variation on their branch surfaces (Giachini and Castellano, 2011). *Phaeoclavulina* was reapproved as a valid genus, following the morphological and molecular data (Giachini et al. 2010, Giachini & Castellano 2011). In this study, we report on a unique species of *Phaeoclavulina* with blue green apical branches, namely *P. aeruginea*. This new species was recently reported from Hunan Province, China (Liu *et al.*, 2022), and our report is the first information outside China.

Morphologically, our specimens resembled the genera of ramarioid fungi in having the corals basidiomata. However, the presence of clamp connection and spiky spores' ornamentation of our specimens placed them in *Phaeoclavulina* (Beug, 2004; Nasim *et al.*, 2008; Giachini *et al*., 2010). Previously, *Phaeoclavulina* were added to the genus *Ramaria* (Corner, 1970; Petersen, 1981) due to the similar morphology of basidiomata. *Phaeoclavulina aeruginea* FIPIA-DEP44 can be delimited by the green to blue apex of dichotomous branches, equipped by 2 stout sterigmata, and having echinulate basidiospores. The branch tips of our specimen were slightly in color form those reported from type locality, which posed a dominant blue of the tips. The basidomata dimension of specimen FIPIA-DEP44 was similar to closely related species reported by Maneevun et al. (2012) from Thailand.

Corner (1970) considered that some species of *Ramaria* are not easy to distinguish from *Phaeoclavulina* due to their similar morphology. After series of discussions between Petersen (1981) and Corner (1983), the latter author noted that field characters from fresh material should be used for delimiting the species of *Phaeoclavulina.* In the current study, we obtained fresh specimens for morphological investigation. *P. aeruginea* has some closely morphological related species including *P. zippelii* which produces smaller basidiomat (brown color) and white tips (Giachini 2004). Conversely, our specimen has bluish green tips. In addition, *P. aeruginea* FIPIA-DEP44 has a bigger basidia and larger hypha compared to *P. zippelii* reported by Giachini (2004). In addition, *P. aeruginea* FIPIA-DEP44 is closely related to *P. cyanocephala* which is difficult to distinguish by morphology. Therefore, we combine morphological analysis with molecular data in the current study. Prior report suggests that the identification of macrofungi should be authenticity validated by molecular and morphological evidence (Ahmad et al. 2024).

The BLAST comparison of our sequence on GenBank showed that specimen FIPIA-DEP44 correlated to *P. aeruginea* from type locality (Hainan, China) as the top hit (96%). Aligned with BLAST, the phylogenetic tree of ITS 1/4 genes revealed that our specimen was highly similar to *P. aeruginea* MHHNU6887 and MHHNU8909 from China, with a 100% bootstrap support value. *Phaeoclavulina aeruginea* FIPIA-DEP44 is a sister clade to *P. pseudozippelii* from Thailand and *P. cyanocephala* from Guyana. The topology of our ITS tree is in line with those reported by Deng et al. (2024) regarding the diversity of *Phaeoclavulina* in China.

Even though *Phaeoclavulina* is considered as cosmopolitan, habitually constant, and common throughout tropical to subtropical areas (Corner 1983), this genus is not well-collected and has never been encountered in Indonesia, perhaps due to the lack of field sampling. Previously, Corner (1950) revealed that *R. cyanocephala* (*P. cyanocephala*) was the most frequent species of *Phaeoclavulina* in Malaysia (near to Indonesia), delimited by its reddish brown basidiomata and bluish green tips. The genus *Phaeoclavulina* are predominantly ectomycorrizal, such as *P. abietina* with *Pinus*, *Betula* and *Pseudotsuga* (Herrera et al. 2002, Norvell & Exeter 2004, Exeter et al. 2006), *P. flaccida* with *Pinus* and *Quercus* (Dong-Hun et al. 2003) and *P. cyanocephala* with *Abies* (Estrada-Torres 2007). In the current study, the rhizomorph can be found near the base of the stipe. However, the host of *P. aeruginea* FIPIA-DEP44 remains unclear with some plants in the surrounding, consist of *Barringtonia*, *Sallacca wallichiana*, *Arrange pinnata*, and *Flacourtia*.

