

Taxonomic divergence of the green naked-stipe members of the genus *Microglossum* (Helotiales)

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ABSTRACT

Four new species of the Ascomycete genus *Microglossum* are recognized, based on morphological characters and DNA sequences of nuc rDNA (ITS region and 28S gene) and the second largest subunit of RNA polymerase II (*RPB2*). They differ from *Microglossum nudipes* by the color of the ascocarps and the sizes and shapes of ascospores, asci, and paraphyses. A lectotype is proposed, and an emended description is provided for *M. nudipes*. Descriptions of new species *Microglossum clavatum*, *M. truncatum*, *M. pretense*, and *M. tenebrosus* are provided. Other closely related species in the group of green earth tongues include *Microglossum viride*, *M. rickii*, and *M. griseoviride*. An identification key to green *Microglossum* species is presented.

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INTRODUCTION

Because of their unusual appearance, earth tongues have interested mycologists for a long time. Previous molecular analyses (Sandnes 2006; Wang et al. 2006; Ohenoja 2010) revealed that these fungi do not have a monophyletic origin. The class Geoglossomycetes was established recently by Schoch et al. (2009) as a sister taxon to Leotiomycetes. Currently, Geoglossomycetes include seven more-or-less well-delimited genera (Hustad et al. 2011; Hustad et al. 2013), but the number of extant species is uncertain. *Microglossum* does not seem to belong within Geoglossomycetes (Schoch et al. 2009; Hustad et al. 2013) and is still considered a member of Helotiales in the polyphyletic class Leotiomycetes (Wang et al. 2006).

Microglossum was described by Gillet (1879) for the green species *Geoglossum viride* Pers. and the olivaceous *G. olivaceum* Pers. *Geoglossum viride* was published by Persoon (1796) and mentioned, but without description and thus not validly published, 2 y before (Persoon 1794). Later, Durand (1908) selected *Microglossum viride* (Pers.: Fr.) Gillet as the lectotype of *Microglossum*. The generic name *Microglossum* has been generally used for green earth tongues since that time. Saccardo (1884) based his *Microglossum* (nom. illegit.; Art. 53.1) on *Geoglossum hookeri* Cooke.

Species of *Microglossum* are usually colorful fungi with an earth tongue appearance, with a sterile stipe

and fertile hymenium, occurring in undisturbed grasslands. About 36 species are reported worldwide (www.mycobank.org, www.indexfungorum.org).


Since 1917, the name *Microglossum nudipes* Boud. was applied to all collections of *Microglossum* with a green naked stipe. Our DNA sequencing analyses, employing the nuc rDNA internal transcribed spacer (ITS1-5.8S-ITS2 = ITS) and the 28S and *RPB2* genes indicated that seven taxa could be differentiated in the material we studied. These assumptions were supported by macro-morphological characters, especially color of the ascomata and to a certain extent by micromorphology.

MATERIALS AND METHODS

Morphological studies.—Macromorphological characters were observed in fresh material. Micromorphological structures were studied in dried material using an AxioScope A1 compound microscope (Carl Zeiss, Göttingen, Germany) at a magnification of 1000× with an oil immersion lens. Fragments of material were examined in 5% KOH, tap water, Melzer's reagent, and a solution of Congo Red in ammonia. Measurements were taken from dry material treated in tap water directly under the microscope using the eyepiece micrometer. Dimensions of micromorphological characters were estimated as an average (av.) ± 1 standard deviation from 30 measurements for each taxon, with 10th and 90th

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percentiles in parentheses and Q value of ascospores present length/width ratio. Acronyms for herbaria follow Index herbariorum (Thiers, continuously updated). All descriptions are based on dried studied collections. Several collections were examined microscopically also in living condition (indicated by an exclamation mark); the data from these fresh collections are included in parentheses in the descriptions. For the list of studied *Microglossum* specimens, see SUPPLEMENTARY TABLE 2. The pH reaction of the substrate was measured directly in the field using HANNA HI 99121.

Molecular studies.—Sixty two recently collected specimens of *Microglossum* were selected for molecular analyses. DNA was isolated from dried fungal material using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, California, USA). DNA fragments encompassing the ITS region and the D1–D2 domains of the 28S gene were amplified using the following primer combinations: ITS5/LR6, or ITS1/ITS4 and LR0R/LR6 in cases of difficult amplification (White et al. 1990; Moncalvo et al. 2000). The polymerase chain reaction (PCR) profile for primers ITS5/LR6 was as follows: touchdown PCR initiated with a 2-min denaturation at 94 °C, annealing temperature for first amplification cycle 60 °C, subsequently incrementally reduced by 1 °C per cycle for the next 9 cycles, followed by 36 amplification cycles each consisting of 30 s denaturation at 94 °C, 30 s annealing at 50 °C, and 1 min extension at 72 °C, concluding with 10 min incubation at 72 °C. PCR regimes using other primer combinations followed the protocols of Kučera et al. (2014b). Amplicons of the domain 6–7 region of the second largest subunit of RNA polymerase II (*RPB2*) were obtained using primers *fRPB2*-5F (Liu et al. 1999) and *RPB2*-P7R (Hansen et al. 2005), following protocols outlined by Hansen et al. (2005).

