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Leucangium microspermum: Re-examination of Japanese *L. carthusianum* reveals its taxonomic novelty

日本産 *Leucangium carthusianum* の再検討結果に基づく新種 *L. microspermum* の記載

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Abstract

The genus *Leucangium* (Morchellaceae, Pezizales) is a truffle-like ascomycete that includes the type species *L. carthusianum* from Europe and North America, as well as a variety from China. Two specimens collected from subalpine conifer forests in Hokkaido in 2004 and 2011 are the only records of the genus in Japan. Since they were identified as *L. carthusianum* without detailed examination, in-depth morphological observation and phylogenetic analysis were necessary to confirm their taxonomic placement. In this study, we critically re-examined the Japanese specimens. Morphologically, the length of ascospores of the Japanese *L. carthusianum* was found to be much shorter than that indicated by the original descriptions of the type species and its variety. Phylogenetic analyses based on two nuclear ribosomal DNA regions showed significant genetic divergence between the Japanese specimens and other specimens of *L. carthusianum*, including the variety from China. Accordingly, we described the second species of the genus *Leucangium*, *L. microspermum*, more than a century after the establishment of the genus.

要旨

Leucangium はヨーロッパおよび北米に分布する基準種 *L. carthusianum* および中国産の一変種からなる、チャワンタケ目アミガサタケ科の地下生子嚢菌類である。国内では北海道の亜高山帯針葉樹林において、2004 年と 2011 年の二度本属菌が採集され、*L. carthusianum* と同定されていたが、その信頼性について系統学的手法を含む再検証が必要な状況にあった。そこで今回、筆者らは日本産標本の詳細な分類学的検討を行った。その結果、日本産標本の子嚢胞子長径は *L. carthusianum* およびその変種の原記載より著しく小さかった。また、核リボソーム DNA の 2 領域の塩基配列に基づく系統解析の結果、日本産と欧米・中国産標本との間に顕著な遺伝的分化が見られた。以上の結果より、日本産標本を *Leucangium* に属す第二の独立種とみなし、新種 *L. microspermum* として記載した。なお、従来日本産標本には和名と

してセイヨウイモタケが充てられていたが、本種は日本固有の新種と判明したため、和名をコキイロイモタケと改称する。和名は濃色（こきいろ：黒みを帯びた濃紫色）を帯びた外皮に基づく。今後、本属の和名および日本未報告種である *L. carthusianum* の和名として、セイヨウイモタケを用いることを提案する。

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Introduction

The genus *Leucangium* (Morchellaceae, Pezizales) is a truffle-like ascomycete. This genus is characterized by a black peridium, a solid, white gleba that forms gray to dark gray pockets of fertile tissue, and distinctive, eye-shaped olivaceous ascospores (Trappe et al., 2009). The type species, *L. carthusianum* (Tul. & C. Tul.) Paol., was originally described as *Picoa carthusiana* Tul. & C. Tul. (Tulasne & Tulasne, 1862). Later, Quélet (1883) established the genus *Leucangium* and described the type species, *L. ophthalmosporum* Quél. Paoletti (1889) accepted *Leucangium*, and *P. carthusiana* was transferred to this genus. Several researchers suspected that *L. ophthalmosporum* and *L. carthusianum* were conspecific (Dumée & Burlet, 1923; Knapp, 1951), and the former species was eventually regarded as the synonym of the latter (Szemere, 1965; Trappe, 1971). Thereafter, *Leucangium* has been a monotypic genus.

Li (1997) examined the ultrastructure of *L. carthusianum* ascospores and septa of hyphae and assumed that *Leucangium* is closely related to members of Morchellaceae. Phylogenetic studies confirmed this view, illustrating that *Leucangium* was nested within Morchellaceae (Kovács et al., 2008; Trappe et al., 2010). By contrast, a recent phylogenetic study showed that *Picoa* was nested within Pyronemataceae (Sibissi et al., 2010).

Leucangium carthusianum was originally reported in France (Tulasne & Tulasne, 1862) and, thereafter, mostly in the mountainous areas of Southern Europe (Knapp, 1951). In addition, Gilkey (1939) recorded this species in Oregon, USA, while Liu & Tao (1990) recorded it in Shanxi Province, China. Recently, this species was also collected from Hokkaido, Japan, and was illustrated in a field guide (Sasaki et al., 2016). Thus, *L. carthusianum* is considered to be broadly distributed across the mid-latitude regions of the Northern Hemisphere.