Our report may be a key point in triggering the following research of the diversity and distribution of *Phaeoclavulina* in Indonesia. The information of new record of and or recent record of a hiatus macrofungi would contribute to the development of fungal taxonomy in Indonesia (Putra *et al*., 2022). In the current study, the record of *P. aeruginea* in Indonesia is fascinating since it is the first record of *Phaeoclavulina* in the country. This species is a unique and peculiar coral fungus, which should not be overlooked by mycologists and foragers due to its diminutive stature and coloration. We expect that the additional distribution information as well as the record of other species of *Phaeolcavulina* will be in flux when more field sampling has been done in Indonesia. Our herbarium is now deposited at the Herbarium Bandungense and can be used for further study of *Phaeoclavulina* in Indonesia.

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Table 1 Species, Collection Code, and ITS GenBank Accession Numbers used in this Study

No	Species	Voucher/Isolate	Accession No.	References	Country
1	*Phaeoclavulina aeruginea*	MHHNU6887	ON262784	Liu et al. 2022	China
2	*P. aeruginea*	MHHNU8909	ON262785	Liu et al. 2022	China
3	***P. aeruginea***	**FIPIA-DEP44**	**PQ824460**	**This Study**	**INDONESIA**
4	*P. abietina*	OSC 134649	JX310378	Unpublished	USA
5	*P. abietina*	OSC 140661	JX310379	Unpublished	USA
6	*P. alboapiculata*	AMB 18590	MT055971	Unpublished	Italy
7	*P. alboapiculata*	AMB 18613	MT055964	Unpublished	Italy
8	*P. bicolor*	MHHNU10702	PP809798	Deng et al. 2024	China
9	*P. bicolor*	MHHNU10703	PP809799	Deng et al. 2024	China
10	*P. cinnamomea*	MHHNU10376	ON262786	Liu et al. (2022)	China
11	*P. cyanocephala*	TH9064	KT339249	Unpublished	Guyana
12	*P. coniferarum*	AMB 18562	MT055942	Unpublished	Italy
13	*P. coniferarum*	AMB 18531	NR_176718	Unpublished	Italy
14	*P. carovinacea*	AMB 18533	NR_176719	Unpublished	Italy
15	*P. carovinacea*	AMB 18551	MT055933	Unpublished	Italy
16	*P. clavarioides*	PRM:945441	LR723647	Kříž et al. (2019)	Czech
17	*P. clavarioides*	PRM:945440	LR723646	Kříž et al. (2019)	Czech
18	*P. curta*	AMB 18641	MW115423	Unpublished	Italy
19	*P. curta*	AMB 18605	MT452501	Unpublished	Italy
20	*P. echinoflava*	HKAS 45984	PP809801	Deng et al. 2024	China
21	*P. echinoflava*	HKAS 45992	PP809800	Deng et al. 2024	China
22	*P. flaccida*	AMB n. 18209	MF288928	Unpublished	Italy
23	*P. jilinensis*	MHHNU9149	PP809802	Deng et al. 2024	China
24	*P. jilinensis*	MHHNU9164	PP809803	Deng et al. 2024	China
25	*P. minutispora*	AMB 18588	MT055969	Unpublished	Italy
26	*P. minutispora*	AMB 18586	MT055965	Unpublished	Italy
27	*P. macrospora*	AMB 18614	MT452510	Unpublished	Italy
28	*P. pseudozippelii*	BBH 43575	MG214661	Wannathes et al. (2018)	Thailand
29	*P. roellinii*	PRM:945445	LR723649	Kříž et al. (2019)	Czech
30	*Ramaria admiratia*	TENN 69114	NR_137862	Hughes et al. (2014)	USA
31	*R. calvodistalis*	TENN 69095	KJ416132	Hughes et al. (2014)	USA

Figure 1. Field photograph of *Phaeoclavulina aeruginea* FIPIA-DEP44. A: Young basidiomata. B: Blue to green pical branches of basidiomata. C: White rhizomorph on the stalk base. D: Side view of Basidiomata. Bars: B & D = 5 cm.

