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# A new species of *Chlorociboria* (Helotiales, Ascomycota) on herbaceous stems from China

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## Abstract

A new species, namely *Chlorociboria herbicola*, is discovered on herbaceous stems in central China. Morphologically, the new fungus is distinctive by the combination of light blue-green apothecia, rectangular cells in ectal excipulum, and elongate-ellipsoidal ascospores with rounded ends. Phylogenetic analyses of the internal transcribed spacer and large subunit of nuclear ribosomal DNA sequences confirm its ascription in *Chlorociboria* and distinction from the known species of the genus.

**Key words:** Morphology, Sequence analyses, Taxonomy

## Introduction

*Chlorociboria* Seaver ex C.S. Ramamurthi, Korf & L.R. Batra is a wide-spread genus in the world. It is characterized by apothecia cupulate, infundibuliform or discoid, stipitate, with blue-green pigmentations; ectal excipulum composed of angular, subglobose to elongate cells; hyaline and interwoven hyphae in medullary excipulum; cylindric-clavate asci with a J+ apical ring in IKI or Melzer's reagent; and hyaline ascospores of various shapes (Ramamurthi *et al.* 1957, Dixon 1975, Johnston & Park 2005, Ren & Zhuang 2014). *Chlorociboria* species produce green stain in dead and decayed wood in forests (Ramamurthi *et al.* 1957, Dixon 1975, Johnston & Park 2005). The green-stained wood caused by *Chlorociboria* species was used as decoration since the 15th century (Blanchette *et al.* 1992). The blue-green pigment produced by *Chlorociboria* species is xylindein, which has been studied extensively (Edwards & Kale 1965, Saikawa *et al.* 2000, Donner *et al.* 2012). Using *Chlorociboria* species and xylindein for wood spalting to increase their commercial value has been explored recently (Robinson 2012, Robinson *et al.* 2012; Robinson *et al.* 2014).

About 23 species are currently accepted in *Chlorociboria* (Kirk *et al.* 2008, Huhtinen *et al.* 2010; Pärtel *et al.* 2017; Index Fungorum 2017), among which *C. aeruginascens* (Nyl.) Kanouse ex C.S. Ramamurthi, Korf & L.R. Batra and *C. aeruginosa* (Oeder) Seaver ex C.S. Ramamurthi, Korf & L.R. Batra are most commonly seen. The morphology of sexual and asexual states, culture features and sequence analyses of the two fungi were elaborated based on North American materials (Tudor *et al.* 2014). Taxonomy of the genus was mainly based on morphology (Seaver 1936, Ramamurthi *et al.* 1957, Dixon 1975, Trierveiler-Pereira *et al.* 2008). Johnston & Park (2005) described 13 new species and one new subspecies upon morphological features and tested their reliability using sequence analysis of the internal transcribed spacer (ITS) of nuclear ribosomal DNA.

Three *Chlorociboria* species, *C. aeruginascens*, *C. aeruginosa* and *C. poutoensis* P.R. Johnst., have been reported from China (Teng 1963, Tai 1979, Ren & Zhuang 2014). During our field trip in 2014, a small light blue-green discomycete growing on herbaceous stem was collected and tentatively identified as *Chlorociboria* sp. in the field notes. Further microscopic examinations and sequence analyses of ITS and large subunit (LSU) of nuclear ribosomal DNA indicate it is a novel species of *Chlorociboria*.

## Materials and methods

### Morphological observation

The fresh specimen was collected and photographed by a Canon PowerShot G16 digital camera in September 2014 from Hubei Province, China. After taking a field note, the specimen was dried and deposited in the Herbarium Mycologicum

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Academiae Sinicae (HMAS). Dried apothecia were rehydrated in distilled water and sectioned at a thickness of 20−23 µm with a Yidi YD-1508A freezing microtome (Jinhua, China). Measurements were taken from longitudinal sections and squash mounts in lacto-phenol cotton blue solution using Olympus BH-2 microscope (Tokyo, Japan). Iodine reactions of ascus apparatus were observed in Melzer's reagent and Lugol's solution with or without 3% KOH solution pretreatment according to Baral (2009). Images were captured using Leica M125 stereomicroscope (Wetzlar, Germany) for gross morphology and Zeiss Axio Imager A2 microscope (Göttingen, Germany) for anatomical structure.