Trappe et al. (2009), however, suggested that the North American population might be a different species from the European one. Indeed, recent phylogenetic analyses of North American and Italian samples revealed that *L. carthusianum* sequences diverged into several lineages (Chen & Fan, 2018). Moreover, Chinese collections were morphologically distinguishable from the specimens from other regions, and were therefore designated as *L. carthusianum* var. *purpureum* L. Fan & M. Chen (Chen & Fan, 2018). Further cryptic lineages may be uncovered from specimens traditionally identified as *L. carthusianum*, especially those from outside of Europe, including Japanese specimens. Thus, we conducted detailed observations and phylogenetic analyses of *L. carthusianum* from Hokkaido, Japan.

Materials and methods

Morphological observations

Two dried specimens collected on September 2004 (*H. Sasaki 481*; TNS-F-79665) from Furano-shi and on September 2011 (*K. Yamamoto 110912-1*; KPM-NC 27881) from Shikaoi-cho were used. The distance between two sites was ca. 50 km. Microscopic observation was conducted based on Yamamoto et al. (2019). The length : width ratio were presented as *Q* values. All specimens were freeze- or oven-dried at 60°C overnight and deposited in the Kanagawa Prefectural Museum of Natural History (KPM) and National Museum of Nature and Science, Tokyo (TNS) in Japan.

DNA sequencing and phylogenetic analyses

Two ascomata (TNS-F-79665 and KPM-NC 27881) were used for sequencing of the internal transcribed spacer (ITS) region and the large subunit (LSU) of nuclear ribosomal DNA. DNA extraction and PCR amplification were performed in accordance with Yamamoto et al. (2019). Newly generated sequences were deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp) under LC516713 (ITS) and LC516714 (LSU) for TNS-F-79665, and LC514671 (ITS) and LC514672 (LSU) for KPM-NC 27881. DNA sequence similarity was examined using National Center for Biotechnology Information (NCBI) BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

For phylogenetic analysis, thirty sequences of ITS and twenty-five sequences of LSU from species in Morchellaceae, including all available sequences of sequestrate species, i.e., *Fischerula macrospora* Mattir., *F. subcaulis* Trappe, *Imaia gigantea* (S. Imai) Trappe & Kovács, *Kalapuya brunnea* M.J. Trappe, Trappe & Bonito, *L. carthusianum* var. *carthusianum*, *L. carthusianum* var. *purpureum*, were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/nucleotide) and included in each dataset. *Disciotis venosa* (Pers.) Boud. and *Verpa conica* (O.F. Müll.) Sw. were selected as outgroups. Multiple alignment and phylogenetic analyses using the maximum likelihood

(ML) and maximum parsimony (MP) methods were conducted based on Yamamoto et al. (2020). The final alignment was deposited in TreeBASE (accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S25487). ML analysis was conducted under the general time-reversible model of nucleotide substitution, with a discrete gamma distribution (+G) and invariant sites (+I) for LSU dataset and with +I for ITS dataset selected by MEGA X (Kumar et al., 2018).

Results

The two specimens temporarily identified as *L. carthusianum* were both collected from subalpine forest floors dominated by *Picea* or *Abies* in September (autumn) (Fig. 1A, B). Ascomata were covered with almost black to blackish purple, asperate to partially warty peridium (Fig. 1C). The gleba was rather solid, composed of almost-white sterile veins and olivaceous pockets of fertile tissue (Fig. 1D). Peridial tissue was pseudoparenchymatous (Fig. 1E, F). Glebal tissue was composed of interwoven hyaline hyphae and irregularly distributed asci (Fig. 1G). Asci were globose (Fig. 1H) and inamyloid. Ascospores were eye-shaped and brownish at maturity (Fig. 1I). These morphologies agreed well with descriptions of *L. carthusianum* from Europe (Montecchi & Sarasini, 2000; Tulasne & Tulasne, 1862) and North America (Gilkey, 1939; Luther, 2009). On the other hand,

