

The Genus *Chlorociboria*, Blue-Green Micromycetes in South Korea

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Abstract The species of the genus *Chlorociboria* Seaver are very common on the forest floor, and can be easily distinguished by small and numerous blue-green fruitbody, especially the blue substrate dyed with xylindein produced by this group. This genus has rather high species diversity in the Southern Hemisphere, while a little attention was paid to this group in East Asia area. During a field survey in South Korea, several *Chlorociboria* specimens were collected. Based on morphological and phylogenetic analyses, three species of *Chlorociboria* were reported, including one new record in South Korea and one new record in Jeju Island. The key to the species of *Chlorociboria* from South Korea is provided.

Keywords *Chlorociboria*, Molecular phylogeny, New record, South Korea

The genus *Chlorociboria* Seaver belonging to Helotiales is distributed worldwide and is very common on the hardwood debris of sugar maple, poplar, birch, beech, alder, and oak, as well as the softwoods pine and cedar [1, 2]. This genus characterized by its small and numerous blue-green fruitbody typically grows on green-blue-stained wood. The green-blue straining of wood debris caused by infection with *Chlorociboria* species makes this fungus easy to recognize on the forest floor. The blue-green pigment (xylindein) produced by *Chlorociboria* species has no important applications because of the lack of easily obtainable natural or synthetic xylindein [2].

The genus *Chlorociboria* was erected by Seaver (1936) [3] and then placed in the family Helotiaceae, tribe Helotieae [4]. Later, Dixon [1, 5] monographed three genera *Chlorosplenium*, *Chlorociboria*, and *Chlorencoelia*, and accepted four species in *Chlorociboria*, with one divided into two geographically

distinct subspecies. Recently, 15 species were recorded in New Zealand, including 13 new species and 1 new subspecies; while phylogenetic analyses revealed that the genus *Chlorociboria* is a monophyletic group belonging to the Helotiales clade based on neighbor-joining and parsimony analysis [6]. Approximately 19 species have been discovered worldwide mainly based on the spore size and shape [1, 6-

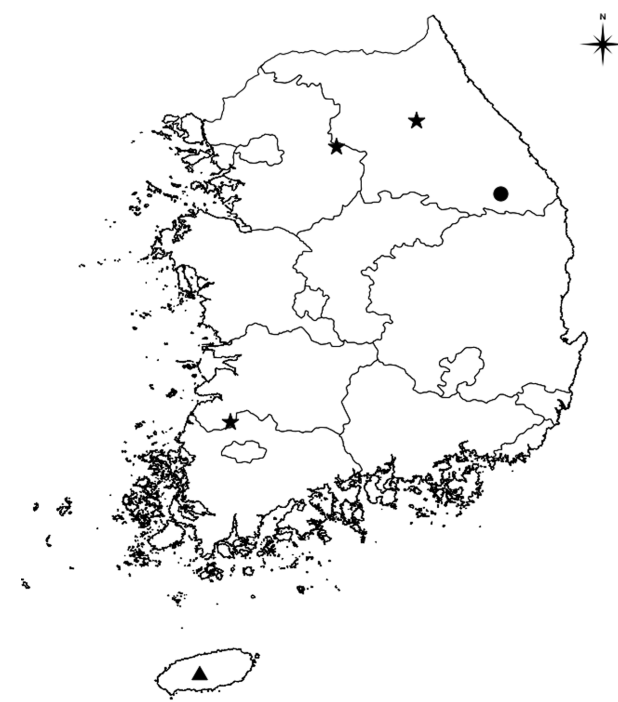


Fig. 1. Distribution of *Chlorociboria* species in Korea. ●, *C. aeruginosa*; ▲, *C. aeruginascens*; ★, *C. poutouensis*.

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8], but only two species have been recorded in the South Korea mainland and Ulleung Island [9, 10]. During a field survey in 2016, several specimens of *Chlorociboria* were collected. Based on morphological and phylogenetic analyses, three species were confirmed, including one new record in South Korea. The distribution (Fig. 1) and key to the *Chlorociboria* species in South Korea are described here.