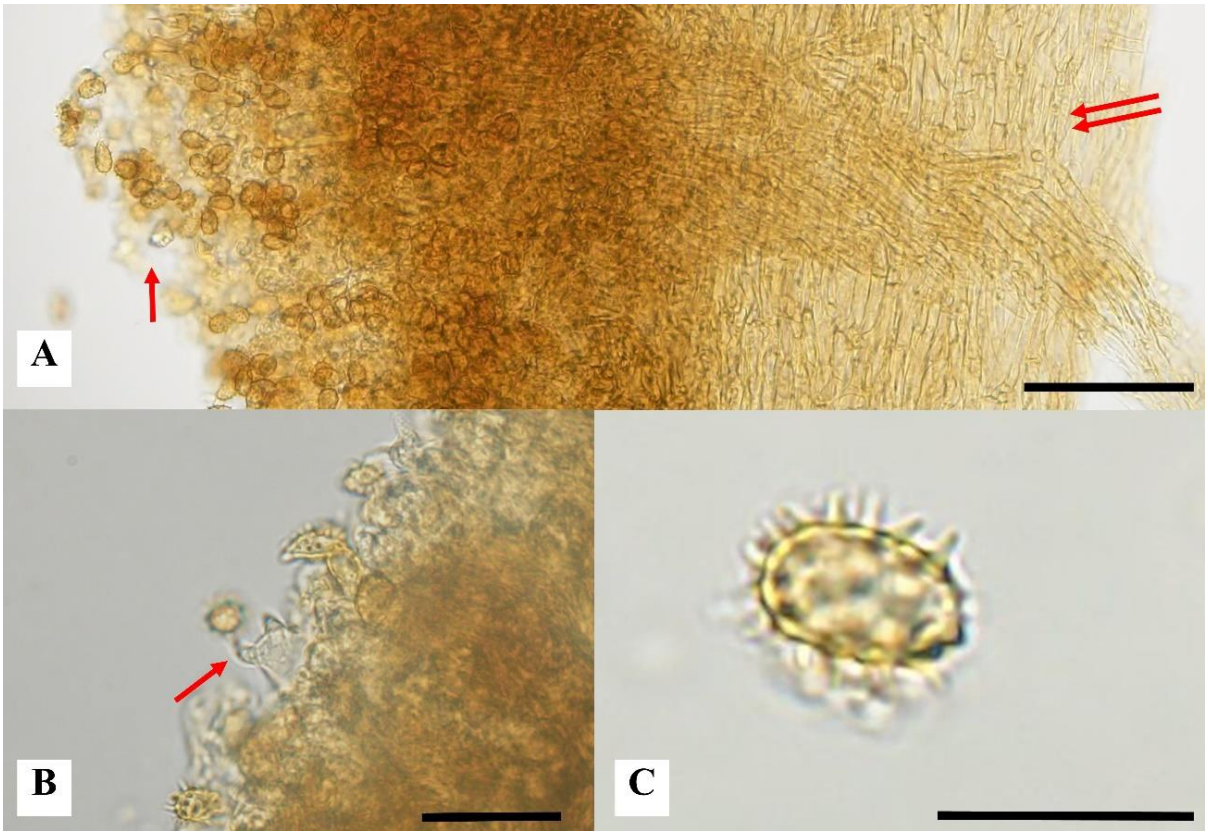

Figure 2. Microscopic characters of *Phaeoclavulina aeruginea* FIPIA-DEP44. A: Subhymenial trama (double arrow) and cluster of spores (arrow). B: Basidium with 2 sterigmata (arrow). C: echinulate basidiospore. Bars: A = 50 µm, B = 20 µm, C = 10 µm.

