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Three New Anamorph of *Ceramothyrium* from Fallen Leaves in Vietnam

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Abstract

Three new anamorph of *Ceramothyrium aquaticum* sp. nov., *Ceramothyrium exiguum* sp. nov., and *Ceramothyrium phuquocense* sp. nov. are described and illustrated. These fungi were isolated from submerged decaying leaves collected from Phu Quoc National Park, Phu Quoc province, Viet Nam. The phylogeny based on ITS region and D1/D2 of the 28S rDNA gene showed that these fungi nested in the *Ceramothyrium*. Morphologically, *C. aquaticum*, *C. phuquocense* sp. nov. and *C. exiguum* sp. nov. are characterized; they were different from known anamorph species of *Ceramothyrium* by having one main axis and two lateral arms with 70 - 90, 33.5 - 72.5 and 70 - 130 µm long main axis, respectively. The table to compare *Ceramothyrium* anamorph is also given.

Keywords

Aquatic Fungi, Annamorph, Telemorph, Litter Fungi

1. Introduction

The genus *Ceramothyrium* was erected by Bat. & Maia based on type species *Ceramothyrium paiveae*; it was characterized by the lack of setae and by the hyaline, transversely pluriseptate ascospores [1]. No anamorph stage of this fungus was discovered until the study of [2]. In this study, they reported *Stanhughesia* as anamorph of the *Ceramothyrium*. There were 38 species of *Ceramothyrium* were reported; among them, three species *C. carniolicum*, C. *linnaeae* and *C. lycopodii* had *Stanhughesia* asexual stage [2]. More two anamorph of *Ceramothyrium* were reported under the older telemorph name: *C. melastoma* and *C. podocarpi*. While *C. melastoma* has *Trisulcosporium* morph, *C. podocarpi* has its own anamorph stage [3].

During an investigation of microfungi in Vietnam, three anamorh of *Ceramothyrium* were isolated from fallen leaves which were collected in Phu Quoc National Park of Vietnam. Conidia of these fungi produced when they were submerged in distill water after 2 - 3 days. They have tri-radiate spores, consisted chains of sausage-shaped cells, one main axis and (one)-two arms at the basal cell. Phylogeny of these fungi based on nrDNA large subunit (LSU D1/D2) and ITS region showed that these fungi belong to *Ceramothyrium*. They are differed from each other in the celled-numbers and the lengths of each cell.

The purposes of this study is to characterize these fungi not only morphologically but also phylogenetically and as well as to describe and illustrate two new anamorph of *Ceramothyrium*: *C. aquaticum* sp. nov., *C. phuquocense* sp. nov. and *C. exiguum* sp. nov.

2. Materials and Methods

2.1. Isolation and Morphological Identification

Fallen leaves were collected in Phu Quoc National Park of Viet Nam in 2011. The samples were kept in moist chamber for 3 - 10 days in a laboratory; leaf was cut in to pieces, 1 - 1.5 × 3 - 3.5 cm, and spread on surface of a low nutrient carbon agar medium (LCA) [4]. A single spore on the LCA was isolated by a Skerman's micromanipulator under a light microscope to obtain the pure culture. Cultures have been deposited to the Vietnam Type Culture Collection, Institute of Microbiology and Biotechnology, Vietnam National University, Ha Noi, Vietnam (VTCC) and to the National Institute of Technology and Evaluation, Biological Resources Center, Chiba, Japan (NBRC).

2.2. Morphological Study

The isolates were cultured at 25˚C on a potato carrot agar medium (PCA, extract from 20 g/L potato, extract from 20 g/L carrot, 15 g/L agar), LCA and potato dextrose agar (PDA, Nissui, Japan) for morphological observations. Observations were made under a differential interference contrast microscope (DIC: Axioplan 2, Zeiss, Jena, Germany) and a scanning electron microscope (JSM-6060: JEOL, Tokyo, Japan).

2.3. DNA Isolation and PCR Amplification

A Small pieces of a colony (3 × 3 mm) grown on malt extract agar (MEA) medium at 25˚C for 10 d were put into 2 mL Cryo tubes. DNA was extracted using the PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). PCR was performed by using KOD-Plus Kit (Toyobo, Osaka, Japan), following the manufacturer's protocol. The nrDNA large subunit region (LSU D1/D2) was amplified with primers NL1 and NL4 [5]. To amply the ITS region, the combination ITS1 and ITS4 [6] were used. Amplification of the DNA fragments was performed using the GeneAmp PCR System 9700 (Applied Biosystems) under the following thermal cycling programme: an initial

denaturation at 94˚C for 2 min, 35 cycles of denaturation at 94˚C for 15 s, annealing at 56˚C for 30 s, extension at 68˚C for 1 min 30 s, a final extension at 68˚C for 10 min, and a 16˚C soak. PCR products were checked by agarose gel electrophoresis, and were purified by using AMPureKit (Agencourt Biosciences, Beverly, MA, USA). Sequencing reactions were performed by using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) and the primers of the PCR. The newly generated sequence data were deposited in GenBank.