**DNA extraction, PCR amplification and sequencing**

Genome DNA was extracted from pure culture using the CTAB procedure (White *et al.* 1990) with some modification. Primer pairs used for amplification and sequencing included ITS1/ITS4 (White *et al.* 1990) for ITS, LR0R (Moncalvo *et al.*1995) and LR5 (Vilgalys & Hester 1990) for D1/D2 domain of LSU. PCR amplification had a final volume of 25 µl, containing 12.5 µl of 2×Taq MasterMix (Beijing CWBiotech, China), 1.25 µl of each primer (10 mM) and 2 µl of DNA template. PCR reactions were conducted using an Applied Biosystems 2720 thermocycler (Foster City, CA, USA) under the following conditions: an initial step at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 30−45 s at 72 °C, and by a final extension at 72 °C for 10 min. The PCR products were purified and sequenced at Beijing Tianyi Huiyuan Bioscience and Technology, China.

**Sequence assembly, alignment and phylogenetic analyses**

Forward and reverse sequences were assembled and edited using BioEdit 7.0.5.3 (Hall 1999). The newly generated sequences were deposited in GenBank and additional sequences were retrieved from GenBank (Table 1).

**TABLE 1.** Sequences used in this study.

| Species | Voucher | ITS | 28S |
|---|---|---|---|
| *Chlorociboria aeruginascens* subsp. *australis* P.R. Johnst. | D1347 | JN943459 | JN939931 |
| *C. aeruginascens* subsp. *australis* | D923 | JN943460 | JN939939 |
| *C. aeruginosa* (Oeder) Seaver ex C.S. Ramamurthi, Korf & L.R. Batra | AFTOL-ID 151 | DQ491501 | AY544669 |
| *C. argentinensis* J.R. Dixon | ICMP:16994 (SA86) | EF520124 | JN939919 |
| C. argentinensis | ICMP:16995 (SA188) | EF520123 | JN939930 |
| *C. awakinoana* P.R. Johnst. | D1575ss2 | JN943461 | JN939921 |
| C. awakinoana | D1549asc1 | JN943462 | JN939922 |
| *C. clavula* P.R. Johnst. | D1594asc2 | JN943465 | JN939924 |
| C. clavula | D1611 | JN943466 | JN939941 |
| *C. duriligna* P.R. Johnst. | D518 | JN943467 | JN939925 |
| C. duriligna | D1814 | JN943468 | JN939934 |
| *C. halonata* P.R. Johnst. | D1530asc1ss1 | JN943469 | JN939933 |
| C. halonata | D2137 | JN943471 | JN939935 |
| C. halonata | D1553 | AY755354 | JN939936 |
| *C. herbicola* H.D. Zheng & W.Y. Zhuang | HMAS 273905 | **KY498614*** | KY498616 |
| *C. spathulata* P.R. Johnst. | D1822 | JN943463 | JN939923 |
| C. spathulata | D1725 | AY755342 | JN939942 |
| *Crocicreas coronatum* (Bull.) S.E. Carp. | F-176,601 | FJ005106 | FJ005129 |
| *Cudoniella clavus* (Alb. & Schwein.) Dennis | AFTOL-ID | DQ491502 | DQ470944 |
| *Dicephalospora huangshanica* (W.Y. Zhuan) W.Y. Zhuang & Z.Q. Zeng | KUS-F52405 | JN033408 | JN086711 |
| *D. rufocornea* (Berk. & Broome) Spooner | KUS-F52274 | JN033401 | JN086704 |
| *Hymenoscyphus caudatus* (P. Karst.) Dennis | 1105 | AY348576 | KJ472234 |
| *H. fructigenus* (Bull.) Gray | HMAS 275508 | **KY498615** | KY498617 |
| *Lambertella corni-maris* Höhn. | CLX2872 | KC958553 | KC964849 |
| *Rhizoscyphus ericae* (D.J. Read) W.Y. Zhuang & Korf | UAMH 6735 | NR_111110 | AM887699 |
| *Rutstroemia firma* (Pers.) P. Karst. | KL292 | LT158450 | KX090832 |
| *Sclerotinia sclerotiorum* (Lib.) de Bary | 1980 | DS267914 | DS267914 |
| *Vibrissea truncorum* (Alb. & Schwein.) Fr. | CBS_258.91/CUP-62562 | EU434854 | AY789402 |

