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Research Article

Cryptothecia austrocoreana (Arthoniales, Arthoniaceae), a New Species from South Korea

Jung-Jae Woo[1], László Lőkös[2], Edit Farkas[3], Chan-Ho Park[1] and Jae-Seoun Hur[1,*]

[1]*Korean Lichen Research Institute, Sunchon National University, Suncheon 57922, Korea*
[2]*Department of Botany, Hungarian Natural History Museum, H-1476 Budapest, Hungary*
[3]*Institute of Ecology and Botany, Centre for Ecological Research, Hungarian Academy of Sciences, H-2163 Vácrátót, Hungary*

Abstract *Cryptothecia austrocoreana* is a new lichen species from South Korea. The species is characterized by the presence of a heteromerous thallus and faveolate ascigerous area (ascomata) immersed in a slightly raised thallus. The species has muriform ascospores, (4)6–8–spored 8–11 × 3–4 septate, (34)36–48(51) × (17)19–23(25) µm. Atranorin, chloroatranorin, and barbatic acid are present. In the phylogenetic tree, *C. austrocoreana* belongs to the arthonioid clade in Arthoniaceae.

Keywords Arthoniaceae, Arthoniales, *Cryptothecia*, New species, South Korea

The genus *Cryptothecia* is distributed in tropical or subtropical areas worldwide. Approximately 68 species of *Cryptothecia* have been reported to date, of which 65 were provided with a world key [1, 2]. General morphological characteristics of *Cryptothecia* are (1) byssoid thallus lacking isidioid outgrowths; (2) medulla I+ blue in patches; (3) trentepohlioid photobiont; (4) broadly clavate to globose thick-walled *Cryptothecia*-type asci, aggregated in ascigerous areas or loosely dispersed on thallus; (5) muriform ascospores with wavy septa [1]. *Cryptothecia* is morphologically similar to other genera belonging to Arthoniaceae. A distinguishing character is that *Arthonia* and *Arthothelium* have distinct fruiting-bodies, whereas *Cryptothecia* and *Stirtonia* lack distinct fruiting-bodies. Additionally, *Arthonia* and *Stirtonia* have transversely septated spores, whereas *Arthothelium* and *Cryptothecia* have muriform spores [3]. Phylogenetic studies based on DNA sequencing have indicated that *Cryptothecia* belongs to Arthoniaceae. Furthermore, *Cryptothecia* is reported to be polyphyletic [4-6].

In South Korea, major genera such as *Arthonia* and *Arthothelium* of Arthoniaceae have been studied, but *Cryptothecia* is undiscovered. The genus *Cryptothecia* has been studied through a program for the undiscovered taxa of South Korea initiated by the National Institute of Biological Resources. Members of *Cryptothecia* usually grow on rough bark in shaded and humid subtropical forests. In South Korea, Jeju Island and the southern coasts have a subtropical climate [7]. *Cryptothecia* was collected from the vicinity of the Seonam Temple, which is located in Jo-gye Mountain Provincial Park in Jeollanam Province. The temple is surrounded by valleys that contain *Carpinus tschonoskii*, *C. laxiflora*, *Acer pseudosieboldianum*, and *Meliosma myriantha*. The aim of this study was to investigate the phylogenetic position of new species in Arthoniaceae through morphological, chemical, and molecular analysis.

MATERIALS AND METHODS

Morphological and chemical studies. The lichen specimens were collected from Seonam temple, Jeollanam Province, South Korea from 2016 to 2017 and deposited in the Korean Lichen Research Institute (KoLRI) (Fig. 1). External morphological characteristics of lichen specimens were examined using a stereomicroscope (SZX-7; Olympus, Tokyo, Japan). Macro photographs were taken with an Olympus E450 camera using the Quick Photo Camera 2.3 software. The anatomy of the thalli and ascigerous areas was examined using a microscope (Eclipse Ni-U; Nikon, Tokyo, Japan) equipped with a Nikon DS-Fi1c camera, which was operated using the NIS-Elements BR software. Calcium oxalate crystals were identified using 25% H_2SO_4. Spot tests

