Arthothelium punctatum (Arthoniaceae, Arthoniales),
A New Lichen Species from South Korea

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Abstract
A total of 121 species of lichens belonging to the genus Arthothelium have been described to date, most of which have been found in tropical regions. Here, we describe the discovery of a novel Arthothelium species for the first time in South Korea. Until now, Arthothelium ruanae was the only Arthothelium species reported in South Korea. Among the 113 specimens collected in this study, we identified A. ruanae and a putative new species, Arthothelium punctatum (J. S. Park & J.-S. Hur, sp. nov.). The diagnostic characters of A. punctatum are as follows: apothecia punctate, shortly elongate to branched, small, 0.1–0.2 mm wide, hypothecium hyaline to pale brown and obovate to broadly ellipsoid, muriform ascospores, 29.5–44.6 × 12.2–18.2 μm. The new species was found in Mt. Seokbyeong at an altitude of 790 m on smooth bark. Upon phylogenetic analysis, the putative new species, A. punctatum, was separated from other Arthothelium species although the specimens analyzed were clustered with Arthoniaceae in phylogenetic trees based on both the mitochondrial small subunit (mtSSU) sequence and combined mtSSU and nuclear ribosomal large subunit sequences. Our data clearly indicate that this species is a new species belonging to the family Arthoniaceae. To elucidate the taxonomic characteristics of the new species, we provide morphological descriptions and a distribution map.

Keywords
Arthothelium, mtSSU, nuLSU, Phylogenetic analysis, RPB2, Taxonomy

Lichen species of the genus Arthothelium A. Massal. are cosmopolitan and mostly distributed in tropical regions. Currently, the genus is represented by approximately 121 species [1, 2]. Arthothelium grows on bark, rock and even living on other lichens (lichenicolous) [3-5]. The genus is lichenized with Trentepohlia Mart. or chlorococcoid algae, or found in the non-lichenized form [6]. Arthothelium and a very similar genus Arthonia belong to the family Arthoniaceae [7]. In 1852, Massalongo was the first to segregate Arthothelium from Arthonia species [8]. The two genera are mainly distinguished by their ascospore morphology, with Arthothelium ascospores being muriform and those of Arthonia are transversely septate [9]. Arthonia and Arthothelium have been described as paraphyletic genera based on their morphological (thallus, ascomatal, meiospore, conidiomatal, amyloid), chemical, and ecological characteristics [6]. Arthothelium is characterized by a smooth, effuse or verrucose thallus; rounded, irregular to lirlline, branched ascomata; branched, anastomosing and coherent, anastomosing coherent paraphysoids forming a thick epithecium above; Arthonia-type 8-spored asc, and muriform ascospores [9].

The molecular phylogeny of the genus Arthothelium has not been studied in great detail. Arthothelium species have primarily been used for comparison with the closely related families, Opegraphaceae and Roccellaceae [10, 11]. In a study of Dothideomyceta, Arthothelium was grouped among Arthonia species when phylogenetic analysis was performed using nuclear ribosomal large subunit (nuLSU) and mitochondrial small subunit (mtSSU) sequences [12]. Interestingly, Arthonia ruana has morphological features typical of Arthothelium species such as muriform ascospores, but was found to cluster with Arthonia [12]. Finally, Arthonia ruana was re-named Arthothelium ruana, indicating that morphological and molecular analyses are both essential for taxonomic identification. In a recent study, Arthonia and Arthothelium were assumed to be paraphyletic although these two genera are more closely related to each other than to other taxa based on their morphological and RNA polymerase II subunit (RPB2) sequences [13].

Most investigations of Arthothelium sp. have been
conducted on European species. Indeed, prior to this study, only one species, A. ruanum, was collected and described in South Korea by Joshi et al. [14] and Kondratyuk et al. [15]. In the present study, we extensively collected and identified Arthothelium specimens in Korea, leading to the discovery of a putative new Arthothelium species named A. punctatum, as well as an already known species. Here, we present a detailed morphological description and molecular analysis of these two species using mtSSU, nuLSU, and RPB2 sequences. We also provide a current taxonomic description of each species with mapping information.

MATERIALS AND METHODS

Morphological examination. A total of 113 specimens were collected in South Korea from 2015 to 2017. Air-dried samples were observed using a stereomicroscope (SMZ-645; Nikon, Tokyo, Japan) and a compound microscope (BX-50; Olympus, Tokyo, Japan). The color reaction was conducted as described by Yoshimura [16] and Baral [17]. Briefly, sections of the ascomatal structure were mounted in water and lactophenol cotton blue was used as a stain. The ascomatal structure was then observed in 10% aqueous solution of potassium hydroxide (K), while the amyloidity of the ascomatal structure were tested by Lugol’s iodine solution (I), and with and without pretreatment with 10% aqueous potassium hydroxide (KI).

