




## *Geoglossum subdifforme* sp. nov. and *G. simile*, Two New Earth Tongues from South Korea

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### ABSTRACT

During an investigation of Korean Ascomycetes in 2023, we found two undescribed species from South Korea. We analyzed them using a combined approach, including morphological and phylogenetic analyses of the rDNA regions (internal transcribed spacer and large subunit). The two species were identified to belong to the genus *Geoglossum*; the species *G. simile* and a new species named *G. subdifforme* sp. nov. The phylogenetic tree constructed using the ITS region showed that *G. subdifforme* is closely related to *G. difforme*. These species are distinguishable by certain morphological characteristics, particularly the size and septae of ascospores. Morphologically, *G. simile* is related to *G. glabrum*, but it is distinguishable by the morphological characteristics of paraphyses as well as ITS sequences. In this study, the descriptions, photographs, and phylogenetic relationships of these *Geoglossum* species are presented.

### ARTICLE HISTORY

Received 1 October 2024  
Revised 2 December 2024  
Accepted 6 December 2024

### KEYWORDS

Geoglossales;  
Geoglossaceae; ITS; LSU;  
morphology; phylogeny

Traditionally, the family *Geoglossaceae*, commonly known as earth tongues, included several genera, such as *Geoglossum* Pers., *Trichoglossum* Boud., *Microglossum* Gillet, *Spathularia* Pers., *Leotia* Pers., *Gudonia* Fr., *Mitrla* Fr., among others [1]. However, recent molecular phylogenetic approaches revealed that the genera formerly considered characteristic earth tongue genera do not represent a monophyletic group [2–5]. Thus, Schoch et al. [5] established the class *Geoglossomycetes* Zheng Wang, C.L. Schoch & Spatafora and order *Geoglossales* Zheng Wang, C.L. Schoch & Spatafora derived from an analysis of a six-gene sequence dataset. Today, according to the studies of several fungal taxonomists, eight genera are included in this class: *Geoglossum*, *Glutinoglossum* Hustad, A.N. Mill., Dentiger & P.F. Cannon, *Hemileucoglossum* S. Arauzo, *Leucoglossum* S. Imai, *Nothomitra* Maas Geest., *Sabuloglossum* Hustad, A.N. Mill., Dentinger & P.F. Cannon, *Sarcoleotia* S. Ito & S. Imai, and *Trichoglossum* [5–8].

The genus *Geoglossum* in the family *Geoglossaceae* Corda was originally described by Christian Hendrik Persoon in 1794; it currently contains 28 species commonly known as “dark earth tongues” or “black earth tongues” [5, 9]. Although associations between several *Geoglossum* species and various plants and mosses have been hypothesized [10–12], the ecology of *Geoglossum* species and their phylogenetic relationships has yet to be fully understood [7, 13]. Generally, *Geoglossum* species are saprophytic on humus, soil, moss, and

occasionally rotten wood. They are characterized by black or dark brown fruiting bodies consisting of a fertile club- or tongue-shaped head and a sterile stipe. Because of their undifferentiated macro-morphology, their microscopic characters are critical for species discrimination, especially in terms of the size and shape of ascospores, color and number of septae, and morphology of paraphyses [7, 13, 14].

Several *Geoglossum* species are of conservation significance and many are found on regional red lists of several European countries [7]. In South Korea, only two *Geoglossum* species have been recorded (*G. fallax* and *G. glabrum*, according to the ‘Index of Korean Mushroom’; <http://www.nature.go.kr/kfni/index.do>); however, molecular phylogenetic confirmation of these specimens has not been carried out. Therefore, it is considered necessary to collect and reevaluate these Korean species.

During an investigation of Korean Ascomycetes in 2023, we found three *Geoglossum* specimens from South Korea. The three specimens were deposited in the herbarium of the Korea National Arboretum (KH). Macromorphological descriptions were obtained from field notes and color photographs of the fruiting bodies. Micromorphological data were obtained from the dried specimens using a light microscope after sectioning and rehydration. DNA was isolated from fresh fruiting bodies (samples of approximately 0.1 g) using a DNeasy Plant Mini DNA Extraction Kit (Qiagen, Inc., Valencia, CA, USA). Two primer sets were used

