Research group
MYCOLOGY
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NEW SPECIES OF *LEOTIA* FROM AFRICA

(FUNGI, ASCOMYCOTA)

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FACULTEIT WETENSCHAPPEN

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Abstract

In this bachelor thesis three presumed new species in the genus *Leotia* (Leotiomycetes: Leotiaceae) will be formally described. Fungi in this genus are stipitate cup fungi within the Ascomycota, characterised by their gelatinous appearance of the ascomata. It is therefore not without reason that these fungi are commonly called *jelly babies.* Although these Fungi belong to the cup fungi, the name cup fungi is a little misleading for these representatives of the class. Rather than exhibiting a cup-shaped ascoma, *Leotia* species form a distinctive stalk with a convex, brain-like cap. They mostly grow in woodlands on bare soil or among grass, moss, and/or plant detritus. The genus is placed in the family Leotiaceae, where it shares a close relationship to *Microglossum*, according to SSU and LSU ribosomal DNA sequence analyses. The species described here were collected in Bénin and Mozambique on soil in (sub-)tropical forests. The diversity of this genus in Africa has thus far received no attention. In this bachelor thesis I present macromorphological and microscopic descriptions of the widespread European species *Leotia lubrica* and three presumably new African species. The new species are illustrated with photographs and hand drawings. In addition, a phylogenetic tree of *Leotia* based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene is presented.

Introduction

The two best studied phyla of among the Fungi are Ascomycota and Basidiomycota. Of those, Ascomycota, with its more than 83,000 described species (Kirk, 2019), is the most diverse and best studied phylum. Members of this group are named after their unifying ability to produce sexual spores, ascospores, inside specialized elongated cells, known as asci. A subdivision in the Ascomycota brings about the group Pezizomycotina, which makes up most of the Ascomycota fungi and includes the vast majority of filamentous ascomycetes (Spatafora et al., 2006; Ekanayaka et al., 2017). This subphylum accommodates the artificial group of inoperculate discomycetes, characterized by unitunicate asci in an open apothecial ascomata (Zhang & Wang, 2015). “Inoperculate” refers to the absence of an operculum , which is a lid covering the opening in the ascus and opening at maturity to release the fungal spores. Conversely, these inoperculate fungi possess an (in-)amyloid pore or ring structure. Within this group, the Leotiomycetes are the most diverse class, both from ecological and morphological perspective (Ekanayaka et al., 2017; Johnston et al., 2019; Quandt & Haelewaters, 2021). Leotiomycetes fungi encompass endophytes in stems and leaves, plant and mammalian pathogens, saprobes, mycorrhizae, and coprophilous fungi. Some often manifest high host specificity. Moreover, in terms of habitat intake, this class comes out on top. They occupy fresh-water habitats, dry terrestrial environments, amphoteric bogs, animal dung, soils, and decomposing materials (Zhang & Wang, 2015). The reason of their successful habitat-intake runs parallel with their varied range in sexual fruiting bodies, according to Ekanayaka et al. (2017). Most representatives in the class are apothecial, that is, they have an exposed hymenium, variations in sexual forms such as cleistothecia and perithecia often occur. Additionally, several lineages reproduce (exclusively) asexually (Ekanayaka, 2019; Johnston et al., 2019).

with a total of 6,440 described species across 53 families and 630 genera (Quandt & Haelewaters, 2021) are known in the class Leotiomycetes. Nonetheless, they are anything but well understood and often overlooked. According to Johnston et al. (2019), only 5–7% of the species diversity is currently known. Mainly taxa that are difficult to culture and occur in undersampled geographic areas and habitats, stay under the radar. The poorly understood systematics can also be assigned to deficiencies in classification and phylogenetic reconstruction of the class (Quandt & Haelewaters, 2021). For a long time, phylogenies were solely based on ribosomal DNA loci, which provide insufficient support for Leotiomycetes higher classification. Johnston et al. (2019) and Haelewaters et al. (2021) presented phylogenies based on up 15 loci and still deep nodes lacked support. The genome-based phylogeny of Johnston et al. (2019) was highly supported except near the base of the tree—referring to taxa outside of Helotiales, As a result, despite recent improvements (e.g., Quijada et al., 2020, 2022; Haelewaters et al., 2021), the classification of Leotiomycetes is not yet resolved. Improved tools and techniques should slowly but steadily reveal undescribed clades of Leotiomycetes, improving taxon sampling and thus potentially resolving the deepest nodes.