DNA extraction and nrDNA amplification. Five representative specimens were selected and used for further molecular analyses. Apothecial discs were mainly used for DNA extraction. Samples were ground with a Mini-Beadbeater-16 (3450 RPM, 115 V, 10 A; Biospec products) and then extracted with a NucleoSpin Plant II Kit according to the manufacturer’s instructions (Macherey-Nagel, Duren, Germany). PCR amplifications were conducted using AmpliTaq DNA polymerase with buffer conditions. The following primers were used for PCR amplifications: mtSSU1 and mtSSU3R (sequences as designed by Zoller et al. [18]) for amplification of mtSSU; nuLSU_arthro_2F (5’-CCTTCTGA CGAGTCTGA GTTG-3’), nuLSU_arthro_2R (5’-GTGAGTTG TTTACACTCCT-3’) for nuLSU; RPB2-7cF and RPB2-11aR (as designed by Liu et al. [19]) for RPB2. PCR conditions for nuLSU and RPB2 are as described in a previous study [13]. The following program was used for amplification of mtSSU: initial denaturation for 5 min at 94°C followed by 30 cycles of 94°C for 30 sec, 54°C for 39 sec, 72°C for 7 sec, and then final extension at 72°C for 7 min. The amplified DNA was concentrated and purified using a PCR quick-spin PCR Product Purification Kit (INTRON Biotechnology, Inc., Seongnam, Korea). Sequencing analysis was performed.

Sequence alignments. Obtained sequencing reads were processed using Bioedit. Sequence alignment was performed using Clustal W ver. 1.83 [20] and phylogenetic analysis was conducted with MEGA 6.0. The maximum likelihood (ML) method was selected to construct a phylogenetic tree. The Kimura 2-parameter model was selected and the reliability of the inferred tree was tested by 1,000 bootstrap replications. Dothisa sambuci (mtSSU, AY544739; nuLSU, AY544681) and Pleospora herbarum (mtSSU, FJ190610; nuLSU, DQ247804) were selected as outgroups based on BLAST comparisons of the sequences available in the GenBank database.

RESULTS AND DISCUSSION

Collection, distribution, and taxonomic analysis of Arthothelium in South Korea. We collected a total of 113 putative Arthothelium specimens in Korea from 2015 to 2017 that belong to family Arthoniaceae (Fig. 1). Samples were collected from the southwestern portion of the Korean peninsula (41 specimens) and a northeastern mountainous area (25 specimens), Jeju province (41 specimens) and Ulleung Island (6 specimens).

Morphological identification was conducted according to Zahlbruckner’s taxonomic identification [7]. All putative Arthothelium specimens were identified as belonging to section Euarthothelium based on the presence of black ascomata. In our survey, we identified two species, Arthothelium ruanum and a putative new species Arthothelium punctatum. A. ruanum is dominantly distributed in Asia,
including Korea [7, 14, 15], Europe, and North America [1]. Unlike the previously described morphological characteristics of *A. ruana* [14, 15], 20 specimens from Mt. Seokbyeong showed punctate apothecia, hyaline hypothecium, and ascospores of 9 to 10 transverse septa with 1–4 longitudinal septa. The putative novel species, *A. punctatum*, showed punctate to shortly elongate apothecia. Morphological characteristics of the identified species are provided in the description section.

**Phylogenetic analysis using mtSSU and nuLSU.** For molecular phylogenetic analysis, we applied three markers, Table 1. Specimens included in the phylogenetic analysis with GenBank accession numbers.
RPB2, mtSSU, and nuLSU. We selected three representative specimens for *A. ruanum*, KoLRI 038018, KoLRI 038257, and KoLRI 038261, and two specimens for *A. punctatum*, KoLRI 044205 and KoLRI 044206. Despite considerable efforts to obtain sequences from the loci, nuLSU sequences from *A. ruanum* and RPB2 sequences from *A. punctatum* could not be obtained. Thus, we conducted the analysis using only mtSSU for *A. ruanum* and either mtSSU alone or combined mtSSU nuLSU sequences for *A. punctatum*.