for the amplification of the internal transcribed spacer (ITS) and partial large subunit (LSU) regions of ribosomal DNA: ITS5 and ITS4 [15] for ITS, and LR0R and LR5 for LSU [16]. PCR amplification was conducted based on the protocols provided in Kim et al. [17]. The PCR products were purified using an ExoSAP Kit (USB, Cleveland, OH, USA). The purified double-stranded PCR fragments were directly sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The same primer sets used for the amplification of ITS and LSU were employed for sequencing. Capillary electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Since many reference sequences of *Geoglossum* species were available for the ITS region, phylogenetic analyses were conducted using PhyML and Bayesian inference using only the sequences of the ITS region (Figure 1). In total, 31 ITS sequences were used, including two sequences of *Trichoglossum* species as an outgroup. The ITS sequences data set (including 31 taxa, 464 characters) were aligned using ClustalX 1.81 [18] and trimmed at both terminal ends using Phylit 3.2 [19]. Maximum likelihood (ML) analysis was conducted using PhyML v. 3.0 [20], employing a HKY85+G model of site substitution with 1,000 bootstrap replicates [21]. Bayesian inference (BI) analysis was conducted based on the DNA dataset from the results of MrModelTest v. 2.4 [22] using the Markov Chain Monte Carlo (MCMC) algorithm in MrBayes v. 3.1.2 [23]. Two parallel runs were performed using one cold chain and three heated chains for 3 million generations, starting with a random tree. Trees were sampled every 100 generations. We assumed that the two independent runs converged when the average standard deviation (SD) of the split frequencies decreased below 0.01. The trees obtained before convergence were discarded using the burn-in command, and the remaining trees were used to calculate a 50% majority consensus tree and estimate Bayesian posterior probability (BPP). A BPP below 0.95 was not considered significant.

In the ITS tree, three sequences obtained in this study clustered within the genus *Geoglossum* clade and formed two groups, one group containing KA23-0600 and KA23-0714 and the other containing KA23-0432. KA23-0600 and KA23-0714 were closely related to *G. difforme* but clearly formed a separate lineage with strong support (MLBS/BPP = 97/1.0). KA23-0432 formed a group with *G. simile* with high support values (MLBS/BPP = 73/0.95). Additionally, the LSU of the rDNA region was investigated to determine its similarity with those of previously reported *Geoglossum* species. After the BLAST

searches in NCBI, the results showed that KA23-0600 and KA23-0714 had 99% similarity to *G. difforme* ILLS67349 (GenBank No.: KC222137), and KA23-0432 had 99% similarity to *G. simile* ILLS 61039 (GenBank No.: JQ256437). In addition, the LSU sequences of our specimens were registered in GenBank.

## Taxonomic description

***Geoglossum simile*** Peck, Bull. Buffalo Soc. Nat. Sci. 1(2): 70 (1873) [1873-1874], Figs. 1 and 2.

**Macroscopic description:** Fruiting body 40–70 mm tall, solitary, tongue-shaped to clavate head, stipitate (Figures 1 and 2). Fertile part lanceolate to spatulate, less compressed, 10–25 mm long, 2–4 mm thick, apex subacute to obtuse, black to blackish-brown, differentiated from the sterile stipe. Stipe gracile, cylindrical, terete, glabrous, brownish-brown to ash-gray base, 30–45 mm long, 2–3 mm thick.