Gene fragments were amplified in a Mastercycler_ep thermocycler (Eppendorf, Hamburg, Germany). Amplicons were custom-purified and sequenced at Macrogen (Seoul, Korea). Sequences were deposited in the European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database (K1513002–KJ513011, KX382834–KX382893).

Two data sets were subjected to phylogenetic analyses: (i) one including ITS of all specimens; and (ii) a combined ITS-28S-*RPB2* data set of selected representatives of each lineage of the *M. nudipes* complex (SUPPLEMENTARY TABLE 1). Sequences were aligned using MAFFT, version 7, utilizing the Q-INS-i option (Katoh and Toh 2008). The ITS data set was supplemented with sequences of *M. cyanobasis* Iglesias & Arauzo, *M. griseoviride* V. Kučera, Lizoň & M. Tomšovský, *M. olivaceum* (Pers.) Gillet, *M.*

rufescens (Grélet) Bon, *M. rufum* (Schwein.) Underw., and *M. viride* obtained from GenBank. The ITS sequence of *Leotia lubrica* (Scop.) Pers. strain ZW-GEO54-Clark (GenBank no. AY789349), was selected as an outgroup. The combined ITS-28S-*RPB2* data set was 2546 bp long, whereas intron sites (132–178 bp, in some species divided in two sections) occurring in some 28S sequences were excluded from the analyses. *Microglossum griseoviride* (SAV F-9920) and *M. viride* (SAV F-10249) were selected as outgroups for combined analysis.

Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to estimate phylogenetic relationships of both data sets. The combined data set was partitioned into three subsets of nucleotide sites: ITS, 28S, and *RPB2* because of different models relevant to each gene or region. The likelihood settings from the best-fit model for ITS (TiM2+I+G) and the combined data set (TiM2ef+I+G = ITS, TIM1+I+G = 28S, TrN+G = *RPB2*) were selected by the Akaike information criterion in jModelTest2 (Darriba et al. 2012). BI analysis was conducted with MrBayes 3.2.6. (Ronquist et al. 2012). The combined data set was partitioned into three subsets of nucleotide sites: ITS, 28S, and *RPB2*. We ran four chains for 10 million generations. The burn-in value (1 million generations) was estimated using Tracer 1.6 (Rambaut et al. 2014). Sampling frequency was set to every 1000th generation. ML analysis was performed with RAXML-HPC 8 (Stamatakis 2014) with a GTRCAT model of evolution. Nodal support was determined by nonparametric bootstrapping (BS) with rapid bootstrapping option setting number of replicates automatically.

RESULTS

Morphological study.—Analysis of macro- and micromorphological characters of the studied collections showed that combinations of characters are unique for each of the taxa described below. Characteristic for *Microglossum nudipes* is a combination of olivaceous-green hymenium, large asci, and ascospores with thin, apically branched paraphyses. Blue-green or gray-green ascocarps, a flattened stipe, and sometimes apically thickened paraphyses are distinguishing characters for *M. pratense* V. Kučera, Tomšovský & Lizoň. *Microglossum truncatum* V. Kučera, Tomšovský & Lizoň has a brown-green or brown-colored hymenium and truncate ascocarps at least when young, and paraphyses that are branched in the middle and basal parts. *Microglossum tenebrosus* V. Kučera, Tomšovský, Lizoň & F. Hampe has dark green (dark blue-green) ascocarps and short ascospores <15 µm, whereas *M. clavatum* V. Kučera, Tomšovský & Lizoň has clavate, often flattened ascocarps, a green hymenium, short stipe, and relatively large ascospores <20 (–25) µm.

Microglossum parvisporum V. Kučera, Lizoň & Tomšovský has the smallest ascospores in this complex of green naked-stipe species.

Phylogenetic analyses.—The aligned ITS data set was composed of 513 positions and included 325 conserved, 159 variable, and 36 unique sites, as determined by MEGA 6.0.6. (Tamura et al. 2013). The combined ITS-28S-RPB2 data set had 2546 positions, including 2139 conserved, 398 variable, and 333 unique sites. The sequence data sets are deposited in TreeBASE (Submission ID: 19742).

The phylogenetic trees (FIGS. 1, 2) revealed that the *M. nudipes* complex was composed of *M. fuscorubens* Boud., *M. parvisporum*, and four new species: *M. clavatum*, *M. pratense*, *M. tenebrosum*, and *M. truncatum*. *Microglossum nudipes* sensu stricto still seems to be variable and could be an aggregate of more species, so we use the indication *M. nudipes* aff. in this paper. The *M. nudipes* complex is

proximal to *M. rufum*, *M. rufescens*, *M. olivaceum*, and *M. cyanobasis*. The previously reported sequenced specimen *M. cf. nudipes* (SAV 10024) (Kučera et al. 2014b) fell within *M. pratense*. *Microglossum griseoviride* and *M. viride* form a basal clade.

TAXONOMY

Microglossum Gillet, Champignons de France. Discom. 1:25. 1879.