2.4. Phylogenetic Analysis

Sequences were assembled and edited manually using BioEdit ver. 7.09 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA). Sequences were aligned with Gen-Bank sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) by using Clustal X [7]. A phylogenetic tree was inferred with neighbor-joining (NJ) method [8] and the Knuc value [9] by using Clustal X. The topology of the tree was evaluated by the bootstrap resembling method [10] with 1000 replicates. The NJplot programme [11] was used for plotting the phylogenetic tree.

3. Results

3.1. Morphology

All of three fungal isolates were slow growth on LCA and PDA media, spores are easily produced when submerging in water 3 - 4 days. Conidiopphores absent. Conidiogenous cells intercalary in hyphae. Conidia in cultures are holoblastic, tri-radiate. The spore arises from a cell of the mycelium as a lateral bud. This bud is initially unicellular and constricted where it joints the parent hypha, it grows into the main axis of the spore. When the main axis reaches to three- or four-celled, one or two lateral arms are budding out from opposite side of basal cell of the main axis. At first arms are unicellular, and then it extend away from the main axis at near 120˚ until to become three- to six-celled as the main axis itself extends farther to become eight- to ten-celled. Eventually the main axis becomes constricted off from the parent cell and the spore become detached.

3.2. Phylogenetic Analysis

In a BLAST search using the blast from the National Center for Biotechnology Information (NCBI) [12], VTCCF-1206 (LC360294); VTCCF-1209 (LC360295); VTCCF-1210 (LC360296) had 96.68%; 95.23%; 97.03% similarity to the partial rRNA LSU gene sequences of *Ceramothyrium carniolicum* (FJ358232), an *Chaetothyriales* fungus, respectively. The LSU region of the sequences was used to obtain additional sequences from Gen-Bank, which was added to the alignment. The manually adjusted LSU alignment contained 34 sequences (including the one out group sequence). In NJ analysis, the phylogenetic hypothesis highly supports three monophyletic groups, Capnodiaceae (Verrucariaceae (Verruca-

riales) and Herpotrichiellaceae, Trichomeriaceae, Cyphellophoraceae, and Chaetothyriaceae (Chaetothyriales). The sequences of the three newly isolated fungi cluster within the Chaetothyriaceae clade with 75% boostrap value, the clad contains: *Ceramothyrium carniolicum*, *C. ficus*, *C. thailandicum*, *C. podocarpi*, *C. longivolcaniforme*, *Cyphellophora laciniata*, *C. eugeniae*, *C. sesilis* (**Figure 1**).

The aligned ITS region sequences of approximately 600 bases were obtained from isolates were aligned with the ITS region of the sequences obtained from Gen-Bank. The manually adjusted ITS alignment contained 21 sequences (including the one out group sequence). In NJ analysis, the phylogenetic hypothesis showed that VTCCF-1206 (LC360297); VTCCF-1209 (LC360298) and VTCCF-1210 (LC360299) cluster within the *Ceramothyrium carniolicum* clad, with bootstrap support of 98% (**Figure 2**).

3.3. Taxonomy

Ceramothyrium aquaticum VTCCF-1210 Yen L.T.H, Ando K. and Tsurumi Y. (**Figure 3(c)**, **Figure 4(b)**, **Figure 4(f)**).

MycoBank no.: MB824817

Colonies on LCA and PDA are dark brown, plane, 10 - 15 mm in diameter after 7 days at 25˚C. Mycelium was pigmented, 2 - 2.5 µm. Conidia are sporulated easily after 3 - 4 days aerated. Conidiopphores absent. Conidiogenous cells intercalary in hyphae. Conidia in culture are holoblastic, pale olivaceous, tri-radiate, they consist of a multi-septa main axis and two arms, which are much constricted at the middle cell and the septa. Main axis usually extends six- to eight-celled, usually 70 - 90 µm long (some time reaches to 100 µm) × 2.3 - 2.7 µm wide. From the basal cell of the main axis, two arms arise. The arms are shorter than the main axis, (3) - 5 - 6 septa, (30) - 50 - 70 µm × 2.5 - 2.7 µm.