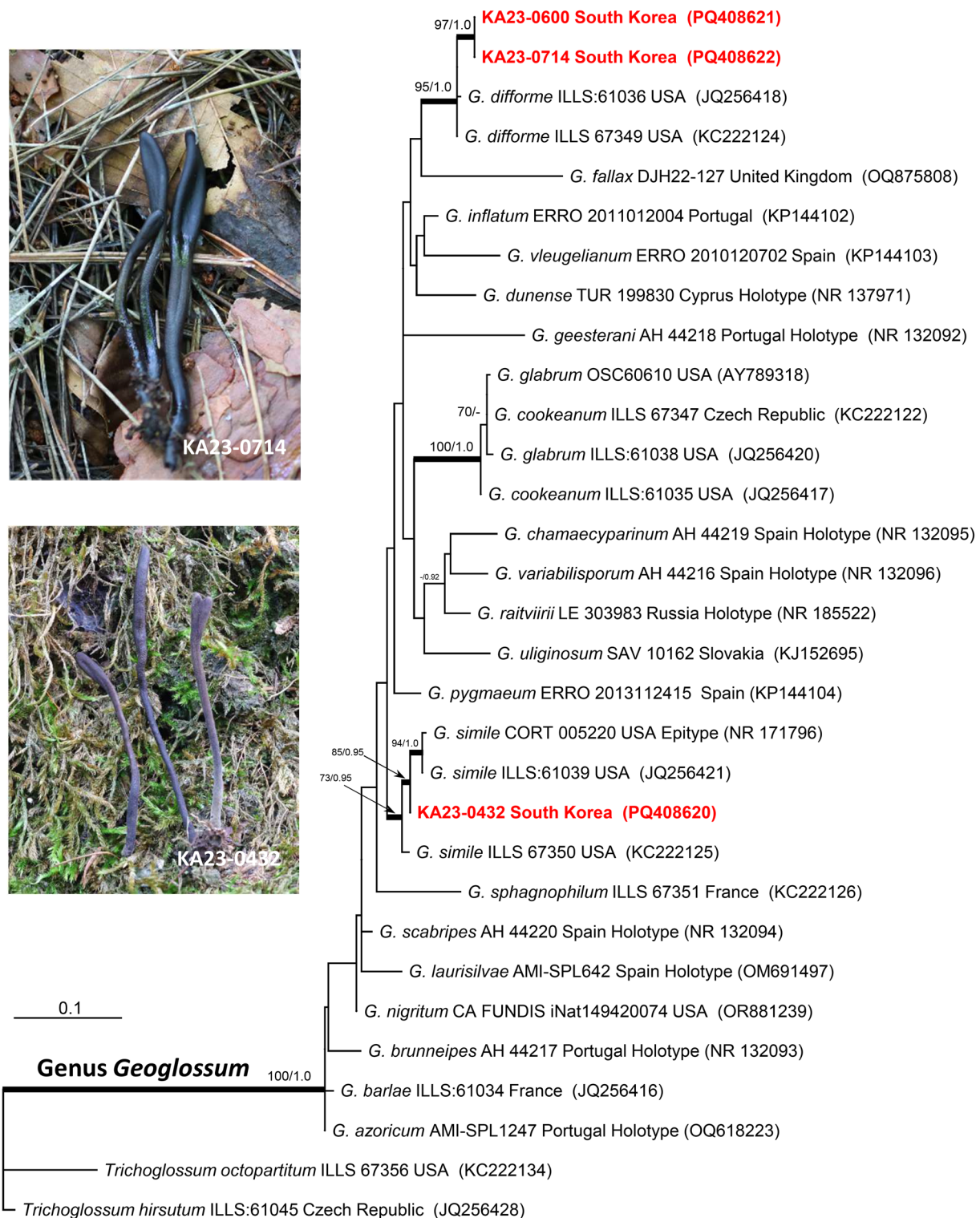
**Microscopic description:** Asci (163.8–) 182.9–213.1 (–227.3) × (18.1–) 23–25 (–25.6) µm ( $n=30$ ), 8-spored, clavate to cylindrical-clavate, unitunicate, rounded apex, narrowed below. Ascospores (65.2–) 77.5–94.4 (–100.8) × (4.8–) 5.3–9.1 (–10.1) µm ( $n=30$ ), in a parallel fascicle, 7-septate, dark brown, cylindric to clavate, slightly curved. Paraphyses filiform, septate, numerous, exceeding the asci, light brown in the upper part, straight or curved.

**Habitat:** Growing on moss-covered soil and humus in mixed forests.

**Distribution:** Asia (China, Japan, South Korea), Europe (Austria, Czech Republic, Denmark, Estonia, France, Germany, Norway, Slovakia, Sweden, Switzerland, United Kingdom), North America (Canada, USA).

**Specimen examined:** South Korea, Gangwon-do, Jeongseon-gun, Imgye-myeon, 22 Jul 2023, 37.5389° N, 128.9189° E (specimen: KA23-0432; GenBank code: ITS PQ408620, LSU PQ408623).