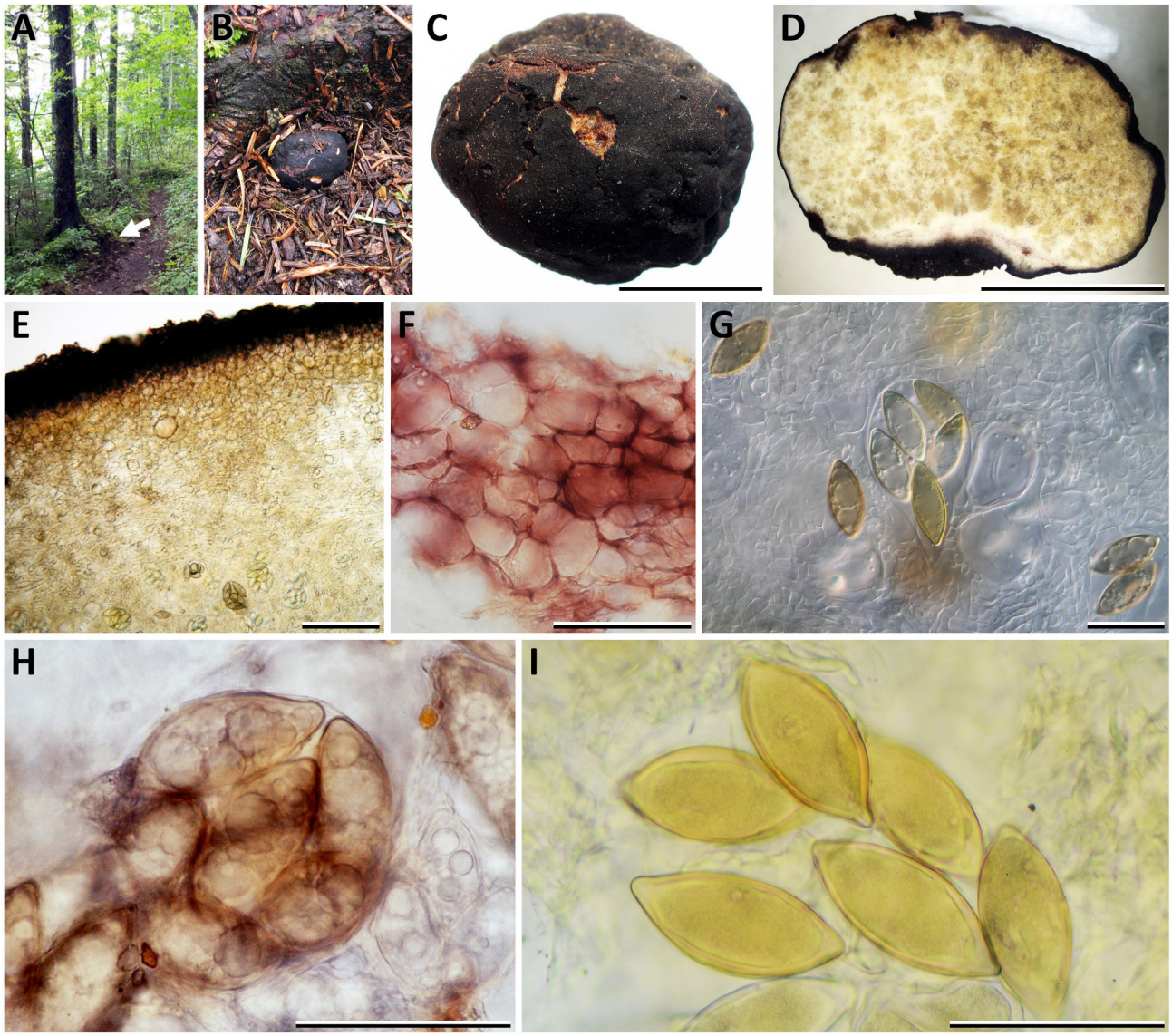

Fig. 1. *Leucangium microspermum* collected from Hokkaido, Japan (A–E, G, I: KPM-NC 27881; F, H: TNS-F-79665). E, G: Differential interference contrast microscopy; F, H, I: Bright-field microscopy. A: Habitat. Arrow indicates the position of ascoma. B: Semi-hypogeous ascoma in a field. C: Ascoma. D: Sectioned ascoma. E: Peridium (upper) and gleba (lower) mounted in lacto-glycerol. F: Surface of peridium composed of textura angularis mounted in water. G: Gleba mounted in lacto-glycerol. H: Ascospores in an ascus mounted in water. I: Matured ascospores mounted in 5% KOH. Bars: C, D = 1 cm; E = 200 µm; F–I = 50 µm.