We obtained ten new sequences (mtSSU, 5; nuLSU, 2; RPB2, 3) (Table 1) for the five aforementioned specimens belonging to two taxa. For phylogenetic analysis, we included sequences of 40 additional specimens from GenBank representing 35 taxa. Therefore, a total of 37 taxa and 45 specimens of Arthoniaceae, Opegraphaceae, and Rocellaceae were used. The mtSSU matrix included 1,140 characters,

![Fig. 2. Maximum likelihood tree of *Arthothelium* species based on mitochondrial small subunit (mtSSU) sequences. mtSSU sequences newly generated in this study are indicated in bold. The numbers at the nodes represent the percentage of their occurrence in 1,000 bootstrap replicates. The family Arthoniaceae is marked by the gray box, *Arthothelium ruanum* is in the light blue box, and the putative new species *Arthothelium punctatum* is delimited by a yellow box.](image-url)
A New Species, *Arthothelium punctatum*, in Korea

259 of which were variable and 389 that were conserved. The phylogenetic tree of *Arthothelium* based on the ML analysis of exclusive mtSSU (Fig. 2), concatenated mtSSU and nuLSU sequence data are presented in Fig. 3.

In the ML tree based on mtSSU sequences, *Arthothelium* species constituted an independent, paraphyletic lineage (Fig. 2). The ML tree based on mtSSU sequences showed that Arthoniaceae is separate from Opegraphaceae and Rocellaceae (Fig. 2). *A. ruanum* and *A. punctatum* were included in Arthoniaceae, which was supported by high-confidence bootstrap values. The three *A. ruanum* specimens were clustered with *A. ruanum* (previously referred to as *Arthonia ruana*) (Fig. 2). Interestingly, the specimens of the putative new species, *A. punctatum*, were clearly separated from other *Arthonia* and *Arthothelium* species in the mtSSU phylogenetic tree (Fig. 2), and this was supported by high bootstrap values. These data clearly indicate that *A. punctatum* could be a new species including Arthoniaceae.

For more detailed analysis, we combined the mtSSU and nuLSU sequences and generated a ML tree. The combined data matrix was composed of 3,727 characters, 2,201 of which were variable and 823 characters that were conserved. As with the ML tree based on the mtSSU sequences (Fig. 2), two specimens from a putative new species, *A. punctatum*,...
were separated from the other *Arthothelium* species (Fig. 3), indicating that *A. punctatum* would be a new species. In summary, we identified two different *Arthothelium* species, *A. ruanum* and *A. punctatum*, based on morphological and molecular analysis. *A. punctatum* specimens showed distinct morphological characteristics and segregation upon molecular analysis. Thus, *A. punctatum* is considered to be a new species in the family Arthoniaceae.

**New species.**

*Arthothelium punctatum* J. S. Park & J.-S. Hur, sp. nov. (Fig. 4).

Mycobank No.: MB 822353.

Similar to *A. scandinavicum*, but differs in having small apothecia, 0.1–0.2 mm, and ascospores muriform with 9 to 10 transverse septa and 1–4 longitudinal septa.

**Type:** Korea, Gangwon Prov., Gangneung, Mt. Seokbyeong, 37°34′41.01″ N, 128°51′32.71″ E, alt. 790 m, on bark, 28 Mar 2017, J. S. Park & J.-J. Woo, 170081 (holotype: KoLRI 044205).

**Etymology:** The epithet "punctatum" refers to its punctate apothecia.

**Morphology:** Thallus is corticolous, dull white to ivory gray, up to 3.5 cm, smooth, no crack, thin, somewhat shiny, effuse or delimited by a black line, corticate, photobiont *Trentepohlia*, cell scattered to grouped, almost spherical, c. 10.1–14.4 × 7.2–9.8 μm. Apothecia 0.1–0.2 mm diam., globose to slightly convex, punctate, shortly elongate, mostly level with thallus, superficial on the thallus surface, black, not pruinose; epithecium carbonized, black, 20.9–25.8(–32.5) μm tall, K+ greenish; hymenium 50.1–87.3 μm tall, hyaline, K+ greenish, I−, apices obscured by dense pigment; hypothecium 17.5–25.2 μm tall, hyaline, rather dense, paraphysoids anastomosing and branched, coherent, 1.1–1.6 μm wide, K+ greenish. Ascii broadly obovoid to ellipsoid, K/I−. Ascospores muriform, 8 per ascus, 29.5–44.6 × 12.2–18.2 μm, hyaline, slightly obovoid to broadly oblong, with 9–10 transverse septa and 1–4 longitudinal septa. Pycnidia not observed.