* Numbers in bold indicating sequences produced by this study.

Alignment was initially generated using Muscle 3.8.31 (Edgar 2004), manually edited with BioEdit 7.0.5.3, and converted to nexus files in ClustalX 1.83 (Thompson *et al.* 1997). Combined dataset of ITS and LSU was used

for phylogenetic analyses. Partition homogeneity test was performed in PAUP* 4.0b10 (Swofford 2003) with 1000 replicates to confirm the combination of the two regions. Maximum parsimony (MP) and Neighbor-joining (NJ) analyses were carried out using PAUP*4.0b10 with parameters used by Zheng & Zhuang (2014, 2016). All characters were treated as unordered and equally weighted, and gaps were treated as missing data. MP analysis was done with the heuristic search option using max trees set to 1000 and auto-increased by 100, TBR branch swapping. NJ analysis was performed using default settings. Branch supports of the MP and NJ trees were evaluated from 1000 bootstrap replications. The resulting trees were viewed in TreeView 1.6.6 (Page 1996).

## Results

### Taxonomy

***Chlorociboria herbicola*** H.D. Zheng & W.Y. Zhuang, *sp. nov.* Fig. 1

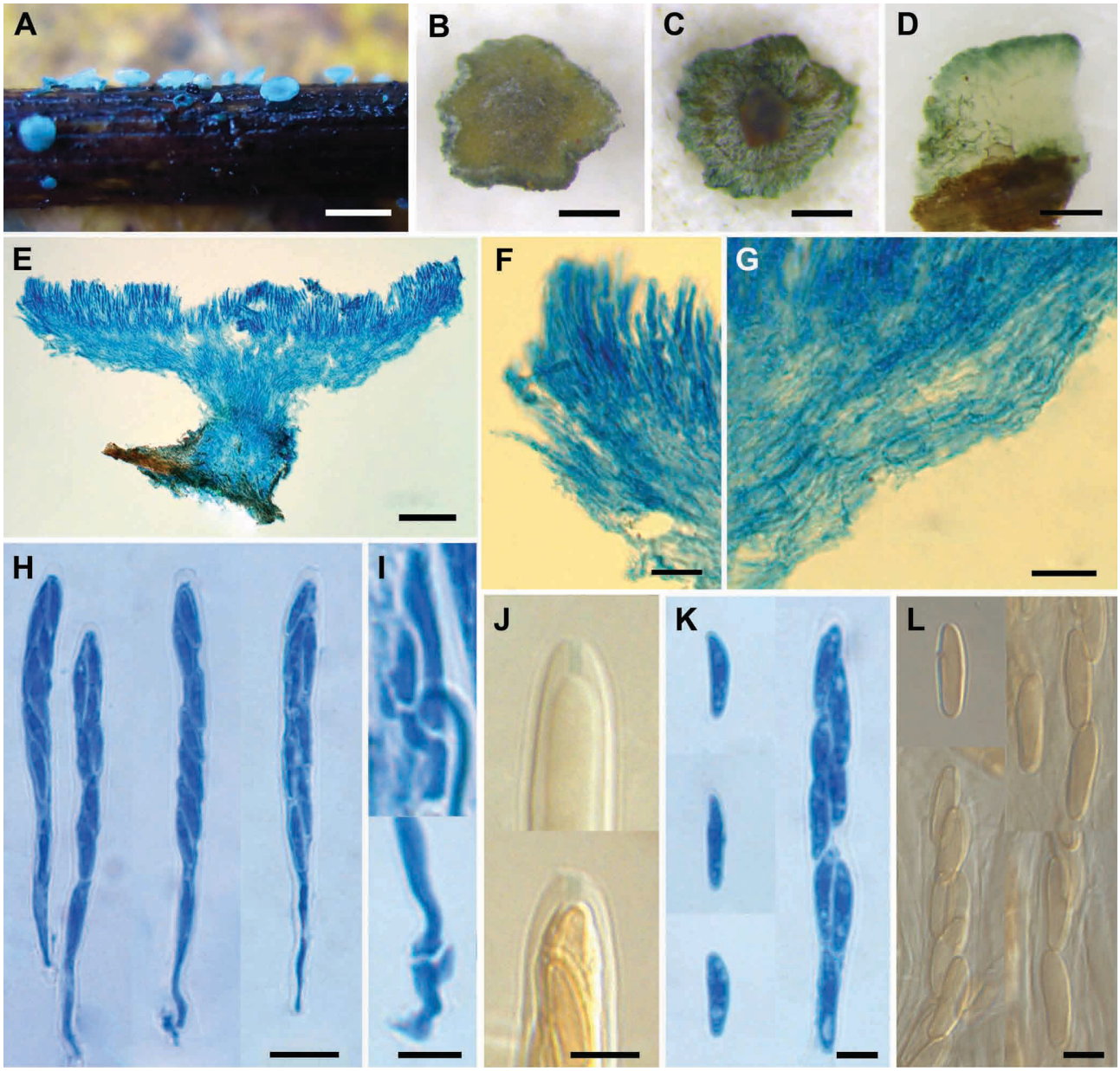


**FIGURE 1.** *Chlorociboria herbicola* H.D. Zheng & W.Y. Zhuang (HMAS 273905). A: Fresh apothecia on natural substrate. B: Dry apothecium (upper surface). C: Dry apothecium (lower surface). D: Partial rehydrated apotheium (lower surface); E: Longitudinal section of apothecium. F: Structure of margin. G: Excipular structure of flank. H: Asci. I: Croziers at ascus bases. J: IKI reaction of apical rings. K & L: Ascospores. *Mounting media*: D water; E–I, K lactophenol cotton blue; J & L Melzer's reagent. *Bars:* A 2 mm; B–D 0.2 mm; E 100 µm; F & G 20 µm; H 10 µm; I–L 5 µm.

Fungal Names FN570380