MATERIALS AND METHODS

Specimens and morphological observation. The samples collected from South Korea were preserved in Korean Lichen Research Institute. Specimen examination and hand-sectioning was conducted under a dissecting microscope (SMZ 745T; Nikon, Tokyo, Japan). All anatomical

Table 1. Materials and Genbank accession number used in this study

Taxon	Collection No.	Accession No.	Location
<i>Botrytis cinerea</i>	isolated, 1423.K	Z73765	Norway
<i>Sclerotinia sclerotiorum</i>	926.P	Z73799	Norway
<i>C. aeruginascens</i>	76435 (PDD)	AY755349	New Zealand
<i>C. aeruginascens</i>	167755 (TRTC)	JX843712	Canada
<i>C. aeruginascens</i>	167754 (TRTC)	JX948104	Canada
<i>C. aeruginascens</i>	19709 (TU)	LT158477	Estonia
<i>C. aeruginascens</i>	BHI-F393 (FH)	KX077900	USA
<i>C. aeruginascens</i>	266501 (HMAS)	KC904942	China
<i>C. aeruginascens</i>	D. Liu 16044 (KoLRI)	KY744737	South Korea
<i>C. aeruginascens</i>	KA12-1719 (KH)	KR673649	South Korea
<i>C. aeruginascens</i> subsp. <i>australis</i>	74101 (PDD)	AY755351	New Zealand
<i>C. aeruginascens</i> subsp. <i>australis</i>		JN943459	New Zealand
<i>C. aeruginascens</i> subsp. <i>australis</i>	74101 (holotype, PDD)	NR119520	New Zealand
<i>C. aeruginascens</i> subsp. <i>australis</i>		JN943458	New Zealand
<i>C. argentinensis</i>	77447 (PDD)	AY755338	New Zealand
<i>C. argentinensis</i>	--	JN943456	New Zealand
<i>C. argentinensis</i>	--	EF520123	Argentina
<i>C. aeruginosa</i>	81292 (PDD)	AY755360	New Zealand
<i>C. aeruginosa</i>	167753 (TRTC), spore culture	JX843710	Canada
<i>C. aeruginosa</i>	167753 (TRTC)	JX948103	Canada
<i>C. aeruginosa</i>	F19715 (UBC)	HQ604856	Canada (British Columbia)
<i>C. aeruginosa</i>	D. Liu 163361 (KoLRI)	KY744738	South Korea
<i>C. pardalota</i>	71611 (PDD)	AY755353	New Zealand
<i>C. poutoensis</i>	60009 (PDD)	AY755352	New Zealand
<i>C. poutoensis</i>	266511 (HMAS)	KC904943	China
<i>C. poutoensis</i>	D. Liu 163383 (KoLRI)	KY744739	South Korea
<i>C. poutoensis</i>	D. Liu 163393 (KoLRI)	KY744740	South Korea
<i>C. macrospora</i>	73994 (PDD)	AY755343	New Zealand
<i>C. macrospora</i>	73994 (PDD)	NR119518	New Zealand
<i>C. duriligna</i>	81278 (PDD)	AY755341	New Zealand
<i>C. duriligna</i>	81278 (PDD)	NR119517	New Zealand
<i>C. duriligna</i>	--	JN943468	New Zealand
<i>C. spathulata</i>	77695 (PDD)	AY755342	New Zealand
<i>C. spathulata</i>	--	JN943463	New Zealand
<i>C. procera</i>	74093 (PDD)	AY755345	New Zealand
<i>C. albohymenia</i>	70089 (PDD)	AY755347	New Zealand
<i>C. campbellensis</i>	74019 (PDD)	AY755357	New Zealand
<i>C. campbellensis</i>	74019 (PDD)	NR119521	New Zealand
<i>C. awakinoana</i>	71672 (PDD)	AY755340	New Zealand
<i>C. awakinoana</i>	--	JN943461	New Zealand
<i>C. clavula</i>	73914 (PDD)	AY755346	New Zealand
<i>C. clavula</i>	--	JN943465	New Zealand
<i>C. aeruginella</i>	104542 (TU)	LT158478	Estonia
<i>C. halonata</i>	71675 (PDD)	AY755354	New Zealand
<i>C. halonata</i>	71610 (PDD)	AY755355	New Zealand
<i>C. spiralis</i>	77771 (holotype, PDD)	NR119519	New Zealand
<i>C. spiralis</i>	77771 (PDD)	AY755348	New Zealand