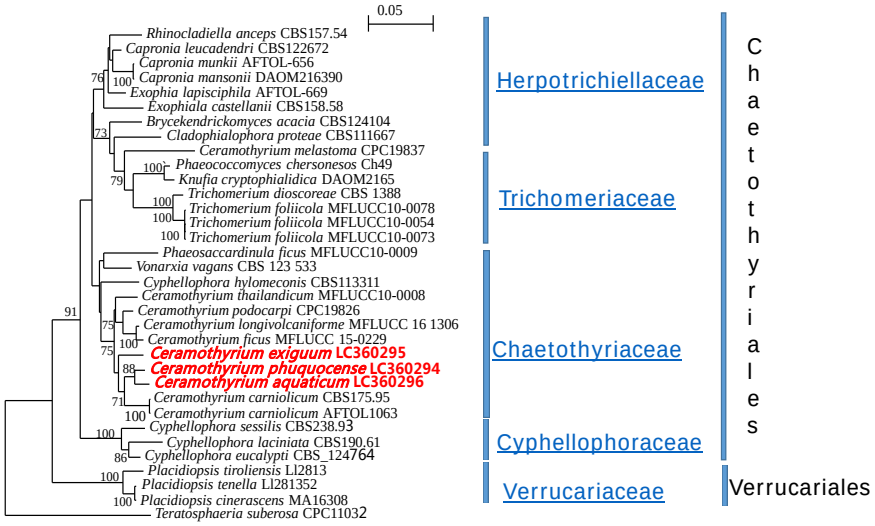

Figure 1. Phylogeny of *Stanhughesia* spp. and their relative species base on 28S D1D2 rDNA sequence. MP bootstrap value ≥ 70%.

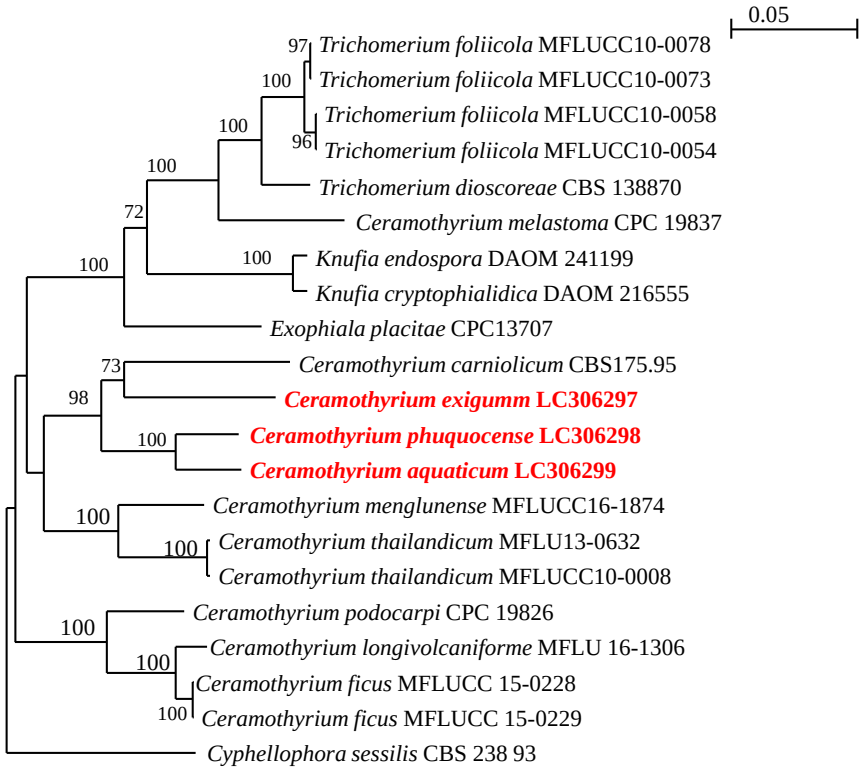

Figure 2. Phylogeny of *Stanhughesia* spp. and their relative species base on ITS rDNA sequence. MP bootstrap value ≥ 75%.