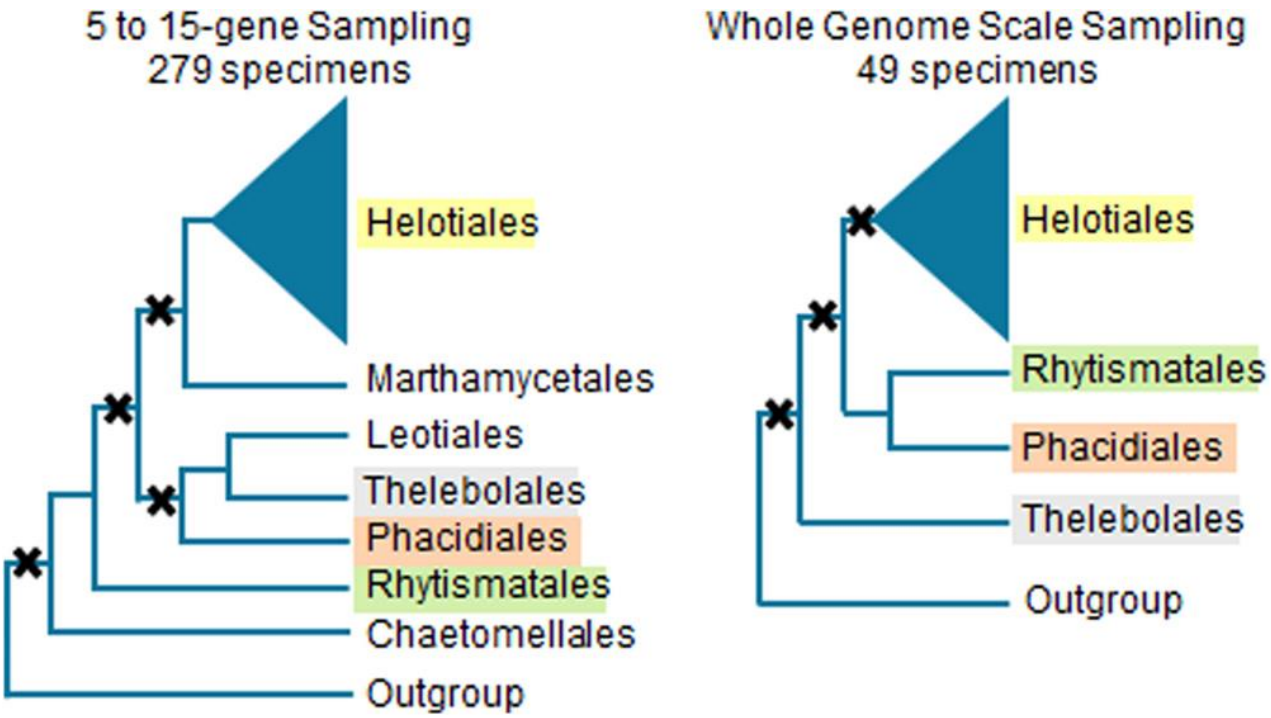

Figure 1: Current evolutionary hypotheses about interordinal relationships within the class Leotiomycetes, based on Johnston et al. (2019). Left, 5–15 locus phylogeny based on 279 isolates; right, whole-genome scale phylogeny based on 49 isolates. Nodes where support is lacking are marked with a black "x". Orders that are represented in both analyses are highlighted in color (Helotiales in yellow, Phacidiales in peach, Rhytismatales in green, Thelebolales in gray), showing major topological disagreement between the two analyses. Other orders have thus far not been considered in Leotiomycetes-wide multilocus or genome-scale analyses. Modified from Johnston, P.R., Quijada, L., Smith, C.A., et al., 2019. A multigene phylogeny toward a new phylogenetic classification of Leotiomycetes. IMA Fungus 10, 1. (Quandt & Haelewaters, 2021, p. 287, fig. 1)