Letters in bold indicate newly generated sequences in this study.

descriptions are based on observations of these preparations under an Olympus BX 50 microscope (Tokyo, Japan) and photos were acquired under a Carl Zeiss MicroImaging microscope with Axio Cam ERc 5s imaging system (Jena, Germany). All measurements of the sections were conducted in water. The dimension range of ascospores was determined by measuring 30–50 ascospores.

DNA isolation, PCR, and DNA sequencing. Total genomic DNA was extracted from freshly collected specimens using the NucleoSpin Plant II Kit (Clontech Laboratories, Mountain View, CA, USA) following the manufacturer's instructions. The internal transcribed spacer (ITS) region was generated for new records. Primers pairs ITS1F [11]/ITS4 [12] were used to amplify the ITS. Protocols for PCR amplification and sequencing were conducted as described by Liu *et al.* [13].

Sequence alignment. Newly generated ITS sequences and selected sequences of *Chlorociboria* from GenBank (Table 1) were assembled using SeqMan (DNASTAR packages) and edited by BioEdit 7.09 [14], and then automatically aligned with MAFFT v7.273 [15]. Ambiguous regions were identified and excluded using Gblocks [16] with the following parameters: for a conserved position, 24; minimum number of sequences for a flanking position, 25; maximum number of contiguous non-conserved positions, 3; minimum length of a block, 2; and allowed gap positions, with half.

Phylogenetic analysis. Maximum likelihood (ML) optimality criterion and Bayesian inference were used to construct phylogenetic trees. Gaps were regarded as missing. TIM + I + G were selected best-fitted substitution models based on the Akaike Information Criterion using jModelTest 3.7 [17]. Bayesian inference analyses were performed with MrBayes v3.1.2 [18] using 4 chains and run for 1 million generations. Trees were sampled every 100 generations. Chain convergence was determined using Tracer v1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) to ensure sufficiently large estimated sample size values. The stop rule was used when parallel Markov chain Monte Carlo runs converged (estimated sample size value > 200). Phylogenetic trees were summarized using the sumt and sumt commands with burn-ins discarded. Bayesian posterior probabilities were estimated from the frequencies of branches among all trees, and clades with posterior probabilities ≥ 0.95 were considered as significantly supported. ML inferences were made using RAxML v7.2.6 [19] with the GTR-GAMMA model. Bootstrap frequencies were estimated from the consensus tree built with 2,000 trees obtained from nonparametric bootstrapping pseudoreplicates. *Botrytis cinerea* and *Sclerotinia sclerotiorum* were selected as the outgroup.

RESULTS AND DISCUSSION

The ITS matrix aligned by MAFFT, contains 47 sequences,

including 16 species and 1 subspecies of *Chlorociboria*, and the matrix contains 496 sets. After Gblocks, 44 positions were ambiguously aligned and 452 (91%) positions were reserved for phylogenetic analyses. The phylogenetic tree is shown in Fig. 2. In contrast to previous studies [2, 6], the ITS phylogenetic tree in this study indicated that the species of *Chlorociboria* forms a strong supported monophyletic clade except for with *C. argentinensis*. *Chlorociboria poutouensis* from Korea is more closely related to the China collection (KC904943) than the New Zealand specimen (AY755352). Based on morphological and phylogenetic analyses, three species were confirmed in South Korea, and *C. poutouensis* was newly reported in South Korea.

Taxonomy.

Chlorociboria aeruginascens (Nyl.) Kanouse ex C.S. Ramamurthi, Korf & L.R. Batra (Fig. 3A, D, G, J).

≡ *Peziza aeruginosa* Pers per Pers.: Fr (var.) b. subgrisea Fr. Syst. Mycol 2: 130 (1822).

≡ *Peziza aeruginascens* Nyl., Not. Sällsk. Fauna F1. Förh. 10: 42 (1869).