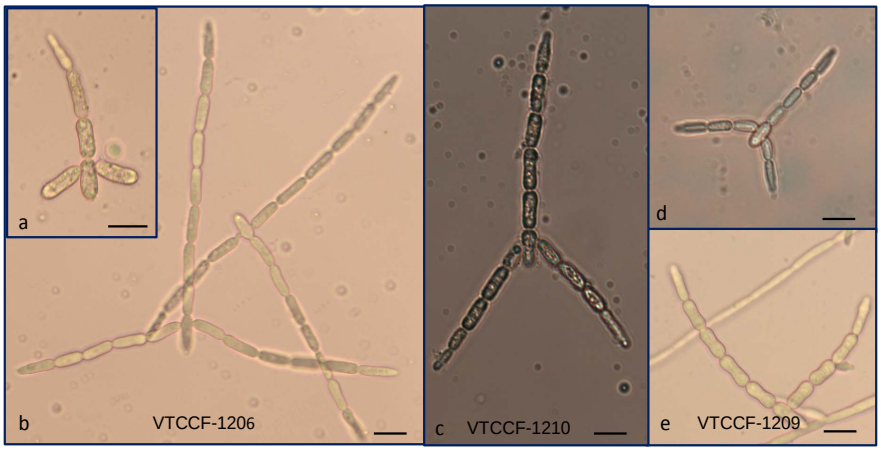

Figure 3. Morphology of *Ceramothyrium* under microscopic: (a) Young conidium of *C. phuquocense*; (b) mature conidium of *C. phuquocense*; (c) mature conidium of *C. aquaticum*; (d) mature conidium with 2 arm of *C. exiguum*; (e) detach conidium of *C. exiguum*, *bar* = 10 µ.

Habitat: isolated from fallen leaves of unidentified deciduous broad-leaved tree, Phu Quoc National Park, Kien Giang Prov., Vietnam, Nov. 2011, collected by L.T.H Yen. Culture is deposited in the Vietnam Type Culture Collection, Hanoi and National Institute of Technology and Evaluation-Japan (VTCC-1210 = NBRC 111199) VTCC-F-EH-1210

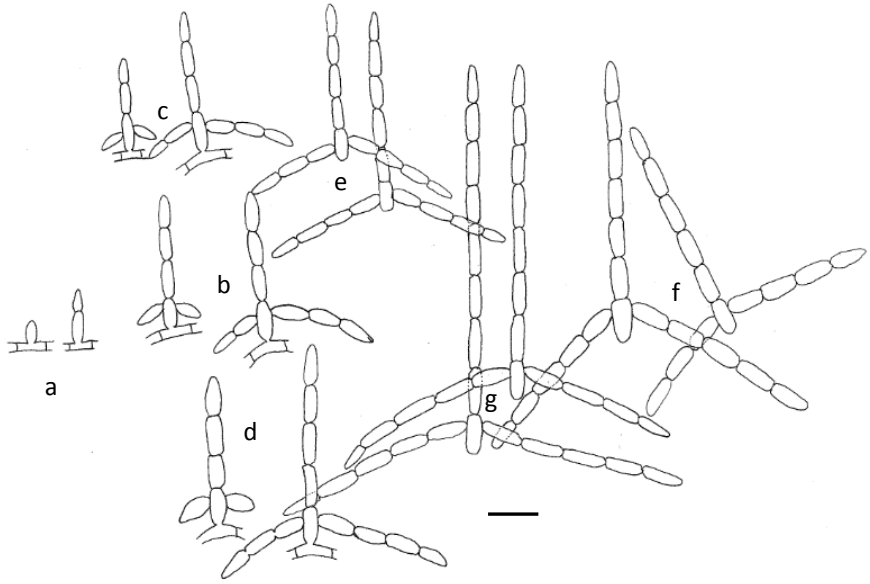

Figure 4. The conidia autogeny of *Ceramothyrium*: a. main axis start budding out from hyphae; (b)-(d) development of the main axis and two lateral arms; (b) *Ceramothyrium phuquocense;* (c) *Ceramothyrium exiguum;* (d) *Ceramothyrium aquaticum*; (e)-(g) mature conidia; (e), *Ceramothyrium exiguum;* (f) *Ceramothyrium aquaticum*; (g) *Ceramothyrium phuquocense*, *bar* = 10 µ.

Ceramothyrium exiguum L.T.H. Yen, K. Ando and Tsurumi sp. nov. VTCCF-1209 (NBRC 111198) (**Figure 3(e)**, **Figure 4(c)**, **Figure 4(e)**).