**Type**:—CHINA. Hubei Province: Shennongjia, Dalongtan, alt. 2000 m, 14 September 2014, on dicotyledonous herbaceous stem, *H.D. Zheng, Z.Q. Zeng, W.T. Qin & K. Chen 9528* (holotype, HMAS 273905).

**Etymology**:—The specific epithet refers to the substrate of the fungus.

Apothecia scattered, discoid, flat to slightly concave when fresh, centrally stipitate, 0.5–1.2 mm in diam.; hymenium surface light blue-green when fresh, orange with blue-green tint when dry, receptacle surface concolorous with hymenium, glabrous or nearly so; stipe concolorous and homogenous with receptacle, 0.3–0.5 mm long. Ectal excipulum of textura prismatica, non-gelatinous, 25–45 µm thick, hyphae parallel to receptacle surface, cells subhyaline or with pale bluish green pigments, with walls thin to slightly thick, 12–20 × 4–6 µm. Medullary excipulum of textura porrecta to textura intricata, 40–110 µm thick, hyphae hyaline, 2–3 µm wide. Subhymenium not distinguishable. Hymenium subhyaline, 70–90 µm thick. Asci arising from croziers, 8-spored, cylindrical-clavate, apex round, J+, apical ring bluing as two lines in Melzer's reagent and Lugol's solution without KOH pretreatment, *Hymenoscyphus*-type, 60–70 × 5.5–7 µm. Ascospores irregularly uniseriate to irregularly biseriate in the asci, elongate-ellipsoidal with rounded ends, equilateral to slightly flattened at one side, non-septate, hyaline, containing a few small to medium-sized guttules, 10.5–13.2 × 2.8–3.5 µm. Paraphyses cylindrical, hyaline, 1.5−2.2 µm wide, not exceeding the asci.