The position of the genus *leotia* Pers. among the Ascomycota was not fully resolved for a prolonged period (Zhong & Pfister, 2004). Also the family Leotiaceae and order Leotiales have undergone several reinterpretations. Based on the most recent conceptions of Johnston et al. (2019), the genus *Leotia* Pers. is included in the family Leotiaceae in the order Leotiales. Members of Leotiales conform to stipitate to clavate apothecial members of Leotiaceae, to which *Leotia* and its close relative *Microgossum* belong. However, what makes these taxa truly distinctive from other taxa is the association of gelatinous tissues within the ectal excipulum. The excipulum is the tissue that surrounds and supports the hymenium within the ascomata. Within *Leotia* it is typically composed of interwoven hyphae in a gelatinous matrix. The structure and composition of the excipulum can be an important characteristic in distinguishing among different species of *Leotia*. However, the morphology and arrangement of the tissue can vary widely among species. Therefore, a combination of macroscopic and microscopic features should be used to accurately identify *Leotia* species (Jordan, 2004; Redhead et al., 2014). Yet, Zhong & Pfister (2004) casted doubt on the use of gelatinized tissue as the single character to distinguish taxa in *Leotia*.

Thus, members of the genus are characterised not only by their tissues consisting of hyphae embedded in a gelatinous matrix (Zhong & Pfister, 2004). They are also distinguished within the cup fungi by their stipitate, globose, irregularly-shaped apothecia. When fresh the fruiting body features a gelatinous, lobed, slightly wrinkled, convex cap and a stem that is smooth or often granulated (Mains, 1956). The margin of the apothecia is often enrolled and undulating. Colour of the ascomata often darkens with age and ranges from bright yellow and yellowish-brown (*Leotia lubrica*) to olivaceous (*Leotia atrovirens, Leotia viscosa* but with a yellow stalk). Zhong & Pfister (2004) concluded that colour is a poor approach of species delimitation and that profound further study is needed. At the microscopic level, *Leotia* distinguishes from *Microglossum* by the inamyloid pore on the ascus apex. Other microscopic features are: ellipsoidal to fusiform hyaline ascospores, cylindrical to

clavate asci containing 8 ascospores, and slender paraphyses that may be simple or branched (Grelet, 1947; Jordan, 2004).

Leotia lubrica is the type species of the genus *Leotia*. It was firstly described by Giovanni Antonio Scopoli in 1772 as *Helvella lubrica* Scop. in his book "Flora Carniolica". Later the species was renamed by Christiaan Hendrik Persoon in 1794 as *Leotia lubrica* (Index Fungorum - Names Record, z.d.). The epithet "*lubrica*" refers to the common slimy appearance of the ascomata. *Leotia* was so far under prevailing discussion whether it can be considered a saprobic or a mycorrhizal member. Fortunately, Kühdorf et al. (2015) provided first evidence for the ecytomycorrhizal status of Leotia in association with *Comarostaphylis arbutoides* (Ericaceae). Further information and a detailed description of *Leotia lubrica* will be given in the Results section.

The objective of this bachelor thesis is to describe new collections of *Leotia* from Bénin, D.R. Congo, and Zimbabwe. In this bachelor thesis, detailed microscopic descriptions will be given as well as photographs and drawings, starting with a Belgian collection of the often reported *L. lubrica*. Furthermore, an ITS-based phylogenetic analysis is provided to determine the placement of the new collections within Leotiaceae.

Materials and methods

Sampling

Specimens of *Leotia lubrica* were collected in Belgium (51.0261 N 3.7150 E). These were first studied as a preparation for the African collections.
Fresh specimens of Leotia were collected during expeditions across Bénin and Mozambique between 1997and 2004. The specimens were dried using silica beads or a food dehydrator. Collections were packaged, labeled, and deposited at the BR and GENT herbaria. The collected specimens were photographed in the field.
The ascomata were searched on soil in woodlands, more specifically gallery and miombo forests.
Miombo forests are a type of woodland ecoregion found in Africa, covering 2,7 million km^2 of southern, central and eastern parts of the continent. Dominant tree species are *Brachystegia sp.* and *Julbernadria sp.* The climate varies depending on the specific location and which season in. In general it ranges from humid to semi-arid, and sub-tropical to even temperate (Gambiza et al., 2000). Miombo forests are categorised into four ecoregions (Angolan miombo woodlands, Central Zambezian miombo woodlands, Eastern miombo woodlands, Southern miombo woodlands) and are distributed among several countries (Fig. 2) The ecoregions include (sub-)tropical grasslands, savannas, and shrublands biome. Miombo woodland is an important ecosystem that provides a range of ecological services, but is like many forest ecosystems under severe anthropogenic threats (Angolan Miombo woodlands | Ecoregions | WWF, z.d.).
A gallery forest, also known as a riparian forest or riverine forest, is a type of forest that grows along the banks of a river, stream, or other watercourse. These forests are typically found in semi-arid and arid regions and occur in narrow strips along the watercourse. Gallery forests are characterized by a dense canopy of trees that provide shade and help maintain a relatively humid microclimate. The trees in gallery forests are typically deciduous and may shed their leaves during the dry season. These forests provide important ecological functions, including stabilizing the banks of rivers and streams, reducing erosion, and providing habitat for a diverse range of plant and animal species. (Beard, 1955; Kellman & Meave, 1997; Chepkemoi, 2017)