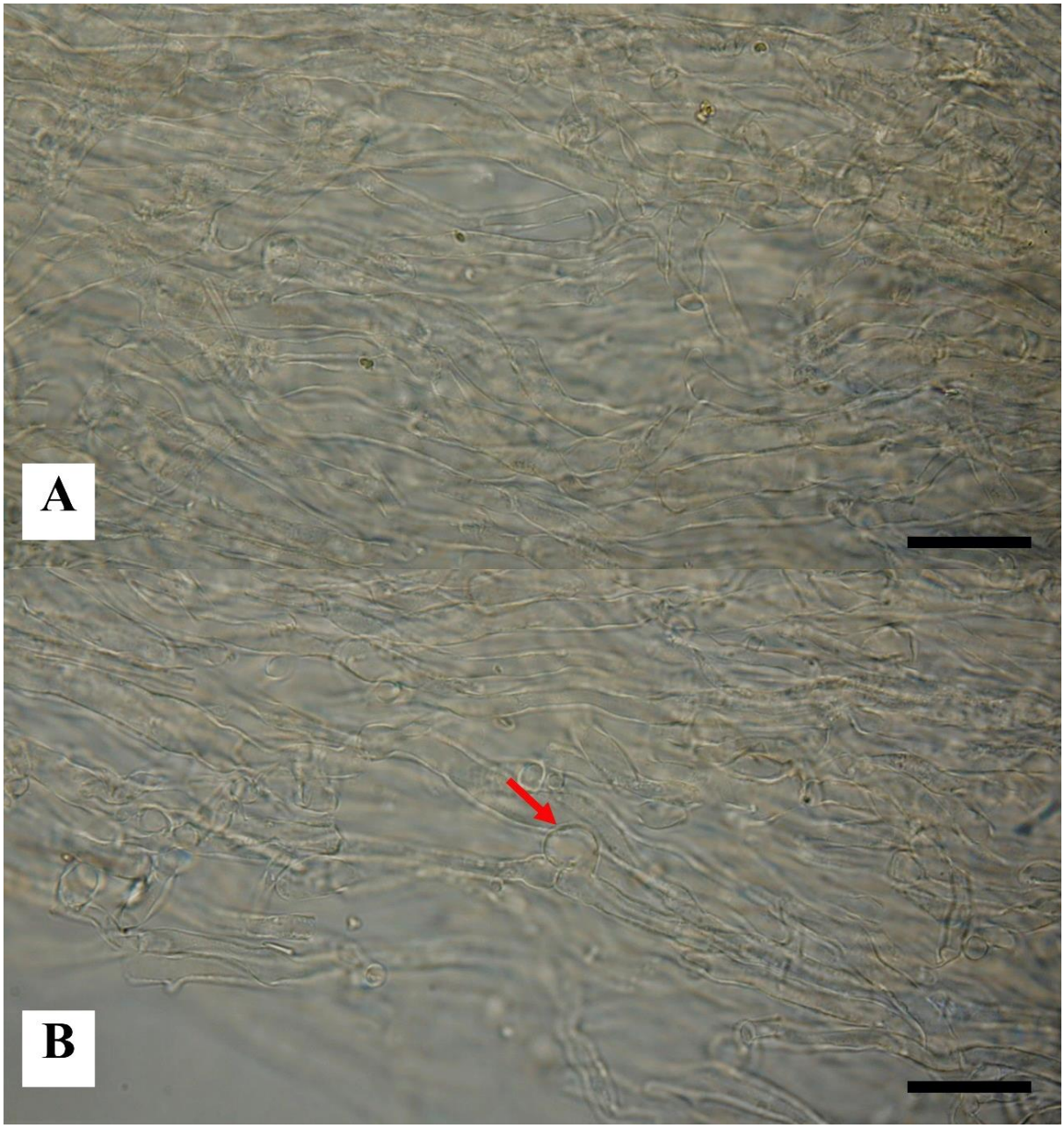

Figure 3. Microscopic characters of *Phaeoclavulina aeruginea* FIPIA-DEP44. A: Subhymenial trama with slightly inflated hyphae. B: Clamp connection. Bars: 20 μm.