Apothecia superficial, develop on decayed or decorticated stump or wood, solitary to gregarious, usually several apothecia arising from the same dark mycelial patch; disc cup-shape and discoid, < 1.5 mm, blue-green to dark blue; receptacle glabrous when fresh and tomentose when dry, concolorous with disc or much more aeruginous; stipe central or slightly eccentric, long, up to 5 mm, concolorous with receptacle, usually become dark towards the base; hymenium hyaline to aeruginous, 66–70 μm ; subhymenium hyaline, textura intricata, 5–18 μm ; medullary excipulum hyaline, textura loosely intricata, 80–98 μm ; ectal excipulum textura angularis, blue-green, 26–44 μm , surface cells giving rise to straight or coiled few to numerous tomentum hyphae; asci cylindric-clavate, slightly tapered towards the apex and long stalk, 55–63 $\times$ 3–3.8 μm , 8-ascospores; ascospores fusiform-elliptic, with blunt apices, hyaline or with green content, prominently biguttulate or with several smaller guttules, 5.6–7.5 $\times$ 1–1.8 μm .

Habitat: It grows on decayed and decorticated wood like *Alnus*, *Betula*, *Carpinus*, *Salix*, *Quercus*.

Specimen examined: Korea, Jeju-do, Seogwipo-si, Namwon-eup, Mt. Halla, Yeongsil trail, 33°21'11" N, 126°29'48" E, 1,290 m, on rotten wood, 4 Jul 2016, D. Liu 16044.

Remarks: This species can be easily distinguished by its longer stalk and frequently several apothecia arising from the same dark mycelial patch. *C. aeruginascens* is most similar to *C. aeruginosa*, but it differs from the later owing to the much smaller ascospores (5.6–7.5 $\times$ 1–1.8 μm vs. [11.7]12.45–16.58[16.98] $\times$ 2.3–3.2 μm), Park *et al.* [9] reported *C. aeruginascens* in Gwangneung and Paldasan, and the size of asci (77.5–110 $\times$ 7.5–10 μm) and ascospores (17.5–25[16.98] $\times$ 4–5 μm) they recorded were much larger than those of our samples and other collections from North America and China [1, 20]; therefore, it may be a misidentification of this species. Comparison with the