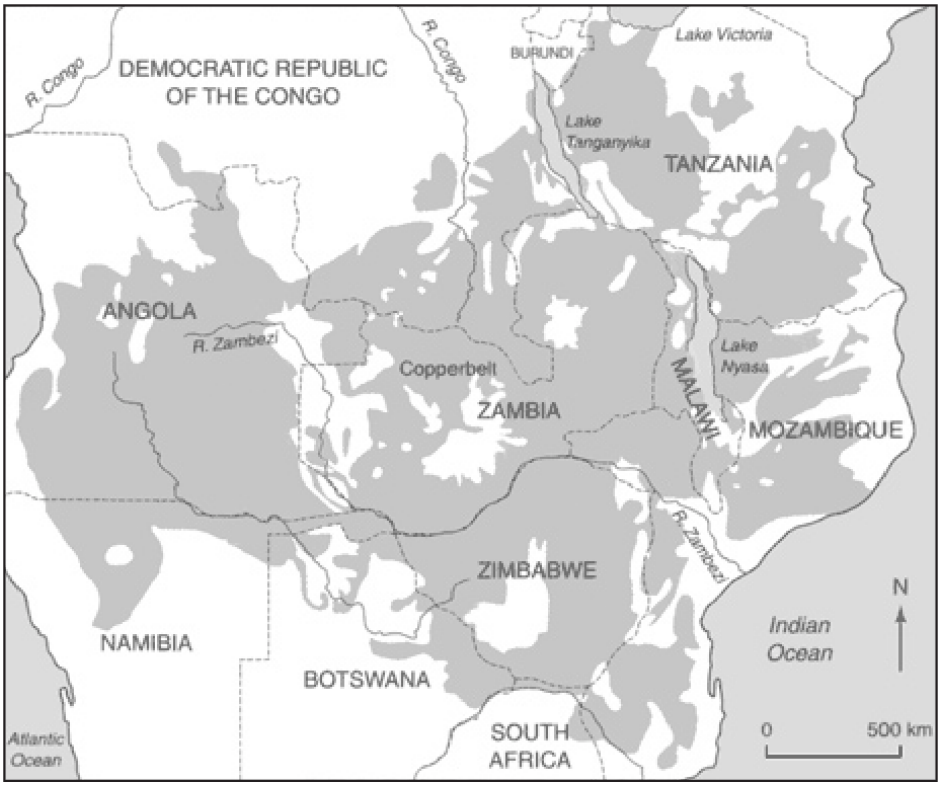

Figure 1 Distribution of miombo woodlands indicated in grey (Abdallah et al., 2007)

Morphological study

For the morphological study isolates from different collections were used. The macromorphological description was based on photographs taken in the field of the fresh material. Since the colour is often considerably changed by drying, macromorphological determination of the dried specimens was not performed due to inaccuracy (Mains EB. 1956). The terminology of Weber et al. (1997) was used for the description of the macroscopic characters. Micromorphological features were described on dried herbarium specimens. The terminology for Orbiliomycetes outlined in Baral et al. (2020) was used for the description. The tissue typology of Hengstmengel (2020) was used to describe the different layers in the excipulum. To examine, draw and describe the microscopic characters, a Kyowa stereo microscope, model SD-2PLQ and a binocular, type Olympus CX31 to which a drawing tube is attached were used. A 400× to 1000× magnification was used to describe the elements. The important elements (ascomata, ascospores, paraphyses, croziers, excipulum) were photographed using a Euromex (type HD-ultra camera VC.3036) camera mounted on an eclipse E600 Nikon compound microscope. Further, the asci (length and width), ascospores (length and width) and the apices of the paraphyses (diameter) were measured using the scale-bars on the photographs. The following formula was uses: (minimal value-) mean minus standard deviation – mean plus standard deviation (- maximal value). Measurements of the ascomata were done using ImageJ (Abràmoff et al. 2004), and noted and further processed in Excel. In the descriptions the following abbreviations were used: CR = congo red SDS, diH_2O = deionized water, KOH = 10% potassium hydroxide, IKI = Lugol's solution, MLZ = Melzer's reagent, LB = lipid body.