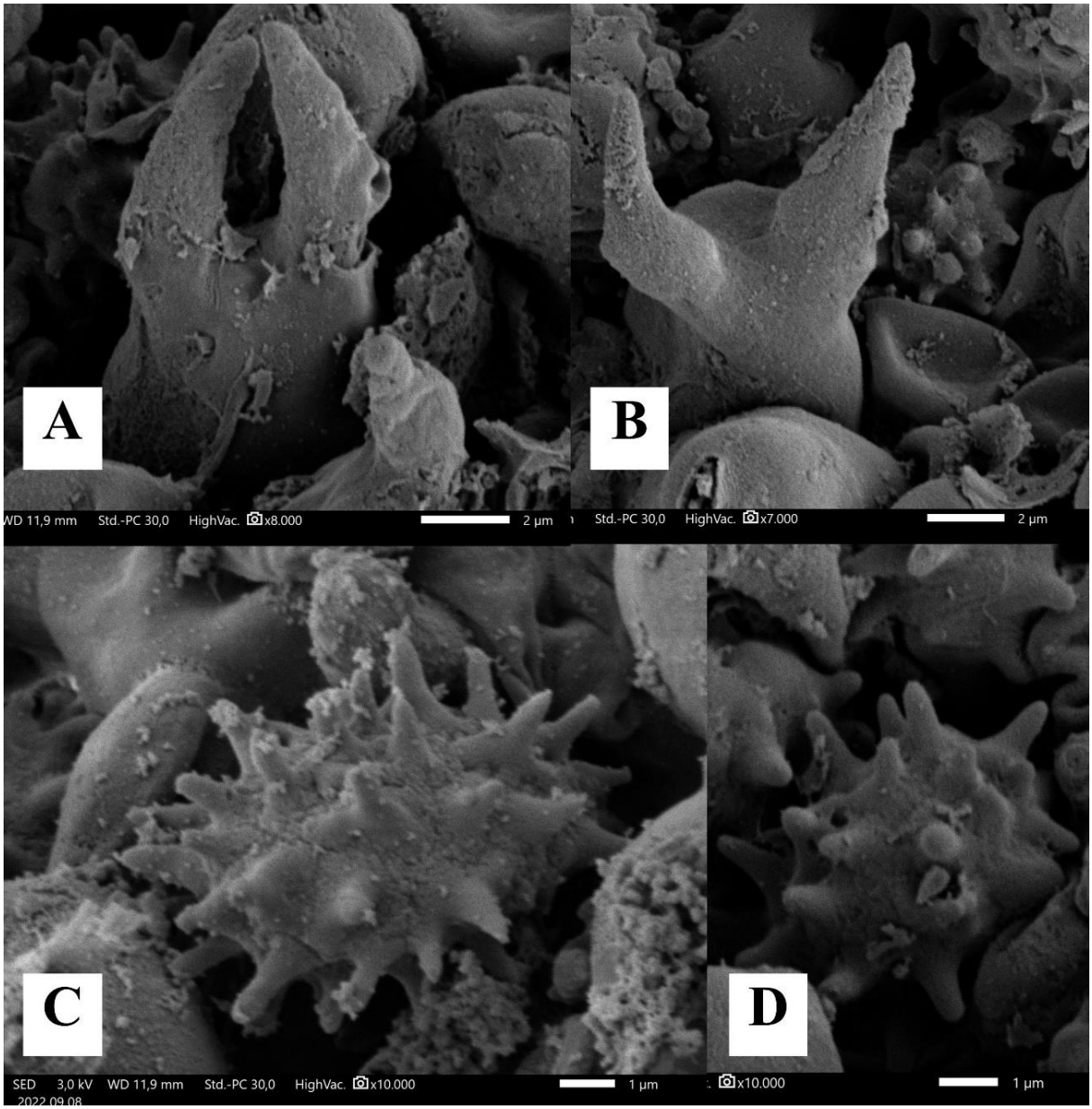

Figure 4. Scanning electron microscope image of *Phaeoclavulina aeruginea* FIPIA-DEP44. A-B: 2-spored persistent sterigmata. C-D: Basidiospores with echinulate spines.