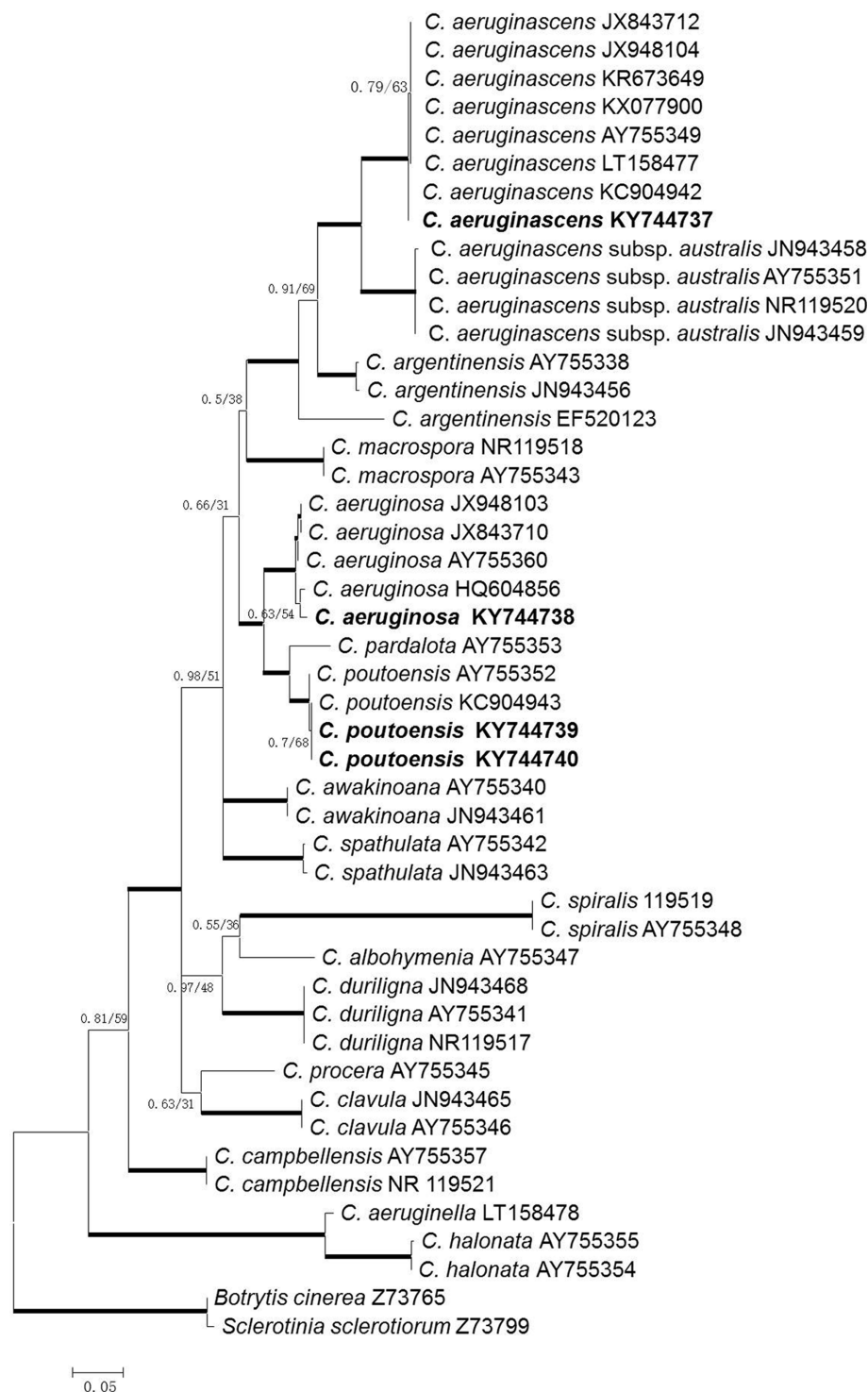


Fig. 2. Phylogenetic relationships between species of *Chlorociboria* inferred from internal transcribed spacer sequences under maximum likelihood criteria. Nodes supported by maximum likelihood bootstrap values > 75% and Bayesian posterior probabilities > 0.95 are identified by thickened branches. Those for which only one value is significant are annotated.

description of Dixon (1975) [1] based on materials from the United States, Canada, and China, the South Korean collections have smaller apothecia (< 2 mm vs. disc < 5 mm in diam.). The blue-green straining into the wood is deeper than that appearing on the *C. aeruginosa*, frequently up to

5 mm; this species is a new record of Jeju Island.

Chlorociboria aeruginosa (Oeder) Seaver ex C. S. Ramamurthi, Korf & L. R. Batra (Fig. 3B, 3E, 3H and 3K).
≡ *Helvella aeruginosa* Oeder, Fl. Danic. 3: tab. 534: 2 (1770).