図 1. 北海道産 *L. microspermum*（A–E, G, I: KPM-NC 27881; F, H: TNS-F-79665）の子実体外部形態、微細構造および発生地．微細構造はラクトグリセロール（E, G）、水（F, H）および 5% KOH（I）での封入時を示す．E, G は微分干渉顕微鏡像，F, H, I は明視野顕微鏡像を示す．A：発生地．矢印は子実体の発生位置を示す．B：発生状況．C：子実体．D：子実体断面．E：外皮（上方）およびグレバ（下方）．F：外皮を構成する多角菌組織．G：グレバ．H：子嚢内部の子嚢胞子．I：成熟した子嚢胞子．スケール：C, D = 1 cm; E = 200 µm; F–I = 50 µm.

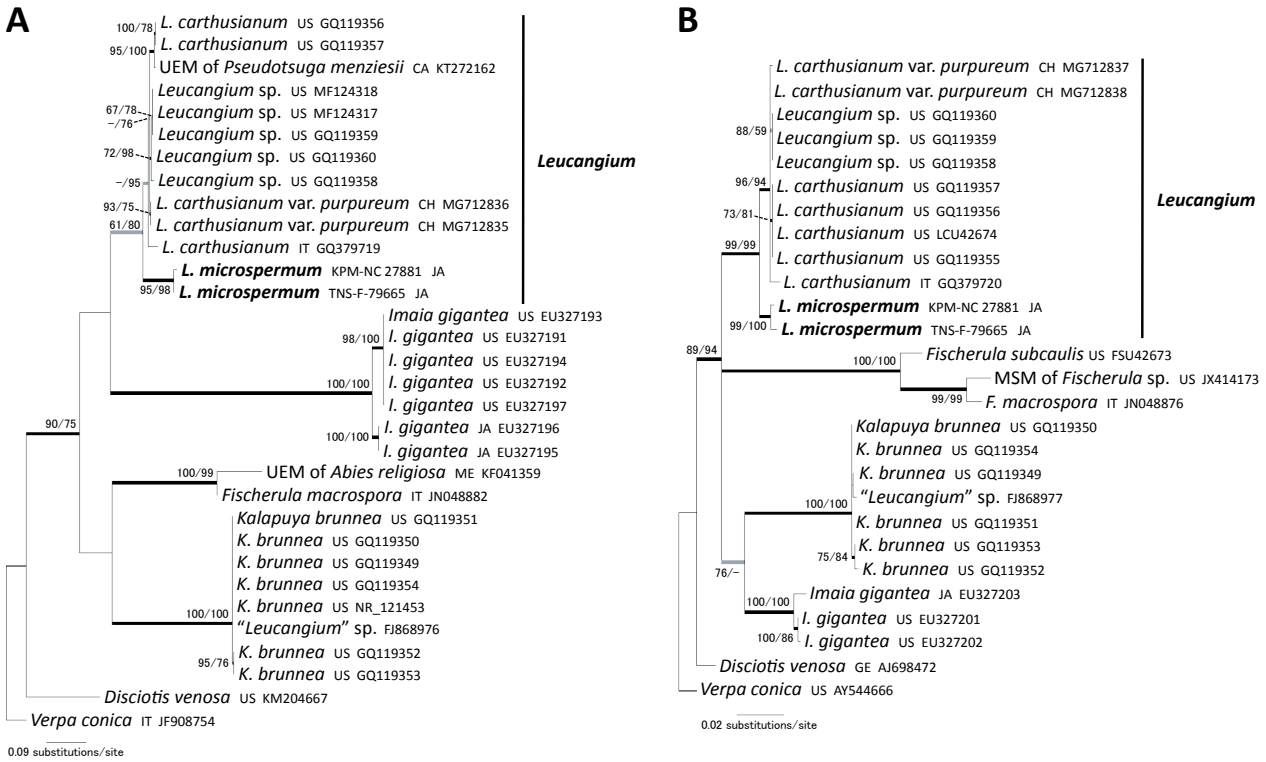

Fig. 2. Maximum likelihood (ML) phylogenetic tree of the internal transcribed spacer (ITS) (A) and large subunit (LSU) (B) sequences of *L. microspermum* and other truffle-like species in Morchellaceae. *Disciotis venosa* and *V. conica* were used as outgroups. Bootstrap (BS) values (1,000 replicates) > 50% from ML (left) and maximum parsimony (MP) (right) are shown near nodes. Branches supported by both BS of ML ≥ 70% and MP ≥ 70% (black), and ML ≥ 70% or MP ≥ 70% (gray) are depicted as thick lines. Abbreviations: UEM = uncultured ectomycorrhiza; MSM = mitospore mat; CA = Canada; US = USA; CH = China; IT = Italy; JA = Japan; ME = Mexico; GE = Germany.

図 2. アミガサタケ科に属する *L. microspermum* とその他の地下生菌の ITS（A）および LSU（B）データセットに基づく最尤法（ML）系統樹．外群には *D. venosa* ならびに *V. conica* を用いた．ノード付近に，最尤法（左）ならびに最節約法（MP）（右）のブートストラップ値（BS）を“MLBS/MPBS”として表示した．MLBS ≥ 70% かつ MPBS ≥ 70% で支持された分岐を黒の太線で，MLBS と MPBS のいずれかが 70% 以上の数値を示した分岐を灰色の太線で示した．UEM = 外生菌根由来の環境配列，MSM = 分生子マット由来の配列，CA = カナダ，US = アメリカ，CH = 中国，IT = イタリア，JA = 日本，ME = メキシコ，GE = ドイツ．

the dimensions of the ascospores in 5% KOH of the TNS-F-79665 specimen were 42.5–56 × 20–24.5 µm (mean, 49.5 × 23 µm; n = 85), Q = 1.9–2.7 (mean, 2.2; n = 85), and that of ascospores of the KPM-NC 27881 specimen was 46–62 × 20–27.5 µm (mean, 53.5 × 24 µm; n = 112), Q = 1.7–2.9 (mean, 2.2; n = 112). Hence, the ascospores of Japanese collections were much smaller than those originally described (60–80 × 25–30 µm) and most other records from Europe and North America (Table 1).