= *Helote* Hazsl., Magyar. Tud. Akad. Értes. 11(19):8. 1881.

= *Ochroglossum* S. Imai, Sci. Rep. Yokohama Natl. Univ. Sect. 2 4:6. 1955.

Type species: *Microglossum viride* (Pers.: Fr.) Gillet, Champignons de France, Discom. 1:25. 1879.

Ascocarps fleshy, erect, stipitate, clavate, ascigerous portion only in upper part, with the general form and

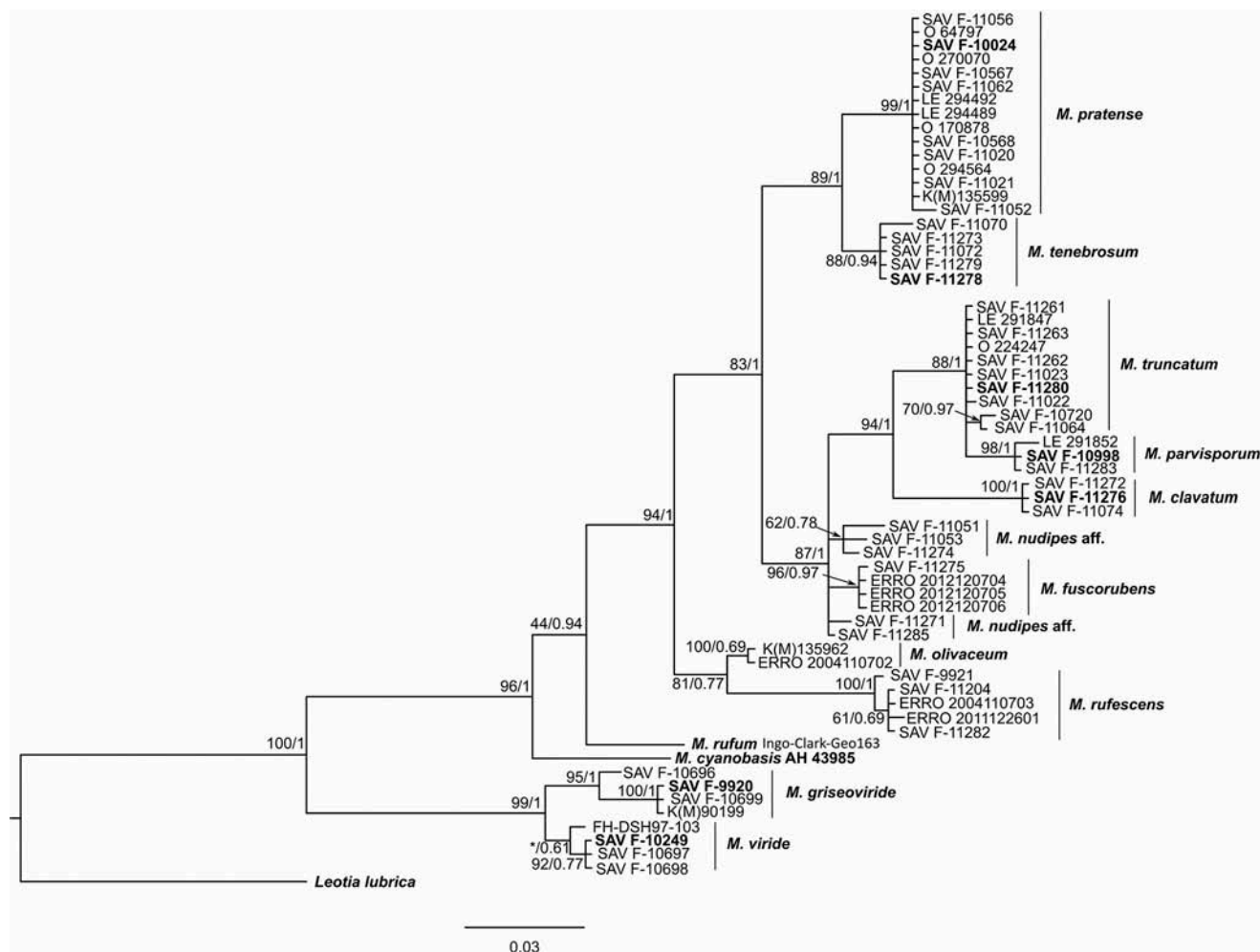


Figure 1. Phylogenetic tree of the internal transcribed spacer (ITS) region conducted by Bayesian analysis (for legends to collection numbers, see SUPPLEMENTARY TABLE 1). Numbers at branches indicate maximum likelihood bootstrap proportion (left) and Bayesian posterior probability (right) values. The type specimens are highlighted in bold. The asterisk (*) indicates different topology in the two analyses. Bar = number of expected substitutions per position.