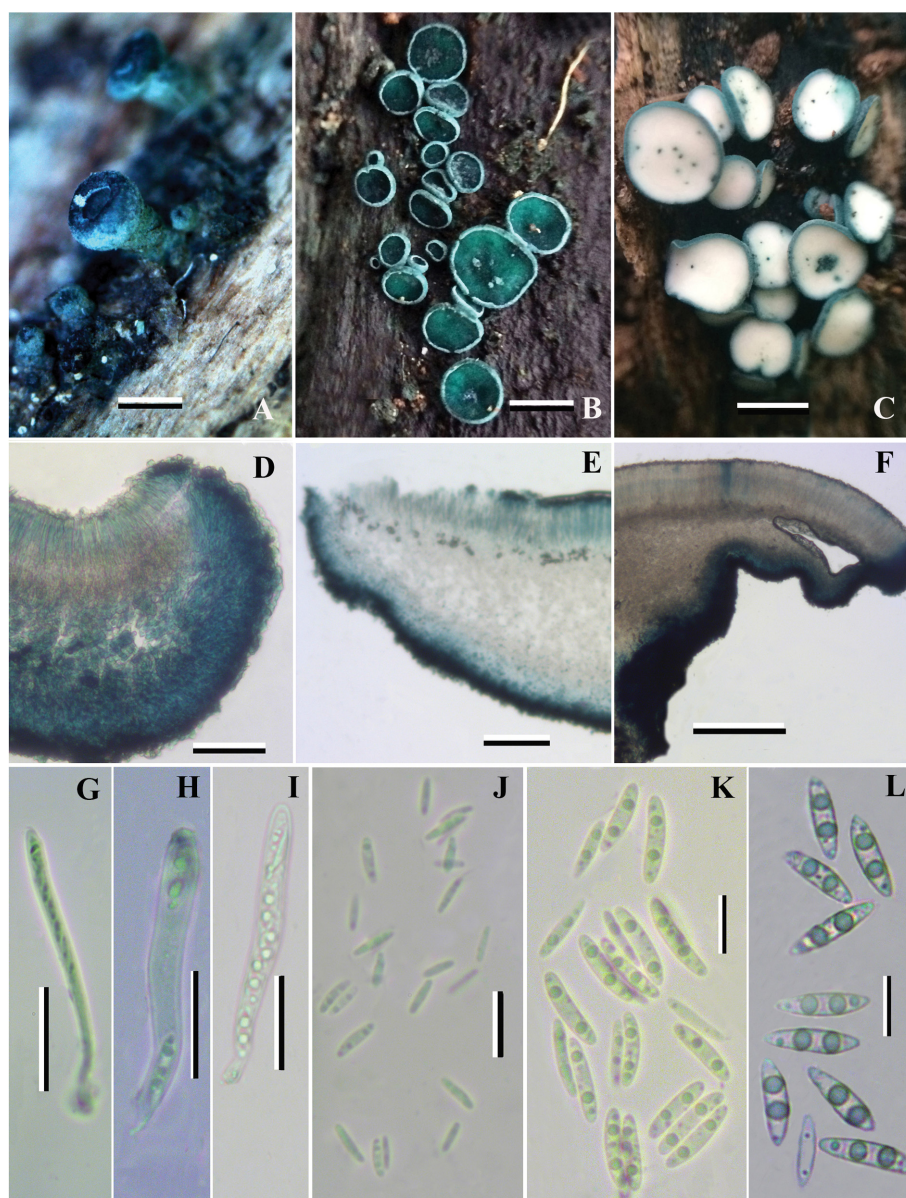


Fig. 3. Photographs of *Chlorociboria* spp. from South Korea. *C. aeruginascens* (D. Liu 16044): A, Habitat; D, Vertical section of apothecia; G, Asci; J, Ascospores. *C. aeruginosa* (D. Liu 163361): B, Habitat; E, Vertical section of apothecia; H, Asci; K, Ascospores. *C. poutouensis* (D. Liu 163425): C, Habitat; F, Vertical section of apothecia; I, Asci; L, Ascospores (scale bars: A = 0.5 mm, B, C = 1 mm, D, F = 200 μ m, E = 100 μ m, G, H = 20 μ m, I = 25 μ m, J–L = 10 μ m).

≡ *Helotium aeruginosum* (Oeder) Gray, Nat. Arr. Brit. Pl. (London) 1: 661 (1821).

≡ *Chlorosplenium aeruginosum* (Oeder) De Not., Discom.: 22 (1864).

≡ *Chlorosplenium discoideum* Masee, Brit. Fung. -Fl. (London) 4: 286 (1895).

Apothecia superficial, develop on decayed or decorticated stump or wood, solitary to gregarious, but never with several apothecia arising from the same mycelial patch; disc cup-shape and discoid, 0.5–3 mm, blue-green to dark blue; receptacle glabrous when fresh and tomentose when dry, concolorous with disc or much more aeruginous; stipe

central or slightly eccentric, up to 1.8 mm, concolorous with receptacle, typically becomes dark towards the base; hymenium hyaline to aeruginous, 68–106.6 μ m; subhymenium hyaline, textura intricata, 6–20 μ m; medullary excipulum hyaline, textura loosely intricata, 125–280.5 μ m; ectal excipulum textura angularis, hyaline to blue-green, 26–40 μ m, surface cells giving rise to straight or coiled, strongly granulate tomentum hyphae; asci cylindric-clavate, slightly tapered towards the apex and long stalk, 58–78 $\times$ 5.3–6.8 μ m, 8-ascospores; ascospores fusiform-elliptic, with blunt apices, hyaline or with green content, prominently biguttulate or with several smaller guttules, (11.7)12.45–16.58(16.98) $\times$

2.3–3.2 µm.