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*Corresponding author
E-mail: jshur1@scnu.ac.kr

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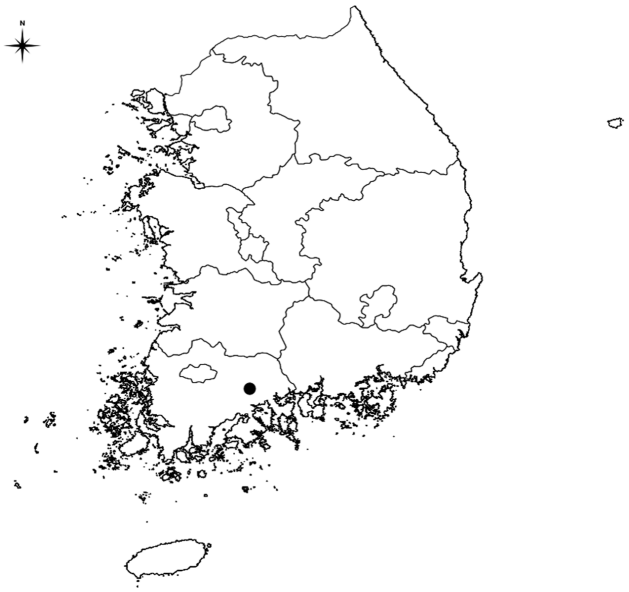

Fig. 1. Collection site of *Cryptothecia austrocoreana*. The collection area is marked with a black star.

of lichen specimens were conducted using 10% KOH (K), saturated $Ca(OCl)_2$ (C), 10% KOH followed by saturated $Ca(OCl)_2$ (KC), and 5% alcoholic p-phenylenediamine (P). Secondary metabolites of lichen specimens were identified using high-performance thin-layer chromatography (HPTLC) and thin-layer chromatography (TLC). HPTLC was performed using solvent systems A, B, and C [8]. TLC was performed using solvent systems A and C per the standard method [9]. Localities from which lichen specimens were collected were mapped using the open source GIS software Quantum GIS 1.7.4 (QGIS).

DNA sequencing and alignment. Genomic DNA was isolated from freshly collected lichen specimens using a NucleoSpin Plant II Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocols. For the mitochondrial small subunit rDNA (mtSSU) region, the primers mtSSU1 (5′-AGCAGTGAGGAATATTGGTC-3′) and mtSSU3R (5′-ATGTGGCACGTCTATAGCCC-3′) were used. The PCR cycle was followed as described by Zoller *et al.* [10]. For the RNA polymerase II (RPB2)