ITS (807 bp and 759 bp) and LSU (635 bp and 859 bp) sequences were obtained from the specimen KPM-NC 27881 and TNS-F-79665, respectively. The sequence homology of them was 99.3% (754/759 bp) for ITS and 99.5% (626/629 bp) for LSU. The BLAST search showed that ITS sequences from Japanese specimens were most similar to sequences of *Leucangium* spp., but the homology was only 89–90%.

Figure 2 shows the ML phylogenies of ITS (Fig. 2A: ln L = − 2879.328967) and LSU (Fig. 2B: ln L = − 2307.632231). In the MP analyses, the statistics of the ITS tree were as follows: tree length = 439, consistency index (CI) = 0.877, retention index (RI) = 0.974, and composite index = 0.86; those of LSU were tree length = 199, CI = 0.786, RI = 0.929, and composite index = 0.756. Both ITS and LSU trees strongly supported the monophyly of each genus of truffle-like fungus in Morchellaceae except for *Leucangium* whose clade was strongly supported only by LSU tree. Both trees showed that the KPM-NC 27881 and TNS-F-79665 specimens were monophyletic and were genetically divergent from the other sequences of *Leucangium* (i.e., *L. carthusianum*–*L. carthusianum* var. *purpureum*–North American *Leucangium* sp. complex) within the *Leucangium* clade (Fig. 2).

Taxonomy

Our morphological comparison and phylogenetic analyses strongly support that the Japanese specimens of *Leucangium* should be treated as a distinct species. Accordingly, we propose

Table 1. Known localities and ascospore dimensions of *Leucangium* spp.

表 1. *Leucangium* 属種の産地および子嚢胞子サイズ.