**Chemistry:** Thallus K−, PD−, C−; apothecia K−; UV−; no lichen compound detected by thin-layer chromatography (TLC).

**Remarks:** This species is characterized by having punctate or somewhat shortly elongate apothecia, hyaline hypothecium, and 9–10 transverse septa. *A. punctatum* may be confused with *A. scandinavicum* Th. Fr., which has globose to round and 0.3–1.1 mm apothecia with 6 to 7 transversely septa and 28–34 × 12–14 μm ascospores, but the latter species differs in having 0.1–0.2 mm apothecia and 29.5–44.6 × 12.2–18.2 μm ascospores with 9 to 10 transverse septa. *A. collosporum* (Vain.) Yoshim. is a similar species that has light brown ascospores measuring 33–48 × 13–18 μm, but *A. punctatum* has a hyaline ascospore color. Like *A. punctatum*, *Arthonia dispersa* Dufour and *A. punctiformis* Ach. also have a smooth, whitish thallus and punctate

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Fig. 4. *Arthothelium punctatum* (holotype). A, Habit and habitat; B, Section of apothecia; C, 8-spored asci; D, Muriform ascospores (scale bars: A = 1 mm, B–D = 20 μm).
apothecia shape. However, both the latter species have 1–3 transversely septate ascospores. Morpho-anatomically the species is usually confused with other genera including Mycoporum Flot. ex Nyl., Tomasellia A. Massal, and Cyrtidula Minks because of its ascospore septation and ascomata shape. Cyrtidula Minks is most similar in having black ascomata with muriform to submuriform ascospores, but all three of the aforementioned genera are pyrenocarpous lichens having hamathecium of pseudoparaphyses [1].

**Substrate and ecology:** This species is confined to Mt. Seokbyeong, where it grows on smooth bark at an altitude of 790 m. The species is associated with Graphis sp.

**Specimens examined:** Korea, Gangwon Prov., Gangneung, Mt. Seokbyeong, 37°34′41.01″ N, 128°51′32.71″ E, alt. 790 m, on bark, 28 Mar 2017, J. S. Park & J.-J. Woo, 170082 (isotype: KoLRI 044206); on bark, 28 Mar 2017, J. S. Park & J.-J. Woo, 170083 (KoLRI 044207).

**Recorded species.** Arthothelium ruanum (A. Massal.) Körb., Parerga lichenol. (Breslau) 3: 263 (1861).

**Morphology characters:** Thallus is corticolous, totally endophloidal, dull white to greenish-brown, smooth, thin, effuse or delimited by a dark line, photobiont Trentepohlia. Apothecia 0.5–2(–3) mm diam., irregularly round to stellate, slightly immersed, level with thallus, rough, often cracked, black, not pruinose. Epithecium carbonized, 13.1–21.4 µm tall, dark reddish-brown, K+ greenish. Hymenium 37.5–48.6 µm tall, hyaline to pale brown, K+ greenish, I+ reddish, apices obscured by dense pigment. Hypothecium 17.5–25.2 µm tall, dark reddish-brown, rather dense, paraphysoids anastomosing and branched, coherent, 1–1.5 µm wide, K+ greenish. Asci 37.5 × 26.2 µm, broadly clavate to obovoid, K/I+ blue. Ascospores muriform, (15.4–)21.5–25.9(–30.1) × 8.2–10(–11.6) µm, hyaline, slightly obovoid to broadly ellipsoidal, with 4–9 transverse septa and 1–3 longitudinal septa. Pycnidia appears commonly at the margin of thalli or periphery of apothecia, punctate, black, conidia bacilliform, 5 × 1 µm.

**Chemistry:** Thallus K−, PD−, C−; apothecia K−; UV−; no lichen compound detected by TLC.

**Remarks:** This species has various distinctive characteristics such as thallus color (white, greenish-gray) and apothecia shape (punctate, stellate, branched, round). A. ruanum is characterized by ascospores of 15–25 µm in size, I+ reddish color in hymenium and asci apically K+/+ with bluish color. This species morphologically resembles A. spectabile, but the latter species has a large spore size (c. 25–37 µm) and I+ blue reaction in the hymenium. This is a suboceanic species known to be distributed in Europe, North America and Asia [1, 8].

**Representative specimens examined:** Korea, Jellanam Prov., Goheung County, Bongnae Township, Singeum Village, 34°28′16.05″ N, 127°28′06.07″ E, alt. 15 m, on bark, 25 Apr 2016, J. S. Park & J.-J. Woo, 160058 (KoLRI 038018); Jeju Prov., Jeju, Mt. Halla, Eoseungsaengak trail, 33°23′33.50″ N, 126°29′42.20″ E, alt. 973 m, on bark, 23 May 2016, J. S. Park & S.-O. Oh, 160122 (KoLRI 038257).

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