Table 1. Voucher and accession numbers for specimens from GenBank

Species		Voucher	mtSSU	RPB2
1	*Arthonia calcarea*	France, Ertz 7540 (BR)	EU704065	EU704029
2	*Arthonia incarnata*	Fonsson FU6271	-	KY983983
3	*Arthonia didyma*	Belgium, Ertz 7587 (BR)	EU704047	EU704010
4	*Arthonia radiata*	Belgium, Ertz s.n. (BR)	EU704048	EU704011
5	*Arthothelium norvegicum*	USA, McCune 31061 (UPS)	KJ851003	KJ851114
6	*Briancoppinsia cytospora*	Luxembourg, Diederich 16849 (BR)	JF830771	-
7	*Briancoppinsia cytospora*	Belgium, Ertz 15244 (BR)	JF830772	-
8	*Chiodecton natalense*	Uganda, Frisch 11Ug324 (UPS)	KF707647	KF707660
9	*Coniocarpon cinnabarinum*	Uganda, Frisch 11/Ug297 (UPS)	KJ850977	-
10	*Crypthonia palaeotropica*	Uganda, Frisch 11/Ug457 (UPS)	KJ850961	KJ851084
11	*Cryptothecia assimilis*	Fiji, Lumbsch 19815l (F)	GU327688	-
12	*Cryptothecia punctosorediata*	USA, Nelsen 4038 (F)	JX046450	-
13	*Cryptothecia austrocoreana*	Korea, KoLRI No.041892	MF769374	163647
14	*Cryptothecia austrocoreana*	Korea, KoLRI No.044721	MF769375	-
15	*Cryptothecia* sp. Ertz 8472	Rwanda, Ertz 8472 (BR)	EU704053	-
16	*Cryptothecia* sp. Ug1	Uganda, Frisch 11/Ug194 (UPS)	KJ850956	KJ851093
17	*Cryptothecia* sp. Ug2	Uganda, Frisch 11/Ug18 (UPS)	KJ850955	KJ851092
18	*Cryptothecia* sp. Ug3	Uganda, Frisch 11/Ug39 (UPS)	KJ850954	KJ851086
19	*Cryptothecia subnidulans*	Reunion, v.d.Boom 40613 (hd v.d.Boom)	KJ850952	KJ851087
20	*Cryptothecia subnidulans*	Guyana, Joensson Guyana 6a (UPS)	KJ850953	KJ851088
21	*Herpothallon inopinatum*	Mexico, Rudolphi 12 (UPS)	KJ850964	KJ857099
22	*Herpothallon kigeziense*	Uganda, Frisch 11/Ug26 (UPS)	KF707644	KF707654
23	*Herpothallon rubrocinctum*	Mexico, Rudolphi 5 (UPS)	KF707643	KF707655
24	*Inoderma byssaceum*	Japan, Thor 25952 (UPS)	KJ850962	KJ857089
25	*Myriostigma candidum*	Gabon, Ertz 9260 (BR)	EU704052	EU704015
26	*Myriostigma candidum*	Uganda, Frisch 11/Ug125 (UPS)	KJ850959	KJ851096
27	*Reichlingia syncesioides*	Uganda, Frisch 11/Ug14 (UPS)	KF707651	KF707656
28	*Stirtonia neotropica*	Brazil, Caceres & Aptroot 11112 (ISE)	KP843611	-
29	*Stirtonia* sp. Ug1	Uganda, Frisch 11/Ug325 (UPS)	KJ850965	-
30	*Tylophoron hibernicum*	France, Diederich 16335 (BR)	JF830779	-
31	*Tylophoron hibernicum*	Uganda, Frisch 11/Ug220 (UPS)	KJ850966	KJ851097

mtSSU, mitochondrial small subunit rDNA; RPB2, RNA polymerase II.

region, the primers f*RPB*2-7cF (5′-ATGGGYAARCAAGC-YATGGG-3′) and f*RPB*2-11aR (5′-GCRTGGATCTTRTC-RTCSACC-3′) were used. The PCR cycle as described by Liu *et al.* [11] was followed. Generated sequences were aligned with those of genera belonging to Arthoniaceae selected from GenBank (Table 1). All raw sequences were assembled and edited using BioEdit 7.09 [12].

Phylogenetic analysis. The TVM + I + G model as best-fitting the data was determined using Akaike Information Criterion as implemented by jModeltest v2.1.10 for MrBayes [13]. Phylogenies were analyzed using maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference. Bootstrap values were obtained in MEGA 7 using ML and MP methods with 1,000 bootstrap estimates. The ML was analyzed using the GTR + I + G model [14]. Bayesian analyses were conducted with the Metropolis-coupled Markov chain Monte Carlo method using MrBayes v. 3.1.2. In the Bayesian inference, two parallel independent analyses were performed using one cold and three heated chains for 1,000,000 generations, and trees were sampled every 100 generations [15].