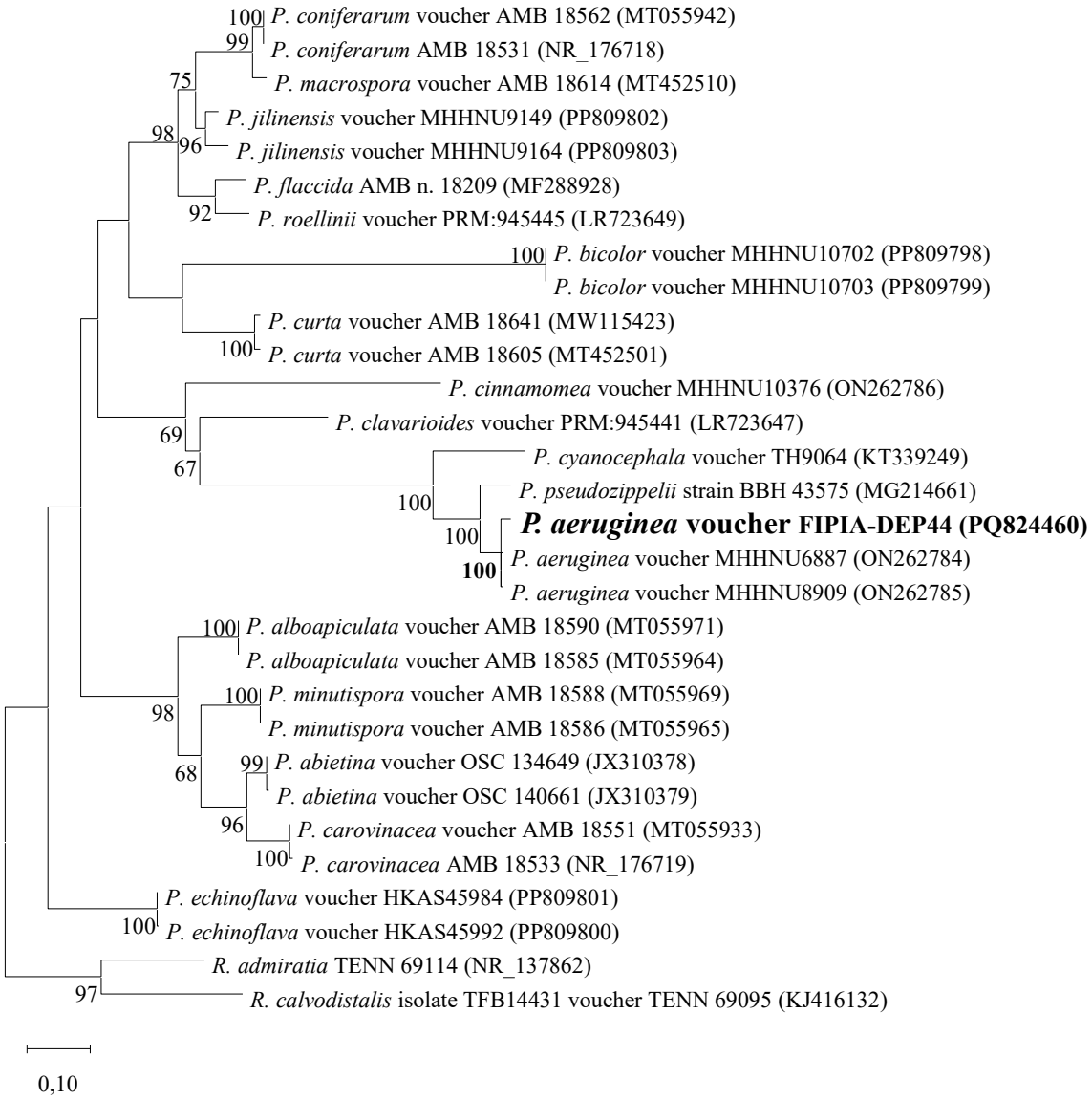

Figure 5. Phylogenetic tree for *Phaeoclavulina aeruginea* FIPIA-DEP44 and related species based on ITS rDNA sequences. Bootstrap values > 60% are indicated at the nodes (1000 replication). The scale bar refers to an evolutionary distance of 0.050