MycoBank no.: MB824818

Colonies are dark brown, plane on LCA; dark grey, twisted in PDA medium, slow growth, 10 - 15 mm in diameter after 7 days at 25˚C. Mycelium was pigmented, 2 - 2.5 µm. Conidia are sporulated easily after 3 - 4 days aerated. Conidiophores absent. Conidiogenous cells intercalary in hyphae. Conidia in culture are holoblastic, pigmented, tri-radiate, they consist of a multi-septa main axis and two arms, which are much constricted at the septa. Main axis usually extends to five-celled, sometimes up to six-celled, 50 - 70 × 2.0 - 2.5 µm with each cell 10 - 12 µm long. From the basal cell of the main axis, (one) - two arms arise. The arms are shorter than the main axis, 3 - 4 septa, 25 - 50 µm × 2.0 - 2.5 µm with each cell 9 - 11 µm long.

Type: VTCC-F-H-1209 (holotype: dried culture specimen, from VTCCF-1209, on LCA) deposited in the Vietnam Type Culture Collection, Hanoi (VTCC). NBRC H-13275 (isotype: dried culture specimen, from VTCCF-1209, on LCA) deposited in the NITE Biological Resource Center (NBRC).

Ex-type culture: VTCCF-1209 (=NBRC 111198), isolated from fallen leaves of unidentified deciduous broad-leaved tree, Phu Quoc National Park, Kien Giang Prov., Vietnam, Nov. 2011, collected by L.T.H Yen.

Ceramothyrium phuquocense L.T.H. Yen, K. Ando and Tsurumi sp. nov. VTCCF-1206 (NBRC111197) **Figure 3(a)**, **Figure 3(b)**; **Figure 4(d)**, **Figure 4(g)**.

MycoBank no.: MB424823

Colonies are dark grey on LCA and PDA media. The mycelium was dark to brown, submerged in agar, twisted together, little aerial mycelium was produced, the colony became convoluted. Twisted mycelia form a restricted colony that makes it difficult to prepare a good permanent slide. Conidia are not produce on the media, after submerging in water 3 - 4 days, they were sporulated easily. Conidiophores absent. Conidiogenous cells intercalary in hyphae. Conidia in culture are holoblastic, pale olivaceous, tri-radiate, consist of a main axis and two arms. Cells of main axis and arms constricted at intervals so that they look like chains of sausages. Main axis extends up to ten-celled, (80) - 95 - 125 × 2.5 - 2.7 μm with each cell 12 - 14 μm long. From the basal cell of the main axis, two arms arise. The arms are shorter than the main axis, (3) - 5 - 6 - (7) septa, (30) - 50 - 80 μm × 2.5 - 2.7 μm.

Type: VTCCF VTCC-F-H-1206 (holotype: dried culture specimen, from VTCCF-H-1206, on LCA) deposited in the Vietnam Type Culture Collection, Hanoi (VTCC). NBRC H-13274 (isotype: dried culture specimen, from VTCCF-H-1206, on LCA) deposited in the NITE Biological Resource Center (NBRC).

Ex-type culture: VTCCF-1206 (= NBRC 111197), isolated from fallen leaves of unidentified deciduous broad-leaved tree, Phu Quoc National Park, Kien Giang Prov., Vietnam, Nov. 2011, collected by L.T.H Yen.

4. Discussion

All of three newly *Ceramothyrium* anamorphs are agree well with *Trisulcosporium acerium* Hudson and Sutton 1964 [13] on the autogeny, morphology and the habitate. They have tri-radiate or tetra-radiate spores which consisted a main axis and two to three arms. Cells of main axis and arms constricted at intervals so that they look like chains of sausages. The main axis is tapering slightly to the apex while two arms arise equidistantly from the basal cell of the main axis. Furthermore, both *Trisulcosporium* and our newly C*eramothyrium* anamorph were isolated from fallen leaves and their spore production was induced by water. But there were some differences between *Ceramothyrium* anamorph and *Trisulcosporium*: Arms of Hudson and Sutton's fungus were budding out from the lower portion of basal cell, whereas, in our Vietnamese fungus the arms were budding out from upper part of the basal cell. Moreover, *Trisulcosporium* had hialyne conidia, while in *Ceramothyrium aquaticum*, *C. minima* and *C. phuquocense,* the conidia were pigmented. These characteristics make our fungi different from *Trisulcosporium*.