RESULTS AND DISCUSSION

A phylogenetic tree of the new species was produced by alignment with 28 sequences for mtSSU and 20 sequences for RPB2 selected from GenBank (Table 1). The genera

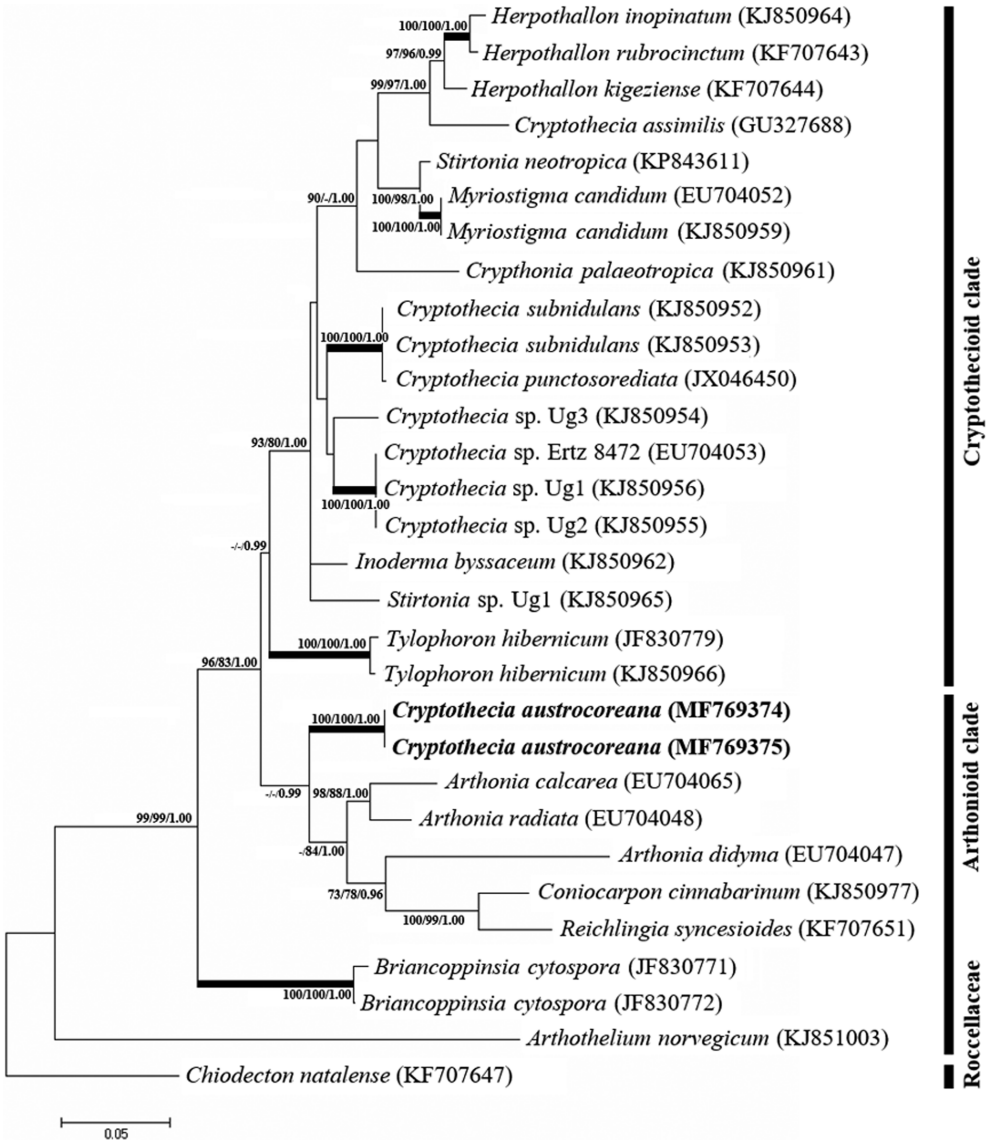

Fig. 2. Analysis of phylogenetic relationship among genus *Cryptothecia* and other related genera based on mitochondrial small subunit sequences. Maximum likelihood (ML), maximum parsimony (MP) bootstrap value ≥ 70%, and Bayesian posterior probabilities (PP) value ≥ 95% are shown above the branches. Bootstrap values are shown in the order of ML, MP, and PP in the tree. If the bootstrap value is 100% (ML, MP) or 1.00 (PP), the branch is shown in bold.