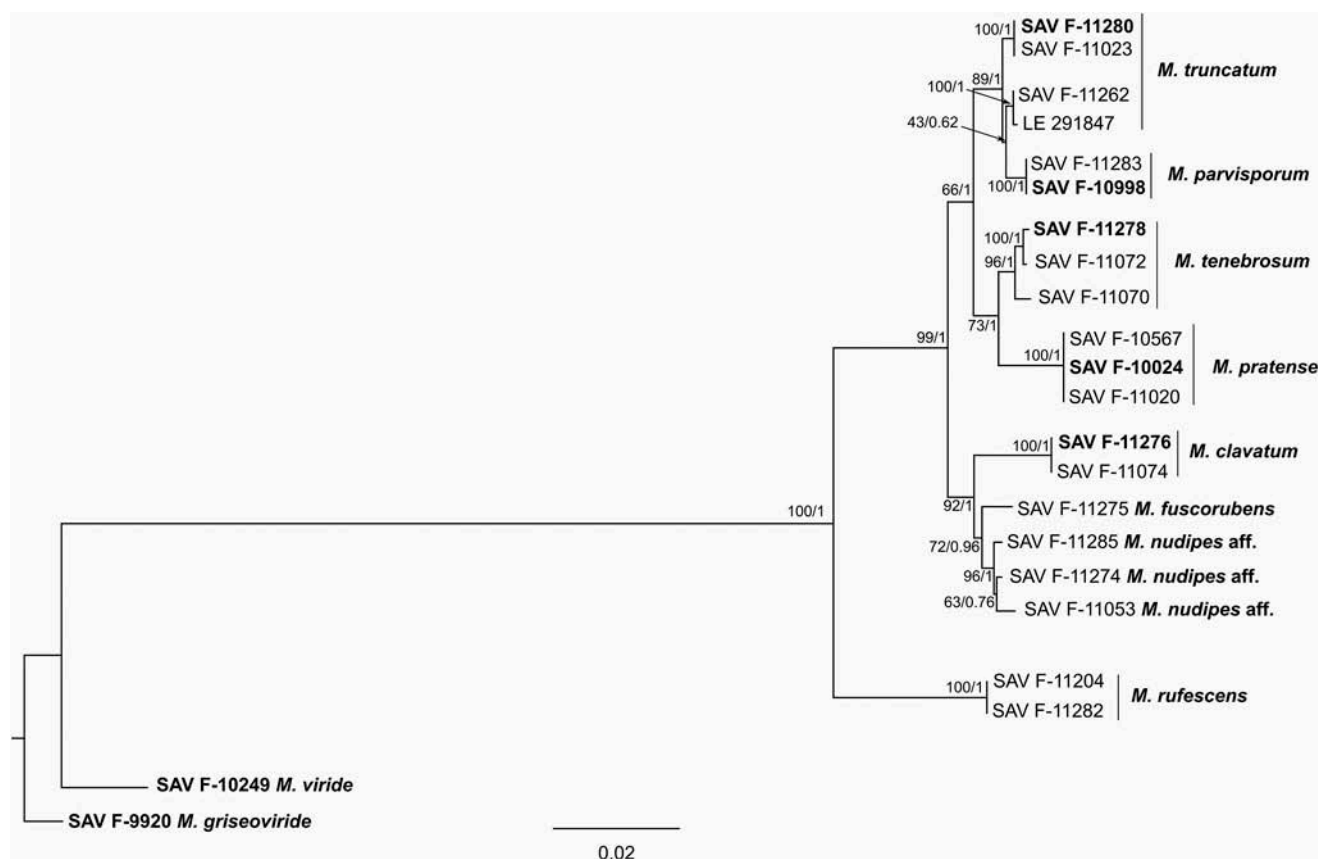


Figure 2. Phylogenetic tree for a combined analysis of the ITS region and 28S and *RPB2* genes, conducted by Bayesian analysis (for legends to collection numbers, see SUPPLEMENTARY TABLE 1). Numbers at branches indicate maximum likelihood bootstrap proportion (left) and Bayesian posterior probability (right) values. Type specimens are highlighted in bold. Bar = number of expected substitutions per position.

habit resembling species of the genus *Geoglossum*, clavate or tongue-shaped, somewhat compressed, always colorful (green, brown or reddish brown, olivaceous, yellow, clay), never dark brown or black. Asci clavate-cylindrical, opening by an amyloid pore. Ascospores 8, biseriate, hyaline, smooth, ellipsoidal, fusiform or cylindrical to oblong, aseptate or septate; paraphyses straight, slightly curved, or circinate at the apex.

Two basic groups of green earth tongues can be distinguished easily: (i) a group characterized by scaly stipes, including *M. viride*, *M. rickii* Imai, and *M. griseoviride* (Kučera et al. 2014b); and (ii) a group with naked smooth stipe, including *M. nudipes* and *M. olivaceum* both in a broad sense.