Squash mount slides from fungal tissues were made in diH_2O, KOH, IKI, and CR. IKI was used to determine amyloidity. Sections of the dried ascomata were made using different methods. The wet method was used by cutting small fragments of the dried apothecium with a scalpel and subsequently rehydrating the material in diH_20 for several minutes to allow swelling. After that, swollen pieces of tissue were sliced in smaller sections and then brought in KOH for several minutes to ensure clear visibility of important microscopic elements. Next, squash mount slides were prepared by staining the sections in CR and IKI. To obtain a clear vision of the distinct

layers of the excipulum, the dry method was often used to make cross sections of the fertile part of the ascoma. In this method, thin slices of the cap were cut with a small razor blade and stained directly in CR. Sometimes a better result was attained by putting a droplet of either diH_2O or KOH on a slide and subsequently dipping the pointy end of the razor blade in the droplet before thinly shaving through the section of interest. Similar to the wet method, squash mount slides were made with CR and IKI. Furthermore, strong squeezing to separate the elements was required for best observations, both for the dry as the wet method. Sometimes the observed material often had to be ruptured by putting pressure on the cover slip and simultaneously moving it in lateral direction to obtain free elements.

To determine amyloidity, IKI was used that initiates a blue iodine reaction of the apical ring of the ascus in case of amyloidity. Sometimes a deviation is acquired, in which is spoken of a false negative or hemiamyloid reaction (Baral 1987). This reaction comes down to a KOH-provoked amyloidity (Baral 2009). Hence, KOH was used due to its serious influence on the iodine reaction regarding colour intensity. (Kohn & Korf 1975, Nannfeldt 1976) A treatment with 10% KOH was used rather than 2% KOH because of a more intense blueing, according to Kohn & Korf (1975). To test for both inamyloid and hemiamyloid reactions, different techniques were applied – following the practical application guide by Baral (2009). Baral 1999 and Kohn & Korf suggest that using IKI without KOH pretreatment is sufficient to prompt a blue iodine reaction, while the use of MLZ requires KOH pre-treatment to confirm amyloidity. When a non-amyloid reaction came out clearly, MLZ was used afterwards for a final additional confirmation.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from rice-sized pieces of tissue using the QIAamp DNA Micro Kit and the DNeasy PowerPlant Pro Kit (Qiagen, Valencia, California). DNA extracts were stored at -20 °C until PCR could be performed. The following loci were amplified: small and large subunit (SSU and LSU) of the nuclear ribosomal RNA gene, and the internal transcribed spacer (ITS) region. Primer combinations were as follows: NS1/NS6 for SSU (White et al. 1990), ITS1f/ITS4 for ITS (White et al. 1990, Gardes & Bruns 1993), and LR0R/LR5 for LSU (Vilgalys & Hester 1990, Hopple 1994). Purification and sequencing with the same primers were outsources to Genewiz (Plainfield, New Jersey). Sequence reads were assembled and edited using Sequencher version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan). DNA extractions and PCR amplifications were done by Jingyu Liu at Purdue University (West Lafayette, Indiana, USA).

Phylogenetic analyses

Newly generated ITS sequences (Table 1) were compared with existing sequences in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using Nucleotide BLAST excluding models and uncultured/environmental sample sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). An ITS dataset was assembled with the newly generated sequences and sequences of *Leotia* downloaded from GenBank (most sequences were from Zhong & Pfister 2004). Sequences were aligned using the command-line version of MUSCLE v.5.1.0 (Edgar 2004). Sequences were trimmed at conserved motifs at the 3' end of the SSU and the 5' end of the LSU (Dentinger et al. 2011). The ITS1 and ITS2 spacers and 5.8S conserved part of the ITS region were treated as different partitions in the phylogenetic analyses, as they have different rates of evolution. Maximum likelihood analyses were done using the command-line version of IQ-TREE v.1.6.7 (Nguyen et al. 2015) under partitioned models (Chernomor et al. 2016). Best-fit models of nucleotide substitution were selected using the IQ-TREE built-in program ModelFinder (Kalyaanamoorthy et al. 2017) based on the Akaike Information Criterion corrected for small sample size (AICc). Ultrafast bootstrapping was done with 1000 replicates (Hoang et al. 2017). Trees were visualized and edited in FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). Phylogenetic analyses were done by promotor Danny Haelewaters.