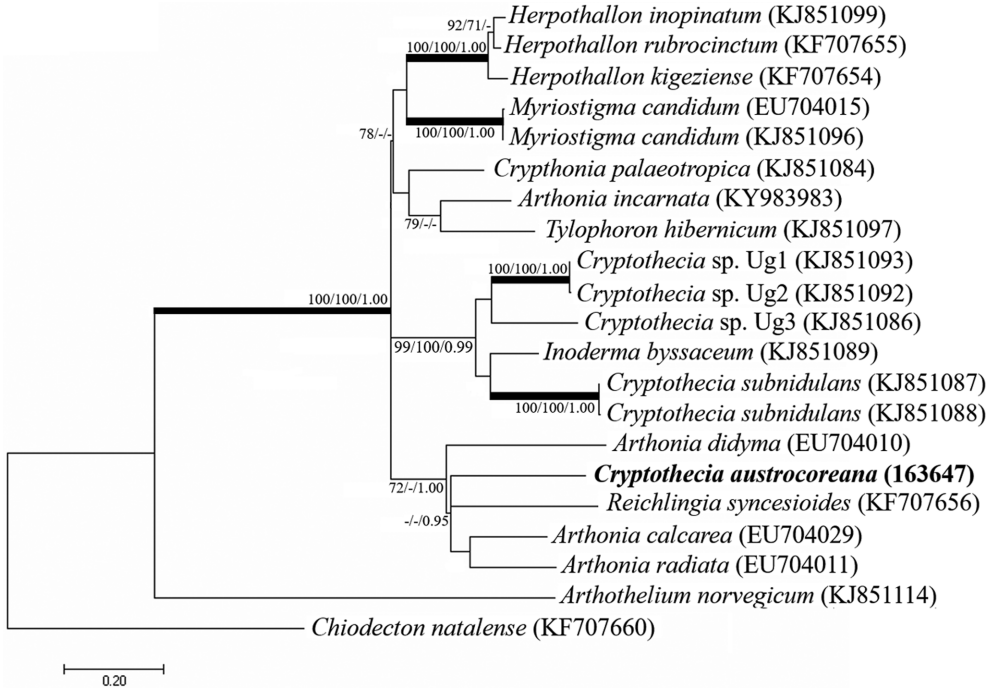

Fig. 3. Analysis of phylogenetic relationship among genus *Cryptothecia* and other related genera based on RNA polymerase II subunit. Maximum likelihood (ML), maximum parsimony (MP) bootstrap value ≥ 70%, and Bayesian posterior probabilities (PP) value ≥ 95% are shown above the branches. Bootstrap values are shown in the order of ML, MP, and PP in the tree. If the bootstrap value is 100% (ML, MP) or 1.00 (PP), the branch is shown in bold.

Herpothallon, *Stirtonia*, *Myriostigma*, *Inoderma*, and *Tylophoron* belonging to the cryptothecioid clade were included in the tree. The cryptothecioid clade consists of genera with a byssoid thallus morphologically similar to that of *Cryptothecia*. *Chiodecton natalense* (Roccellaceae) was included as an outgroup.