Habitat: This species grows on decayed and decorticated wood such as *Abies*, *Alnus*, *Betula*, *Carpinus*, *Salix*, *Pinus*, *Quercus*.

Specimen examined: Korea, Gangwon-do, Jeongseon-gun, Gohan-eup, 37°11'25.03" N, 128°53'31.31" E, 900 m, on rotten wood, 22 Sep 2016, D. Liu 163361.

Remarks: This species is easily distinguished by the size of ascospores and granulate tomentum hyphae. The blue-green straining typically invades the surface of the wood.

Chlorociboria poutouensis P. R. Johnst. (Fig. 3C, 3E, 3I and 3L).

Apothecia superficial, develop on decay or decorticated stump or wood, solitary to gregarious, never with several apothecia arising from the same dark mycelial patch; disc cup-shape and discoid, 0.8–3 mm, white to slightly blue-green when fresh, bright yellow to orange when dry; receptacle glabrous aeruginous or dark blue-green; stipe central or slightly eccentric, up to 1.2 mm, dark blue-green near top, typically becomes black toward the base; hymenium hyaline to aeruginous, 80–110 µm; subhymenium hyaline, textura intricata, 20–31 µm; medullary excipulum hyaline, textura loosely intricata, 170–190 µm; ectal excipulum textura angularis, blue-green, 40–70 µm, surface cells giving rise to straight or coiled few to numerous tomentum hyphae; asci cylindric-clavate, slightly tapered towards the apex and long stalk, 80–100 × 7.5–9 µm, 8-ascospores; ascospores fusiform-elliptic, with blunt or slightly sharp apices, hyaline or with green content, prominently biguttulate or with several smaller guttules, (9.79)12.65–17.48 × 3.1–4.2 µm.

Habitat: This species grows on decayed and decorticated wood such as *Alnus*, *Betula*, *Carpinus*, *Salix*, *Quercus*.

Specimen examined: Korea, Gangwon-do, Hongcheon-gun, Seoseok-myeon, 37°46'38.45" N, 128°13'34.35" E, 726 m, on the rotten stump, 24 Sep 2016, D. Liu 163380, 163383; Gyeonggi-do, Yongmun-myeon, Yangpyeong-gun, 37°33'56.41" N, 127°35'17.85" E, 560 m, on the rotten stump, 25 Sep 2016, D. Liu 163393; Gyeonggi-do, Namyangju-si, Sudong-myeon, Mt. Chungnyeong, 35°22'14.92" N, 126°44'14.21" E, 335 m, on rotten wood, 28 Sep 2016, D. Liu 163425.

Remarks: *Chlorociboria poutouensis* is distinguished by a hymenium more or less white or pale yellow when fresh and yellow to orange when dry. This species was recently recorded in China [20] and New Zealand [6]; compared with their description, specimens in South Korea shared similar characters in disc, stipe, asci, and ascospores, but the hymenium is shorter.

Key to *Chlorociboria* in Korea

1. Never with several apothecia arising from the same dark mycelial patch; the blue-green straining typically invades the wood surface (< 3 mm); ascospores large, 12.45–17.48 × 2.3–4.2 µm 2

1. Frequently with several apothecia arising from the same dark mycelial patch; the blue-green straining typically invades the wood deeper (> 3 mm); ascospores small, 5.6–7.5 × 1–1.8 µm *C. aeruginascens*
2. Disc less white to pale yellow when fresh and bright yellow to orange when dry; ascospores sharper and wider *C. poutouensis*
2. Disc blue-green; ascospores blunter and narrower *C. aeruginosa*

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