Morphologically, our fungi are similar to some known *Ceramothyrium* anamorph: *Stanhughesia carniolica*, *S. linnaeae* [2] and the *Trisulcosorium* anamorph stage of *Ceramothyrium melastoma* [3] by having tri-radiate/tetra-radiate, pigmented spore which consist of one main axis and one-two arms arising at the basal cell. However, there are some different points: *S. carniolica*, *S. linnaeae* and

C. melastoma had a truncate basal cell, 3-5 µm wide, subsequent ones gradually tapered to a 2 - 3 µm wide, apical cell ending in a c. 1 - 1.5 µm wide beak, thin- and smooth-walled, markedly constricted at the main septa, but slightly or not constricted at secondary, very thin septa which sometimes divide the cells. While our *Ceramothyrium* anamorph, septa is constricted at all cells, makes the main axis and arms look like chains of sausages. Furthermore, size of main axes of our new *Ceramothyrium* differs from each other and all of other known *Ceramothyrium* anamorph. Main axes of *Ceramothyrium aquaticum, C. minima* and *C. phuquocense* are 70 - 90, 33.5 - 72.5 and 70 - 130 µm, respectively; while *Stanhughesia carniolica*, *S. linnaeae* and *C. melastoma*'s main axes were 70 - 120, 25 - 40 and 15 - 30 µm, respectively (Table 1).

Table 1. Comparison of conidium morphology in the *Ceramothyrium* anamorph species.

Species	Length conidia (µm) (Numbers of cell/ conidia)				Refference
	Main axis (No of cell)	Branch 1 (No of cell)	Branch 2 (No of cell)	Wide of conidia	
Stanhughesia carniolica	70 - 120 6 - 10	to 60 (to 4)	ND	3 - 4 µm wide at the basal cell, gradually tapered to a (1) - 2 - 2.5 µm at apex cell.	O. Const. 1989
Stanhughesia linnaeae	25 - 40 3 - 6	13 - 30 2 - 3	13 - 30 2 - 3		O. Const. 1989
Stanhughesia lycopodii	25 - 45 (4 - 6)	15 - 30 2 - 4	(rarely)		O. Const. 1989
Stanhughesia nipponica	32 - 40 (3 - 4)	10 - 17	10 - 17	2 - 2.5 µm wide at the basal cell, gradually tapered to a 1 - 1.5 µm	K. Matsush. & Matshu. 1996
Ceramothyrium melastoma					
Stanhughesia morph	40 - 60	7 - 25	ND	2.5 - 3.0 µm	Crous *et al.* 2012
Trisulcosporium morph	15 - 30	15 - 35	15 - 35	(2.5) - 3 - 4 µm	
Ceramothyrium podocarpi	Star - shaped conidium with numerous branches, 25 - 90 µm long, 1 - 9 - septate,			4 - 6 µm wide with hilum 1.5 - 2 µm diam	Crous *et al.* 2012
Ceramothyrium acerinum	70 - 130.3 (6 - 11)	(0) - 43 - 85.9 (0) - (4 - 7)	(0) - 40.8 - 93.5 ((0) - 4 - 7)	2.3 - 2.7	This study
Ceramothyrium exiguum	37.3 - 72.5 (3 - 6)	(0) - 19.3 - 44.3 (0) - 2 - 4	(0) 25.8 - 54.3 ((0) 3 - 4)	2.3 - 2.5	This study
Ceramothyrium phuquocense	67 - 95 (6 - 8)	(0) - 3 - 70 (0) - 3 - 6	(0) - 45 - 60 ((0) - 3 - 5)	2.5 - 2.7	This study

The genus *Ceramothyrium* has *Stanhughesia* asexual morphs [2] [3] and *Trisulcosporium* [3] represents a genus of epiphyllous ascomycetes in the *Chaetothyriales* for which DNA data has been lacking until the recent study of Chomnunti *et al.* (2012), Crous *et al.* (2102), Hongsanan *et al.* (2015) and Zeng *et al.* (2016) [3], [14], [15] and [16]. Phylogeny based on ITS region and D1/D2 of the 28S rDNA gene analysis showed that these new *Ceramothyrium* anamorph were nested in *Ceramothyrium* clad with 98% and 75% bootstrap value, respectively. In this study, only the asexual morph of *Ceramothyrium aquaticum*, *C. minima* and *C. phuquocense* were observed, we choose to name it in the older sexual genus, *Ceramothyrium* which consisted of 41 taxa, accepting *Stanhughesia carniolica, S. linnaeae, S. lycopodii, S. nipponica, C. melastoma, C. podocarpi* [2], [3], [17] and our three new anamorph having existing names in *Ceramothyrium* as synonym.

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