Although *Cryptothecia austrocoreana* has morphological characteristics indicative of *Cryptothecia*, it was found to belong to the arthonioid clade in the phylogenetic tree based on mtSSU and RPB2 (Figs. 2 and 3). Previous studies reported that *Cryptothecia* was polyphyletic and belonged to the cryptothecioid clade [4, 5, 11]. However, this is the first case in which *Cryptothecia* was found to belong to the arthonioid clade rather than cryptothecioid clade. To date, about 68 species of *Cryptothecia* have been reported, but only less than 10 species have sequencing data for comparison of relationships. Therefore, we have identified the phylogenetic position of *Cryptothecia austrocoreana* in Arthoniaceae with related genera. *Cryptothecia* sequences registered in the NCBI database are the results of RPB2, nuclear large subunit (nLSU), and mtSSU. We also used mtSSU, RPB2, and nLSU for phylogenetic investigation of *Cryptothecia austrocoreana*, however, we obtained sequences only for mtSSU and RPB2. The sequencing results of *Cryptothecia* and related genera confirm that *Cryptothecia* can belong to other groups in addition to the cryptothecioid clade. Further, sequence data of *Cryptothecia* and minor groups of Arthoniaceae are not sufficiently complete. Thus, a new marker gene that is advantageous for analysis of the Arthoniaceae phylogeny is proposed for further study.

New species.

Cryptothecia austrocoreana J.-J. Woo, L. Lőkös, E. Farkas & J.-S. Hur, sp. nov. (Fig. 4).

MycoBank No.: MB 822507.

Type: South Korea, Jeollanam Prov., Sunchon-si, Seonam Temple, 34°59′33.8″ N, 127°20′23.9″ E, alt. 160 m, Seonam valley; trees around the valley, on the bark of *Meliosma myriantha*, 11 Apr 2016, J.-J. Woo, 163647 (holotype: KoLRI 041892, accession number: MF769374); the same locality, 9 Feb 2017, J.-J. Woo, 170597 (KoLRI 044721, accession number: MF769375).

Distribution and ecology: *Cryptothecia austrocoreana* has been found in the type locality where it grows on the bark of *Carpinus tschonoskii* and *Meliosma myriantha.*

Etymology: Name refers to the southern region of South Korea.

Morphology: Thallus crustose, corticolous, ecorticate, byssoid, epiphloedal, firmly attached to the substrate, up to 15 cm in diameter, beige to greenish white, lacking soredia and isidia, up to 75 μm thick, heteromerous, with numerous needle-shaped calcium oxalate crystals. Prothallus white, up to 5 mm. The faveolate ascigerous area (ascomata) is mostly spherical and verrucose immersed in a slightly raised thallus. Asci broadly clavate, (4)6–8-spored. Paraphysoids enclosing the asci. Ascospores hyaline, ellipsoid, muriform,