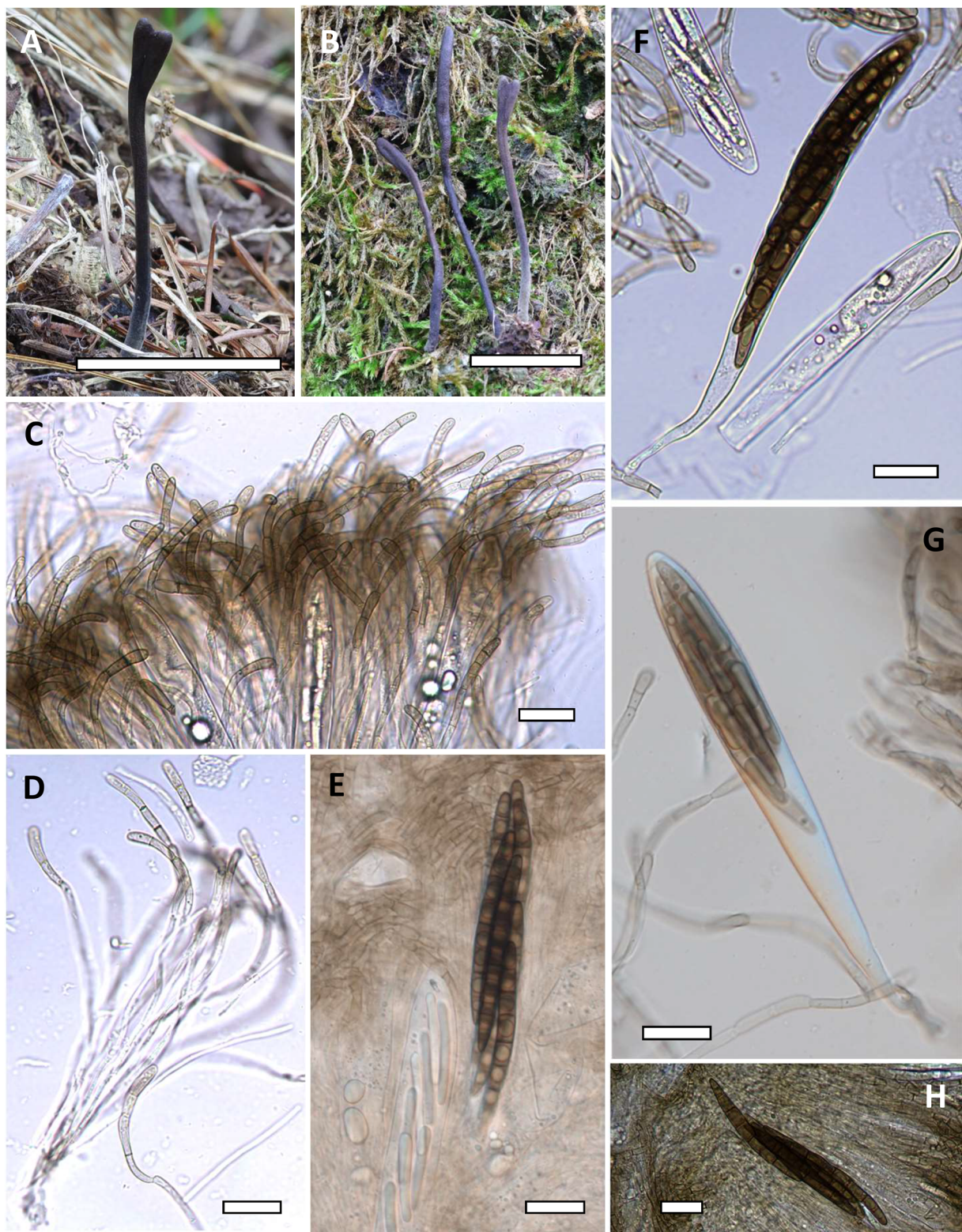
**Note:** Macro-morphologically, this species is similar to *G. glabrum* and *G. cookeanum*. However, they are clearly distinguished by the microscopic characteristics such as lack of dark pigmentation at the terminal cells of the paraphyses, weaker adherence of paraphyses, and greater size variation of terminal cells [24]. Additionally, they are clearly distinguished in the ITS tree (Figure 1). *Geoglossum simile* appears to be distributed worldwide, including East Asia, Europe, and North America. However, molecular phylogenetic studies on samples from East Asia, including Korea, are still lacking [24]. According to Hustad et al. [24], it was revealed that the *G. simile* specimens from Europe and North



**Figure 1.** Phylogram of *Geoglossum* species obtained from a maximum likelihood (ML) analysis based on ITS sequences. Numbers above or below the branches indicate ML bootstrap support values (MLBS >50%) and Bayesian posterior probabilities (BPP ≥0.95). Wide black branches indicate MLBS > 70 and BPP ≥0.95. The tree were rooted with *Trichoglossum hirsutum* and *T. octopartitum* as an outgroup. Our specimens are indicated in red.