KEY TO GREEN MICROGLOSSUM SPECIES

1. Stipe covered with scales, ascocarp green, yellowish-green, grayish-green, apex of paraphyses with a “cap” of pigment. 2

- 1'. Stipe naked, green, pinkish-green, olive-green, blue-green, apex of paraphyses lacking a “cap” of pigment. 4
2. Ascocarp <1.5 cm tall, asci <80 μm (65–75 \times 9–10 μm) long, ascospores 10–14 \times 4–5 μm , known only from Brasilia. *M. rickii*^b
- 2'. Ascocarps <8 cm tall, asci >80 μm long. 3
3. Ascocarp yellow-green, growing in wet places, often with liverworts, asci 106–134 \times 9.5–12 μm , ascospores 18–22 \times 5–7 μm *M. viride*^a
- 3'. Ascocarp gray-green, growing on soil in the forest, asci 105–139 \times 8–10 μm , ascospores 16–20 \times 4–5 μm *M. griseoviride*^a
4. Stipe constituting 1/2–3/4 of the mature ascocarp, dominant color of the hymenium buff, pink, olive, stipe is light brown usually mixed with other colors, ascospores 13–15 \times 4 μm *M. rufescens*^c
- 4'. Stipe equal to or shorter in length than hymenium, ascocarps without pink and olive color. 5

5. Ascospores ≤ 16 μm long, growing in open areas on calcareous bedrock.....6
- 5'. Ascospores > 16 μm long, growing in mesophilous meadows or forests.....7
6. Ascocarps white-green, when old with violaceous color, asci < 80 μm long.....*M. parvisporum*^a
- 6'. Ascocarps dark green, when old dark green with dark violet tint, asci 80–95 μm long.....*M. tenebrosum*
7. Asci > 105 μm long, growing in the forests.....8
- 7'. Asci < 105 μm long, growing on mesophilous meadows, pastures.....9
8. Ascocarp green, hymenium with brownish tint, stipe blue-green, paraphyses branched also in upper 1/3 and in the middle.....10
- 8'. Ascocarp with brown color present also on the stipe, paraphyses tips swollen < 5 μm , branched in basal 1/3.....*M. nudipes* aff.
9. Hymenium concolorous with sterile, ascospores $13.5\text{--}16.5 \times 4\text{--}5$ μm*M. pratense*
- 9'. Hymenium usually brownish-green or brown, stipe blue-green, ascospores $15\text{--}18 \times 4\text{--}5$ μm*M. truncatum*
10. Ascocarp, flattened, hymenium club-shaped, usually longer than the stipe, paraphyses branched at the base and in the middle.....*M. clavatum*
- 10'. Hymenium mace-shaped, as long as stipe, paraphyses branched both at basal and apical part.....*M. nudipes*^d

In the sense of: ^aKučera et al. 2014a, 2014b; ^bImai 1942; ^cMoingeon 2004; ^dBoudier 1917.

Microglossum nudipes Boud. Bull. Soc. Mycol. France 33:16. 1917.

Typification: FRANCE. Vienne: Savigné, Dec 1913, A. Grélet (**lectotype** designated here, ex herb. Boudier, PC 0139818). MBT 373899.

Description of the lectotype: Ascocarps (10–)16.8–31.3 (–38) mm high, tongue-like or club-shaped, stipitate. Hymenium (5–)7.8–15.2(–18) \times 1–3.8(–6) mm, mace-shaped, cylindrical, clavate, truncate or lanceolate, longitudinally grooved, sometimes twisted and almost lacunose or compressed on the side, glabrous, naked, subolivaceous, dark green or grayish-green. Hymenium constituting approximately the upper 1/2 of the ascocarp. Stipe (5–)8.6–16.6(–20) \times 0.5–4 mm, cylindrical, sometimes flexuous, naked, blue-green or concolorous with hymenium, not paler at the base, flesh yellow-green (20 dry ascocarps examined). Asci (80–)91.4–110.3(–130) \times (6–)7.1–9.5(–11) μm , 8-spored, clavate, rounded at the

apex, narrowly tapered towards the base, biseriate above, uniseriate below, pore dark blue in Melzer's reagent. Ascospores (12–)16.6–20.5(–23) \times (4–)4.2–5.2 μm , Q value = 2.4–5.5 (av. = 3.9), usually slightly curved or sigmoid, ends obtuse or tapering, hyaline, without clearly visible lipid bodies, true septa not observed. Paraphyses filiform, 1–2 μm wide, straight, branched at the base, some of the paraphyses branched in apical part, apical cells filiform (1–)1.1–2.5(–3) μm or rarely slightly swollen < 3.5 μm .

Habitat: Growing among the mosses under *Buxus* sp.

Distribution: France.

Additional specimens examined: See SUPPLEMENTARY TABLE 2.

Notes: *Microglossum nudipes* was described by Boudier (1917) from Savigné (Vienne, France) in December 1913, where it was, as noted by him, first collected by D. Arnould and consequently by L. Grélet. The description, presented both in Latin and French, has important data but unfortunately lacks details to allow it to be connected unequivocally to a type specimen. There are four specimens in PC labeled *Microglossum nudipes*, all collected by Grélet: two dated 1913, two collected later in 1938. Arnould is not mentioned as collector on any label. The collections from 1938 are not conspecific with those from 1913 and, according to our observations, represent *M. rufescens*. Moreover, Boudier's description does not fully correspond with any of the specimens. As noted by Van Brummelen (1985), Boudier's microscopic measurements were often exaggerated in his descriptions as a result of an error in the construction of his measuring scale, and his spore measurements after 1885 were usually about 10% too large. When measurement of asci and spore size in his descriptions are adjusted by lowering them by 20%, allowing for shrinkage by drying and Boudier's inaccurate scale, they roughly correspond with our observations of PC 0139818. Thus, we emended the description of *Microglossum nudipes* to include these details. DNA studies using Boudier's original material were not done because of the age of the specimen. Unfortunately, no specimen among recently collected material corresponds with the lectotype of *M. nudipes* and the second collection gathered from the type locality in 1913. Our visit to the original locality in France in 2015 to recollect the species was unsuccessful.