Taxon (種名)	Country (国)	Locality (地域)	Vegetation (植生)[a]	Ascospore (子嚢胞子)[b]			Reference (出典)
				Length (長さ) (μm)	Width (幅) (μm)	Q value (長さ / 幅)	
L. microspermum	Japan	Hokkaido	As, Qc	42.5–56 [49.5]	20–24.5 [23]	1.9–2.7 [2.2]	This study (TNS-F-79665)
			Pj	46–62 [53.5]	20–27.5 [24]	1.7–2.9 [2.2]	This study (KPM-NC 27881)
L. carthusianum	France	Dauphine	F, A	60–80	25–30	n/a	Tulasne & Tulasne (1862)[d]
		Jura	A	60–80	n/a	n/a	Quélet (1883)
		Vosges Mountains	Aa	60–75	20–35	n/a	Laurent (1996)
		Alsace	Pa, Fe	60–80	20–35	n/a	Laurent (1998)
		Vercors	Aa	60–80	20–38	n/a	Astier (1998)
		Cantal	Ps	58–86 [68.6]	(22)25–31 [27.7]	2.1–3 [2.5]	Van Vooren (2017)
	Germany	Schwarzwald	F, Aa	67–81	27–36	n/a	Eichler (1898)
	Italy	Cuneo; Forlì-Cesena; Parma; Reggio nell'Emilia	A, P, F	60–75	20–35	n/a	Montecchi & Sarasini (2000)
		Lucca	Pm, Ca	(46)50–60	(26)30–36	n/a	Gori (2005)
	Romania	Caraș-Severin	F, A	60–80	20–38	n/a	Hollós (1911)
	Switzerland	Kanton Luzern	P	59–71	25–32	n/a	Irlet (1985)
	Tunisia	n/a	Hl, Rs	27[c]	16.5[c]	n/a	Slama et al. (2006)
	USA	Oregon	n/a	74–80	24–32	n/a	Gilkey (1939)
		Washington	Pm	67–82	28–35	n/a	Luther (2009)
		Pacific Northwest	Pt, A, P	60–75(–90)	20–35	n/a	Trappe et al. (2009)
		Western part of the Pacific Northwest	Pm	55–100	19–42	n/a	Beug et al. (2014)
L. aff. *carthusianum*	Spain	Valladolid; Segovia	H, Qi, C	27–60	20–35	n/a	Calonge et al. (1995)
L. carthusianum var. *purpureum*	China	Shanxi Province	Pw	58–65(–70)	19–22	n/a	Liu & Tao (1990)
		Hebei Province	Lp	60–67.5(–70)	20–25(–27.5)	2.4–3.4	Chen & Fan (2018)[d]

[a] A = *Abies*; Aa = *A. alba* Mill.; As = *A. sachalinensis* (F. Schmidt) Mast.; C = *Cistus*; Ca = *Corylus avellana* L.; F = *Fagus*; Fe = *Fraxinus excelsior* L.; H = *Helianthemum*; Hl = *H. lippii* Dum. Cours.; Lp = *Larix principis-rupprechtii* Mayr; P = *Picea*; Pa = *P. abies* (L.) H. Karst.; Pj = *P. jezoensis* (Siebold & Zucc.) Carrière var. *jezoensis*; Pw = *Picea wilsonii* Mast.; Pt = *Pseudotsuga*; Pm = *Ps. menziesii* (Mirb.) Franco; Ps = *Pinus sylvestris* L.; Qc = *Quercus crispula* Blume; Qi = *Q. ilex* L.; Rs = *Rhanterium suaveolens* Desf. [b] Mean value is shown inside brackets. [c] It is highly likely that the length of scale bars of all photographs in Slama et al. (2006) is wrong; their correct length are considered to be about 1/3 length. Therefore, the length and width herein are estimate values based on Fig. 5 in Slama et al. (2006). [d] Original description.

L. microspermum for the Japanese specimens.

***Leucangium microspermum* Koh. Yamam., Hir. Sasaki, Ohmae & Orihara, sp. nov.**

[MycoBank ID: MB 833826]

Fig. 1.

Diagnosis: The species forms much smaller ascospores than other infrageneric taxa of *Leucangium*.

Etymology: From the Greek *micro* = small and *spermum* = spored, referring to the characteristic small ascospore of this species.

Holotype: JAPAN, Hokkaido, Shikaoi-cho, the shore of Lake Shikaribetsu, alt. 817 m, semi-hypogeous under *Picea jezoensis* (Siebold & Zucc.) Carrière var. *jezoensis* standing by a trail, 12 Sep. 2011, *K. Yamamoto 110912-1* (KPM-NC 27881).