America had less than 3% differences in the ITS region but formed a single clade. The KA23-0432 specimen showed slight differences in the ITS region compared to the epitype *G. simile* CORT 005220, but these differences were also less than 3%. Morphologically, the characteristics matched,

leading to the conclusion that they belong to the same species. Further research on whether *G. simile* from East Asia exhibits morphological or ecological distinctiveness compared to samples from Europe and North America would help better understand the ecology of this species.



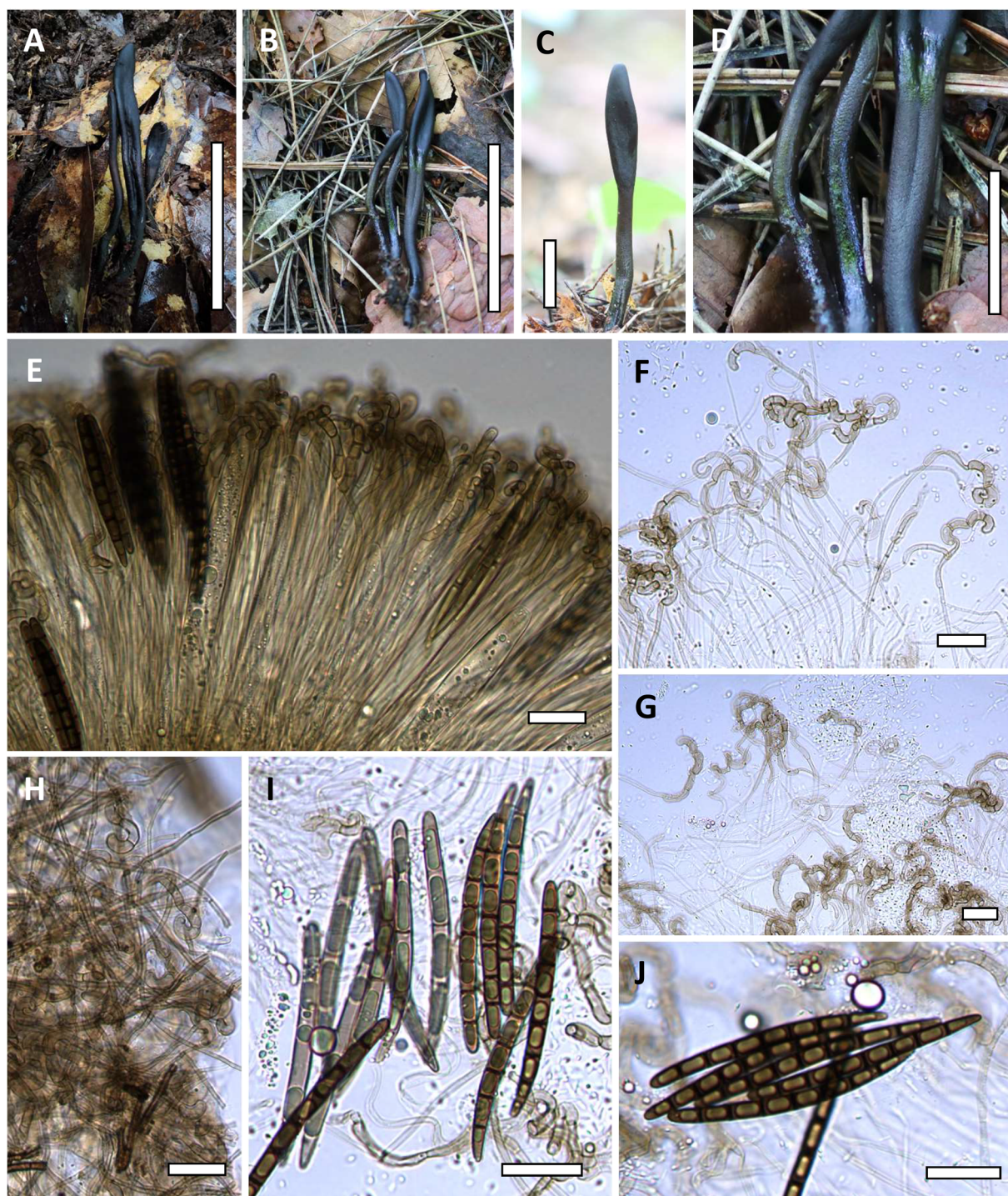
**Figure 2.** *Geoglossum simile*. (A, B) Fruiting bodies; (C, D) Paraphyses in 3% KOH; (E-H) Asci and ascospores in 3% KOH. Scale bars: A, B=20 mm; C-H=25 µm.