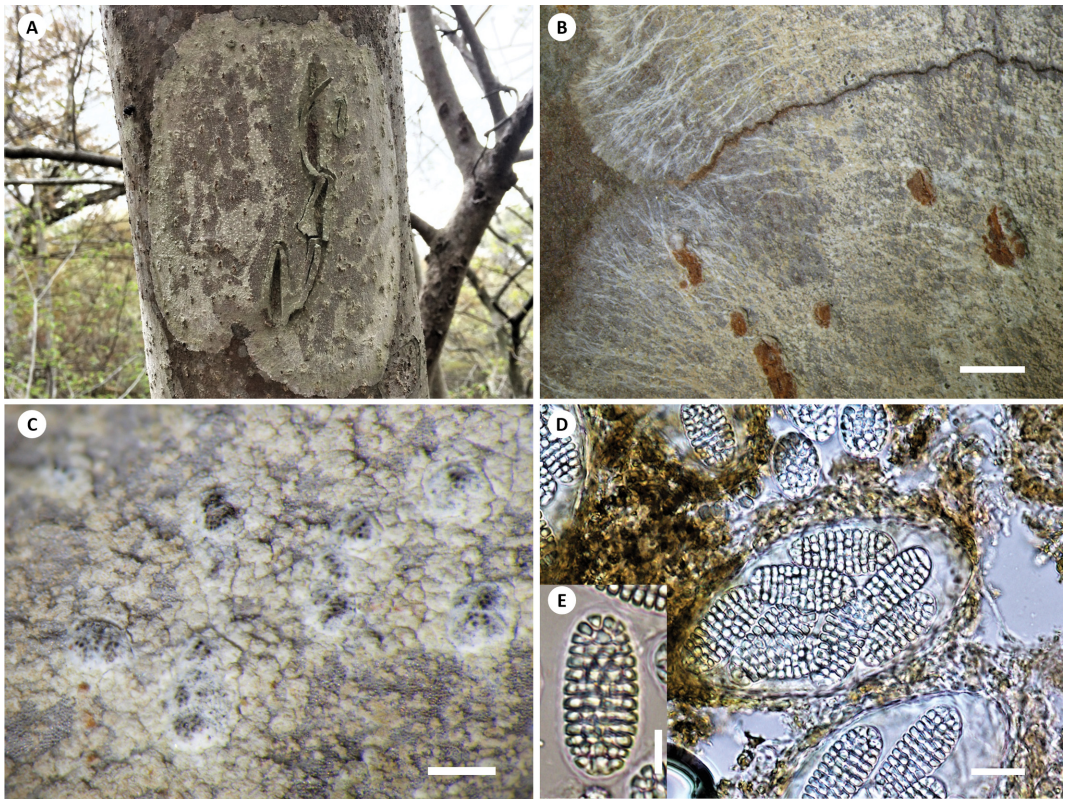

Fig. 4. A, *Cryptothecia austrocoreana* (holotype), part of thallus and substrate; B, Magnified prothallus; C, Faveolate ascigerous area (ascomata); D, Ascus; E, Ascospore (scale bar: B = 2 mm, C = 0.5 mm, D = 20 μm, E = 10 μm).

(34)36–48(51) × (17)19–23(25) μm, 8–11 × 3–4 septate.
Chemistry: K+ yellow, C–, KC–, P–, I+ blue in patches (section), UV–. TLC and HPTLC: Atranorin (minor), barbatic acid (major), and chloroatranorin (trace).
Note: Atranorin, barbatic acid, and chloroatranorin were detected by HPTLC and TLC in *Cryptothecia austrocoreana*. Only six species (*C. albomaculatella*, *C. albata*, *C. aleurina*, *C. caesioalba*, *C. fuscopunctata*, and *C. lunulata*) in *Cryptothecia* contain barbatic acid. *C. fuscopunctata*, which has a lichen substance similar to that in *C. austrocoreana*, differs in the size and shape of spores or the number of septa. In addition, *C. fuscopunctata* has a 135–300 μm thick thallus, which is much thicker than that of *C. austrocoreana*. Table 2 shows a comparison among species containing barbatic acid in the genus *Cryptothecia*. Chloroatranorin was only detected in solvent C in TLC

Table 2. Comparison of morphological characteristics of species containing a barbatic acid in the genus *Cryptothecia*

Species name	Ascospore	Spore size (μm)	Septation	Chemistry
C. austrocoreana	(4)6–8-spored	(34)36–48(51) × (17)19–23(25)	8–11 × 3–4	Atranorin Barbatic acid Chloroatranorin
C. albata	8-spored	17–26 × 7	7–8 × 1–3	Barbatic acid Divaricatic acid Unknown lichen substance
C. albomaculatella	-	50–65 × 22–29	-	Barbatic acid Obtusatic acid
C. aleurina	8-spored	23–36 × 17–19	-	Barbatic acid Unknown lichen substance
C. caesioalba	6–8-spored	50–66 × 20–25	-	Barbatic acid Confluentic acid
C. fuscopunctata	(2)4–8-spored	(23)26–36 × 11–14	8–10 × 3–5	Atranorin Barbatic acid
C. lunulata	8-spored	33–56 × 13–17	-	Barbatic acid Unknown lichen substances

(toluene : acetic acid = 200 : 30). Although *Cryptothecia* and *Arthonia* have different spore types, *C. austrocoreana* is included in arthonioid clade in the phylogenetic trees based on mtSSU and RPB2.

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