Table 1: Herbarium and GenBank accession numbers for studied collections

Species	Strain/isolate	Collection	Herbarium accession	SSU	ITS	LSU
Leotia baculum	DHF-1494a,b,c,d		/	NS1/NS6	ITS1f/ITS4	LR0R/LR5
	DHF-1775a	ADK2010	BR-MYCO 74856-69	/	ITS1f/ITS4	/
	DHF-1776a	ADK2051	BR-MYCO 74856-69	/	ITS1f/ITS4	/
	DHF-1780a	ADK2419	BR-MYCO 112589-69	/	ITS1f/ITS4	/
	DHF-1782a	ADK2715	BR-MYCO 116724-33	/	ITS1f/ITS4	/
	DHF-1783a	ADK3669	BR-MYCO 157073-30	/	ITS1f/ITS4	/
Leotia sp. nov. 2	DHF-1778a	ADK2417	BR-MYCO 112587-67	/	ITS1f/ITS4	/
	DHF-1779a	ADK2418	BR-MYCO 112588-68	/	ITS1f/ITS4	/
	DHF-1785a	ADK5452	BR5020212171270V	/	ITS1f/ITS4	/
Leotia rufa	DHF-1777a	ADK2376	BR-MYCO 112546-26	/	ITS1f/ITS4	/
Leotia sp. nov. 4	DHF-1786a	ADK6060	BR5020212170242V	/	ITS1f/ITS4	/

Results

Phylogeny

The phylogenetic reconstruction of the ITS dataset (Fig. 1) reveals the position of the clade containing *Leotia lubrica, Leotia atrovirens, Leotia viscosa* (with strains from US, UK, Europe, Australia, New-Zealand, China, Canada) as sister to the new African clade (*Leotia baculum*, 2, 3, 4) with a high bootstrap (BS) support of 98%. The monophyletic African clade is subdivided into four putatively species-level subclades. *Leotia* spp. 1 and 2 consist of multiple isolates from multiple collections and multiple countries, and receive BS support of 99% and 100%, respectively. *Leotia* sp. nov. 2 is represented by isolates DH1778a and DH1779a, and DH1785a. Both ITS sequences of DH1778a and DH1779b share 98.10% identity with that of DH1785a. The two other African species are singleton clades, *Leotia* spp. 3 and 4, thus each consisting of a single isolate. These two species receive low support (BS = 71% and 67%, respectively).