*Geoglossum subdifforme* chang S. Kim, *sp. nov.*, MycoBank No.: 855895

**Etymology:** The name refers to the macro-morphologically similar and phylogenetically related to *G. difforme* (Figures 1 and 3).

**Macroscopic description:** Fruiting body 45–80 mm high, growing solitary to gregariously, tongue shaped to clavate head. Fertile part

lanceolate to spatulate, less compressed, 10–30 mm long, 3–5 mm thick, apex subacute to obtuse, black to blackish-brown, differentiated from the sterile stipe. Stipe gracile, cylindrical, terete, glabrous, dark brown to black, the color changes to dark green when the flesh surface is rubbed. Sticky when fresh, 35–50 mm long, 3–5 mm thick, basal mycelium dark gray.



**Figure 3.** *Geoglossum subdifforme* sp. nov. (A) Fruiting bodies of KA23-0600; (B–D) Fruiting bodies of KA23-0714; (E) Asci and ascospores in 3% KOH; (F, G) Paraphyses in 3% KOH; (H) Cauloparaphyses in 3% KOH; (I, J) Ascospores in 3% KOH. Scale bars: A, B=50 mm; C=20 mm; D=10 mm; E–J=25  $\mu$ m.

**Microscopic description:** Asci (170.0–) 181.3–205.8 (–219.9)  $\times$  (14.9–) 15.8–18.4 (–20.3)  $\mu$ m ( $n=31$ ), 8-spored, cylindrical-clavate, unitunicate, slightly rounded apex, narrowed below. Ascospores (67.0) 78.1–90.7 (92.4)  $\times$  (5.3) 5.9–7.2 (8.0)  $\mu$ m ( $n=44$ ), in a parallel fascicle, 7-septate (rarely 3-septate), brown to dark brown, cylindric to clavate, slightly curved. Paraphyses filiform, septate, numerous, exceeding the asci, enlarged and straight to curved to hooked toward the apices, flexuous, 2–4  $\mu$ m wide, light

brown in the upper part, and hyaline toward the base. Cauloparaphyses in a gelatinized layer, 1–3  $\mu$ m wide, light brown, septate, apices curling.

**Habitat:** Growing on humus in mixed and deciduous forests.

**Distribution:** South Korea.

**Holotype:** South Korea, Gyeongsangbuk-do, Ulleung-gun, Buk-myeon, 12 Sep. 2023, 37.5181° N, 130.8695° E (specimen: KA23-0714; GenBank code: ITS PQ408622, LSU PQ408625).

**Other examined specimen:** South Korea, Jeju-do, Seogwipo-si, Sanghyo-dong, San 86-16, 30 Aug 2023, 33.3282° N, 126.5855° E (specimen: KA23-0600; GenBank code: ITS PQ408621, LSU PQ408624).

**Note:** Macro-morphologically and phylogenetically, our specimens are closely related to *G. difforme* (Figure 1). However, they can be easily distinguished by the size of the ascospores and the number of septae (*G. difforme*: ascospores 90–120 × 5–7 µm, 15-septate [25]). Interestingly, our two specimens were both found on islands of South Korea (Ulleung and Jeju islands). It is assumed that the island's climate and vegetation environment are suitable for the growth of this species. It has long been suspected that *Geoglossum* species are closely related to lower plants [11]. Wang et al. [12] investigated the soil fungal diversity in terms of earth tongues, including *Geoglossum* species, by analyzing molecular environmental samples of soil hyphae and root endophytes. Although they did not find a clear relationship between *Geoglossum* species and lower plants, they reported four cases of mycorrhizal association between *Geoglossum* and different plant species. Therefore, in future studies, comparisons of additional specimens and vegetation data will improve our understanding of these species.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This research was supported by the Korea National Arboretum (Project No. KNA 1-1-28-24-1).

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