**Sequence analyses**

A total of eight ingroup taxa and eleven outgroup taxa were included in the analyses. The combined dataset of ITS and LSU consisted of 1516 base pairs with 375 parsimony-informative characters. Two equally parsimonious trees were yielded (TL = 1380, CI = 0.450, RI = 0.695, RC = 0.313) and one of them was shown with bootstrap proportions (BP) of MP and NJ at the nodes (Fig. 2). All the investigated *Chlorociboria* species clustered together with high bootstrap supports (97% MPBP and 99% NJBP). *Chlorociboria herbicola* appeared as a distinct linage within *Chlorociboria* and formed a highly supported group with *C. halonata* P.R. Johnst. (100% MPBP and 100% NJBP).

## Discussion

*Chlorociboria herbicola* is a remarkable species with unique morphological and molecular features different from any other members of the genus. The most distinctive features of the fungus are herbaceous habit and rectangle ectal excipular cells with axes nearly parallel to receptacle surface. All the previously known *Chlorociboria* species are growing on green stained wood or bark, ectal excipular cells with darker greenish pigments or covered by green exudates, if elongate with thickened and somewhat gelatinous walls, and oriented at a high angle to receptacle surface (Dixon 1975; Johnston & Park 2005).

*Chlorociboria campbellensis* P.R. Johnst., originally reported from New Zealand, resembles *C. herbicola* in shape of apothecia and size of asci and ascospores, but differs in larger apothecia (1.2–2 mm in diam.), ectal excipulum composed of gelatinous textura intricata, presence of tomentum hyphae with rough walls on receptacle surface, and growing on bark of blue-green stained fallen branches (Johnston & Park 2005).

The new species is also similar to *C. procera* P.R. Johnst. in ascospore shape, but the latter has a long stipe with scale-like elements on surface, ectal excipulum encrusted with dark green exudates, composed of textura angularis or textura prismatica, cells oriented at a high angle to receptacle surface, larger ascospores [(12–)13.5–15(–17) × (3–)3.5–4 µm], and occurring on blue-green stained bark and decorticated wood (Johnston & Park 2005).

Phylogenetically, *C. herbicola* and *C. halonata* appeared to be closely related (Fig. 2). However, *C. halonata* is quite different in larger apothecia (1.5–3 mm), ectal excipulum composed of textura angularis to textura prismatica oriented at a high angle to receptacle surface and thickly encrusted with dark green matters, coiling tomentum hyphae with rough walls, larger asci (70–100 × 7.5–8.5 µm), larger ascospores (18.5–22 × 2.5–3 µm) with acute ends, and developing on blue-green stained decorticated wood or bark of fallen branches (Johnston & Park 2005).

Combined study of morphological and molecular data plays an important role in the identification of *C. herbicola*. At first glance, the light blue-green color of apothecia indicates that it might belong to *Chlorociboria*, but its herbaceous habit and axes of ectal excipular hyphae nearly parallel to receptacle surface have never been reported in the genus. Phylogenetic analyses of ITS and LSU sequences prove its position in *Chlorociboria*. The genus shows rich diversity of gross morphology and anatomy with the new species added.