Microglossum truncatum V. Kučera, Tomšovský & Lizoň, sp. nov. FIG. 3a, b
MycoBank MB817350

Typification: SLOVAKIA. Biele Karpaty Mts.: Nová Bošáca, ca 1.7 km SE from the church, Španie settlement, Natural Monument Blažejová (48°52'33.3"N, 17°49'03.4"E, alt. 415 m), in a meadow, 7 Nov 2013,



Figure 3. *Microglossum* species with green naked stipes. A. *M. truncatum* SAV F-11261 (M. Krivuš). B. *M. truncatum* SAV F-11280 (V. Kučera). C. *M. pratense* SAV-11020 (P. Marstad). D. *M. pratense* SAV-10024 (V. Kučera). E, F. *M. tenebrosum* SAV F-11278 (F. Hampe). G. *M. nudipes* aff. SAV F-11051 (T. Knutsson). H. *M. nudipes* aff. SAV F-11285 (V. Kaounas). I. *M. nudipes* aff. SAV F-11271 (U. Pera). Bars = 2.5 cm (A) and 5 cm (B–I).

V. Kučera, V. Kautman, and I. Kautmanová (**holotype** SAV F-11280!). DNA sequences from the holotype: ITS = KX382861, 28S = KX382861, RPB2 = KX382875.

Etymology: From *truncatus* (Latin), ending abruptly, ascocarps truncate when young.

Ascocarps (19–)25.4–44.3(–55) [(20–)30.2–55.5(–60)] mm high, tongue-like or club-shaped, stipitate. Hymenium (8–)22.2–28.5(–30) × (1–)3.2–4.1(–7) [(10–)25.3–32.3(–44) × (1–)3.1–5.2(–8)] mm, cylindrical, club-shaped, truncate or lanceolate, only slightly grooved vertically, glabrous, naked, brownish-green or light brown or green-brown, dark green or brownish-green when dry. Hymenium usually constituting more than the upper 1/2 of the ascocarp. Stipe (10–)15.2–20.1(–27) × 1–2.2(–3) [(11–)16.5–23.6(–29) × 1.2–3.1(–4)] mm, cylindrical, blue-green, not paler at the base (15 ascocarps examined). Asci (75–)85.1–98.7(–105) × (8–)8.4–9.6(–10) [(95–)120.6–135.3(–150) × (8–)10.1–11.5(12)] μm, 8-spored, clavate, rounded at the apex, narrowly tapered towards the base, biseriate above, uniseriate below, pore bluing in

Melzer's reagent. Ascospores (14–)15.4–18.3(–20) × 4–4.7 (–5) [(17–)18.2–19.8(–21) × 4–5(5.5)] μm, Q value = 3.3–4.8 (av. = 3.8), hyaline, ellipsoidal to oblong, aseptate. Paraphyses filiform, straight, branched in basal part, some of the paraphyses branched in apical part, apical cells filiform 1–2 μm or only slightly swollen (<3 μm).

Habitat: On soil among grass in mesophilous meadows with soil pH 5.7–6.1.

Distribution: France, Russia, Slovakia, Sweden.

Additional specimens examined: See SUPPLEMENTARY TABLE 2.

Notes: Ascocarps of *Microglossum truncatum* are brown or brownish-green in the hymenium, whereas the stipe is blue-green and oval in cross-section. The most similar species is *M. nudipes* aff., which differs by a brown coloration on the stipe and is usually dumb-bell-shaped in cross-section. *Microglossum truncatum* may be macroscopically confused with *M. parvisporum*, but it clearly differs in the size of asci (85–98 × 8–9 μm vs. 66–79 × 6 μm) and ascospores (15–18 × 4–5 μm vs.

11–14 × 3–4 µm) and acidity of the substrate (pH 5.7–6.1 vs. pH 6.35).

Microglossum pratense V. Kučera, Tomšovský & Lizoň, sp. nov. FIG. 3c, d
MycoBank MB808093

Typification: SLOVAKIA. Stolické vrchy Mts.: Muránska Huta village, Predná Hora recreation area, former ski slope ca. 2 km SSW from the village (48°45'31.24" N, 20°06'26.98"E, alt. 789 m), in grass, 13 Oct 2010, V. Kautman and V. Kučera (**holotype** SAV F-10024). DNA sequences from the holotype: ITS = KC595259, 28S = KC595260, RPB2 = KX382880.

Etymology: from *pratun* (Latin), meadow, growing in meadows and pastures.