Description: Ascomata stereothecia, firm, oblate spheroid, 22–29 × 15–21 mm; surface almost black to dark black-purple, nearly smooth to minutely warty, sometimes becoming rimose, with a fragile basal attachment. Gleba solid, olive brown pockets of fertile tissue separated by sterile veins in maturity, sometimes tissue nearby peridium partially stained purple, sometimes sterile base present. Odor not distinctive. Peridium dark brown or purple-brown in lacto-glycerol, darker toward the outside surface, 300–380 μm thick composed of rounded to polyhedral cells 15–27(–67) μm wide, wall thin or sometimes slightly thickened up to 2.5 μm thick. Glebal tissue textura intricata composed of hyphae hyaline, septate, 3.2–12 μm broad, thin-walled. Asci irregularly distributed, hyaline, ellipsoid, globose or obovoid, with a stipe in youth, 93.5–118.5 × (57–)65.5–82 μm (not including stipe), thin-walled, with 5–8 spores, not reacting in Melzer's reagent with pretreatment in 5% KOH. Ascospores elliptic-fusoid, usually apiculate at both ends, surface smooth, 42.5–62 × 20–27.5 μm (mean, 52 × 23.5 μm; n = 197), *Q* = 1.7–2.9 (mean, 2.2; n = 197) in 5% KOH, hyaline in youth becoming yellow-brown or olive-brown, thin-walled; nonreactive in Melzer's reagent; containing 2–4 large central oil droplets surrounded by tiny ones in youth, single large oil droplet present at maturity.

Distribution, habitat, and fruiting season: hypogeous or semi-hypogeous, solitary under subalpine forest dominated by *Abies* or *Picea* in Hokkaido, Japan. Found in autumn.

Other specimen examined: JAPAN, Hokkaido, Furano-shi, the University of Tokyo Hokkaido Forest, under *Abies sachalinensis* (F. Schmidt) Mast. and *Quercus crispula* Blume, 13 Sep. 2004, E. Sano, *H. Sasaki 481* (TNS-F-79665).

Note: An ascoma and ascospores of the specimen TNS-F-79665 are illustrated in Sasaki et al. (2016) as *L. carthusianum*.

Discussion

Leucangium spp. always form ascomata near pinaceous trees (Table 1), especially *Abies*, *Picea* and *Pseudotsuga*, except in two known cases (Calonge et al., 1995; Slama et al., 2006). *Leucangium* is considered as an ectomycorrhizal mycobiont of Pinaceae based on direct observation of ectomycorrhiza on *Pseudotsuga* (Agerer & Rambold, 2004–2019) and molecular identification of ectomycorrhizal tips (KT272162 in Fig. 2A) (Kranabetter et al., 2015). Nevertheless, Tunisian *L. carthusianum* and Spanish *L.* aff. *carthusianum* were collected from broadleaf evergreen shrub, *Helianthemum*-dominated vegetation without pinaceous plants (Table 1). Moreover, these fungi formed quite small ascospores (Table 1) and the latter lacked apiculi on ascospore (Calonge et al., 1995). Therefore, these fungi may belong to another genus.

The second species of the genus *Leucangium*, *L. microspermum*, is described herein more than a century after the establishment of the genus. *Leucangium microspermum* is hardly distinguishable from *L. carthusianum* by the ascomata morphology, but the ascospores of the former are significantly smaller than those of the latter. Previous records of *L. carthusianum* (Table 1) suggest the presence of further cryptic species. Gori (2005) reported *L. carthusianum* from Italy that formed smaller ascospores similar to those of *L. microspermum*. Likewise, Beug et al. (2014) reported larger ascospores (reaching 100 µ m) in samples from the USA.

Phylogenetically, the LSU tree (Fig. 2B) showed that *L. carthusianum* diverged into the North American- and Italian-lineages, which was also moderately supported by the ITS tree (Fig. 2A). Our study also suggested the presence of another North American lineage (as *Leucangium* sp. in Fig. 2), which was considered as an undescribed species by Trappe et al. (2010). Because the present study indicated that the size of ascospores was a key diagnostic character for the classification in *Leucangium*, detailed morphological comparison of ascospores will probably resolve whether these North American lineages differ from European lineage at the species or infra-species level.

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