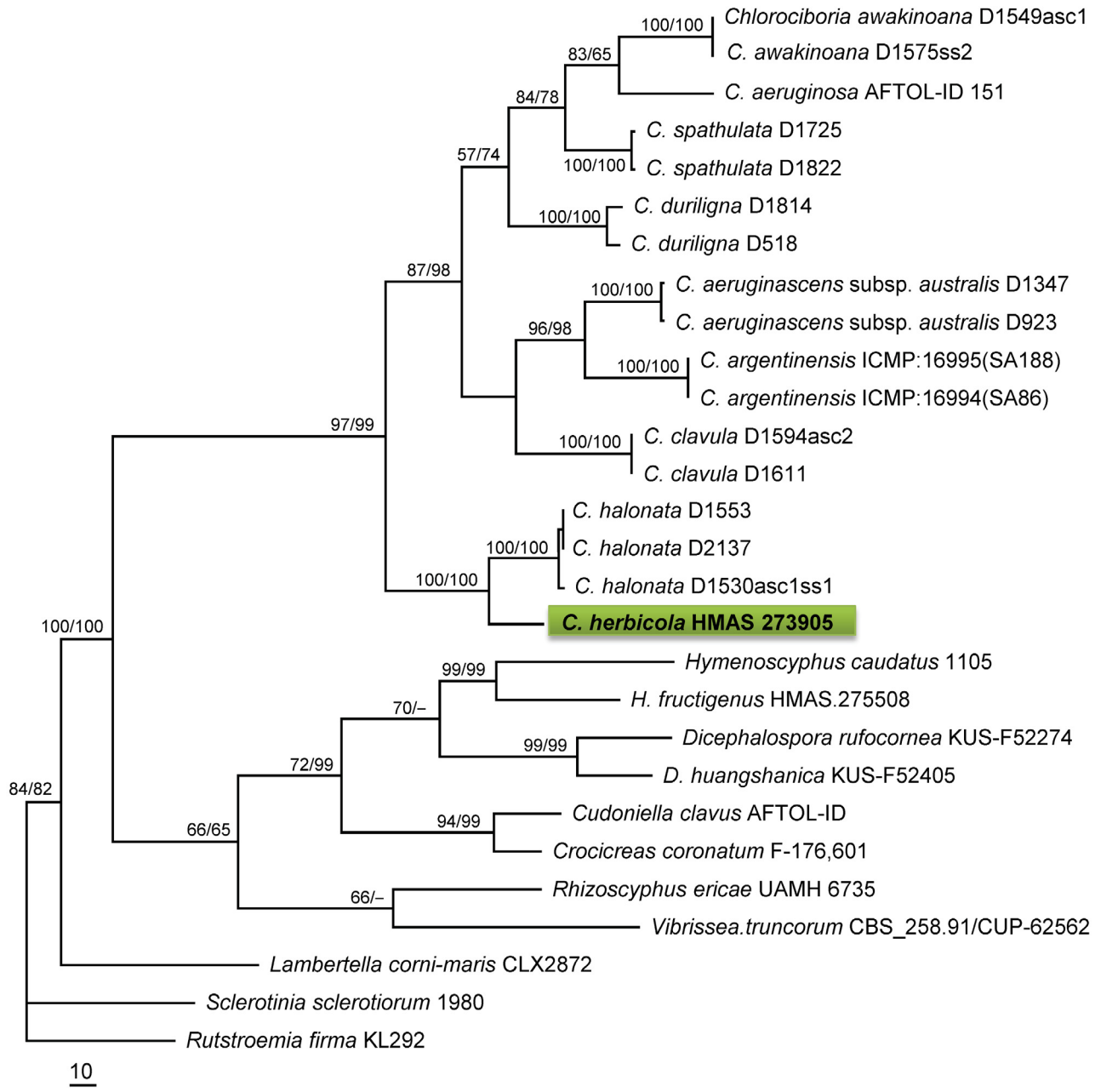


**FIGURE 2.** Maximum parsimony tree inferred from combined dataset of ITS and LSU showing the phylogenetic position of *Chlorociboria herbicola*. Bootstrap values (>50%) of maximum parsimony and neighbor-joining are indicated at the nodes from left to right.


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