Ascomycetes (17–)26.8–49.6(–62) mm high, tongue-like or club-shaped, stipitate. Hymenium (10–)12.4–27.7(–40) × (1.5–)2.2–4.7(–9) mm, mace-shaped, cylindrical, clavate, truncate or lanceolate, vertically grooved, usually twisted, glabrous, naked, blue-green or gray-green, dark green and often with lateral cracks when dry. Hymenium usually occupying the upper 1/2 of the ascocarp or more. Stipe (7–)11.8–24.2(–32) × 1–3 mm, often flattened and flexuous, blue-green or concolorous with hymenium (54 dry ascocarps examined). Asci (67–)78.1–91.5(–105) × (5–)7.1–8.5(–9) µm, 8-spored, cylindrical to clavate, apex rounded, narrowly tapered towards the base, biseriate above, uniseriate below, the pore light bluing in Melzer's reagent. Ascospores (11–)13.5–16.5(–20) × (4–)4.5–5 µm, Q value = 3.2–4.1 (av. = 3.7), ellipsoidal to oblong, usually slightly curved or sigmoid, ends obtuse or tapering, hyaline or with several (<4) lipid bodies, real septa not observed. Paraphyses filiform, straight, branched in basal part, some of the paraphyses branched at apical part, the apical cells filiform 1–2 µm or only slightly swollen <3 µm.

Habitat: Meadows or pastures in north and central Europe, on soil, among grasses. The type collection was found in a site with soil pH 5.2–5.6.

Distribution: Georgia, Norway, Russia, Slovakia, Sweden.

Additional specimens examined: See SUPPLEMENTARY TABLE 2.

Notes: *Microglossum pratense* is characterized by having green or gray-green hymenium lacking a brown tint. Ascocarps are usually produced in bigger clusters of <10. When dry, the ascigerous part often transversely cracked. The hymenium takes more than a half of the whole ascocarp, and the stipe is often flattened. *Microglossum pratense* could be confused with *M. tenebrosum* or *M. clavatum*, but *M. pratense* is paler in color and the ascospores are bigger (13.5–16.5 × 4–5

µm) than in *M. tenebrosum* (12–15 × 4–4.5 µm) and smaller than in *M. clavatum* (15–20 × 4–5 µm). Collections in fungaria are mostly misidentified as *M. olivaceum* or *M. rufescens*. In general, *M. pratense* was the most common green *Microglossum* species with naked stipe in the examined areas.

Microglossum tenebrosum V. Kučera, Tomšovský, Lizoň & F. Hampe, sp. nov. FIG. 3e, f
MycoBank MB817358

Typification: GERMANY. Hessen: Langenthal, Eberschütz (51°36'26.9"N, 09°23'22.6"E, alt. 225 m), calcareous neglected open grassland, shell limestone, with *Juniperus* sp., 12 Oct 2009, F. Hampe (**holotype** SAV F-11278). DNA sequences from the holotype: ITS = KX382845, 28S = KX382845, RPB2 = KX382891.

Etymology: From *tenebrosus* (Latin), dark, ascocarps dark-colored.

Ascomycetes (27–)30.6–48.2(–53) [40–60] mm high, tongue-like or club-shaped, stipitate. Hymenium (14–)15–25.7(–30) × (2.1–)3.2–5 [20–30 × 3–6] mm, mace-shaped, cylindrical, clavate, truncate or lanceolate, vertically grooved, glabrous, naked, at maturity dark green or dark violet-green or bronze-green to brown-green. Hymenium usually occupying the upper 1/2 of the ascocarp or more. Stipe (12–)13.2–25.8(–33) × (1.5–)2.5–3 [20–30 × 2.5–6] mm, often flattened and flexuous, blue-green and often concolorous with hymenium at the base (11 dry ascocarps examined). Asci (70–)79.5–95.4(–120) × (6–)7.3–8.6(–9) [(100–)115.4–124.3(–139.8) × (7.5–)8.6–9.2(–10.4)] µm, 8-spored, cylindrical to clavate, apex rounded, narrowly tapered towards the base, biseriate above, uniseriate below, the pore bluing in Melzer's reagent. Ascospores (10–)11.8–15.4(–20) × (3–)3.7–4.5(–5.5) [(11.6–)13.8–14.8(–17) × (3.1–)4.2–4.6(–5.7)] µm, Q value = 2.5–4.7 (av. = 3.4) [2.4–4.1 (av. = 3.29)], ellipsoidal to oblong, usually slightly curved or sigmoid, ends obtuse or tapering, hyaline or with several (<4) lipid bodies, real septa not observed. Paraphyses filiform, straight, branched in basal part, some of the paraphyses branched at middle part, the apical cells filiform 1–2(–2.5) µm or slightly clavate or capitate (<3 µm).

Habitat: On soil, among grasses or in forest clearings, meadows and pastures with *Juniperus* sp., under *Buxus* sp. and calcareous bedrock, in western and central Europe.

Distribution: France, Germany, Spain.

Additional specimens examined: See SUPPLEMENTARY TABLE 2.

Notes: *Microglossum tenebrosum* is characterized by shades of dark green. The color of the hymenium is generally repeated at the basal part of the stipe. *Microglossum tenebrosum* is distinguished by the Q

value of the ascospores (3.4) compared with *M. nudipes* (3.9), *M. clavatum* (3.8), *M. pratense* (3.7), *M. truncatum* (3.8), and *M. parvisporum* (3.05). Most collections of *M. tenebrosum* are from open spaces and not forests. The combination of relatively large asci ($79.5\text{--}95 \times 7\text{--}9 \mu\text{m}$) and small ascospores ($12\text{--}15 \times 4\text{--}4.5 \mu\text{m}$) is characteristic. The data for fresh collections were taken from Ribes Ripoll (2013).

Microglossum clavatum V. Kučera, Lizoň & Tomšovský, sp. nov.

MycoBank MB817351

Typification: SPAIN. Huesca: Puente de Reina de Jaca ($42^{\circ}34'04.18''\text{N}$, $0^{\circ}45'44.64''\text{W}$, 665 m), under *Buxus sempervirens* in calcareous soil, 6 Dec 2006, J. Hernanz (**holotype** SAV F-11276). DNA sequences from the holotype: ITS = KX382864, 28S = KX382864, RPB2 = KX382884.

Etymology: From *clava* (Latin), club, ascocarps typically club-shaped.

Ascomycetes 15–31(–37) mm high, tongue-like, club-shaped or clavate, usually compressed, stipitate. Hymenium $10\text{--}19.9(25) \times 1\text{--}4$ mm, clavate, truncate or lanceolate, slightly vertically grooved, glabrous, naked, dark green sometimes with a brown tint. Hymenium occupying more than the upper 1/2 of the ascocarp. Stipe $(4\text{--})5.2\text{--}12.5 \times (1\text{--})2.5\text{--}3$ mm, cylindrical or flattened, concolorous with hymenium (14 dry ascocarps examined). Asci $(71.5\text{--})96.6\text{--}117.8$ (130) $\times (6.5\text{--})8.2\text{--}10.1(11) \mu\text{m}$, 8-spored, cylindrical to clavate, apex rounded, narrowly tapered towards the base, biseriate above, uniseriate below, the pore bluing in Melzer's reagent. Ascospores $(14\text{--})15.5\text{--}20.4$ (23) $\times (4\text{--})4.5\text{--}5 \mu\text{m}$, *Q* value = 3–5.7 (av. = 3.8), ellipsoidal to oblong, usually slightly curved or sigmoid, ends obtuse or tapering, hyaline or with several (<4) lipid bodies, true septa not observed. Paraphyses filiform, straight, branched in basal part, some of the paraphyses branched at middle part, the apical cells filiform $1\text{--}2(2.5) \mu\text{m}$ or slightly clavate or capitate ($<3.5 \mu\text{m}$).

Habitat: On calcareous soil under *Buxus* sp. tree.

Distribution: Spain.

Additional specimens examined: See SUPPLEMENTARY TABLE 2.

Notes: *Microglossum clavatum* is similar to *M. nudipes* based on the size of asci and shape of paraphyses. However, the values of all examined characters of *M. clavatum* are slightly smaller, the paraphyses are not apically branched, and the ascus pore does not react as strongly in Melzer's reagent as it does in *M. nudipes*.

Examined specimens of other *Microglossum* spp.: See SUPPLEMENTARY TABLE 2.

DISCUSSION

During our research on the *Microglossum nudipes* complex, we recognized seven well-delimited taxa. Three of them, *M. nudipes* (Boudier 1917), *M. parvisporum* (Kučera et al. 2014a), and *M. fuscorubens* (Boudier 1907), were already described. Four new species are described in this paper, but there is a high likelihood that additional morphotypes and/or taxa in this complex will be discovered.

In all species, we observed that the apical part of the asci and paraphyses are surrounded by a green-brown amorphous matrix that is $<20 \mu\text{m}$ thick. Moreover, ascospores occurring in the apical part of the asci are smaller than others.

Ascomycetes of all taxa in the *M. nudipes* complex lack the pink, ochraceous, or red colors that are typical for *M. rufescens*. Five specimens studied (SAV F-11274, F-11051, F-11285, F-11271, F-11053) are morphologically similar to each other but differ from lectotype of *M. nudipes* and differ from other taxa in the ITS region and *LSU* and *RPB2* genes; we temporarily maintain these entities under the name *M. nudipes* aff. The ascocarps of *M. nudipes* aff. may have, in addition to green, different tints of brown on the stipe, and according to the phylogenetic data, the group is closely related to *M. fuscorubens*.

Occasionally, several species of the *M. nudipes* complex grow in one site. We observed three species (*M. truncatum*, *M. pratense*, and *M. nudipes* aff.) at Grúň in the Biele Karpaty Mts., Slovakia where the ascocarps were growing in a range of less than 2 m.

Taxa of green naked-stipe species preferentially grow among grass on soil with calcareous bedrock, in open spaces such as mowed meadows or pastures. It is also possible to find them in forest or bushes with *Quercus* sp., *Buxus* sp., *Chamaecyparis* sp., or *Laurus* sp., especially in warmer regions. The main period of sporulation is from October to January, depending on geographic location and precipitation.

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