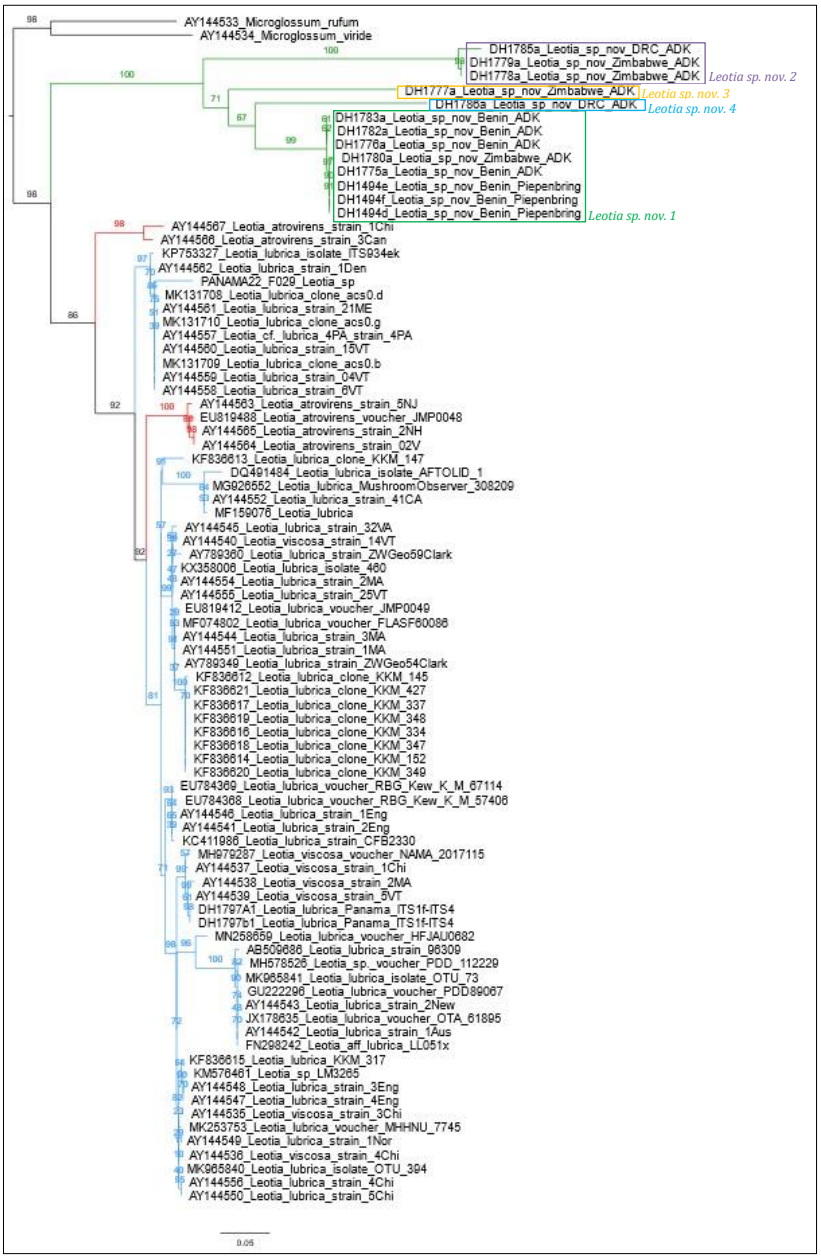

Figure 1: phylogenetic tree made by Danny Haelewaters based on the internal transcribed spacer (ITS) region using maximum likelihood to compare different species belonging to the genus Leotia. For each node, the ML bootstrap support is depicted above the branch leading to that node. Collections are numbered and geographical details are as follows: MA, VT, VA, CA, PA, ME, NJ, NH for states in the USA; Aus = Australia; Can = Canada; Chi = China; Den = Denmark; Eng = England; New = New Zealand. Green branches indicate presumably new species. The tree is artificially rooted to Microglossum rufum and Microglossum viride.

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Taxonomy

Pezizomycotina O.E. Erikss. & Winka, Myconet 1(1): 9 (1997)
Leotiomycetes O.E. Erikss. & Winka, Myconet 1(1): 7 (1997)
Leotiales Korf & Lizoň, Czech Mycol. 52(4): 256 (2001)
Lelotiaceae Corda, Icon. Fung. (Prague) 5: 37 (1842)
Leotia Pers., Neues Mag. Bot.: 31 (1794)

Generic description

"Ascomata stipitate, rounded, thick, more or less wrinkled or lobed, covered by the hymenium, separated from the foot by a fairly wide vallecula. Asci claviform, containing eight ascospores, with emarginated opening or pore. Paraphyses branched. Ascospores oblong-fusiform with small oil droplets inside when young, non-septate or septate when mature." (translated verbatim from Grelet 1947).

Leotia lubrica (Scop.) Pers., Neues Mag. Bot.: 31 (1794)

Specimens examined
BELGIUM. East Flanders Province, Ghent, Ghent University campus Sterre, 51.0261 N 3.7150 E, on soil, 21 November 2022, *coll.* W. Van Caenegem (GENT).

Description

Ascocarps scattered individually or gregarious in small groups, total length 10 – 80 mm. ***Apothecia*** stipitate-capitate, diameter 10 – 30 mm , usually one cap (apothecium) per stipe, sometimes fasciculate with two to three apothecia originating from a common and branching stipe, some separated at the root by a wide but shallow groove, convex, brain-like, lobed, somewhat furrowed or wrinkled, more or less wavy with a overhanging, enrolled, undulating margin, yellowish green to brownish-black to olivaceous, darker with age, when fresh jelly-like and with a smooth surface, somewhat viscid (especially when wet). ***Stipe*** distinct and long, diameter 3 – 10 mm, cylindrical or tapering, , subequal or slightly swollen at the base, sometimes slightly compressed, bright ochraceous to olivaceous, viscid, smooth or slightly granulated; flesh gelatinous, paler, white-yellowish. ***Asci*** eight-spored, elongate, cylindrical to clavate; apex rounded, blunt to slightly conical, gradually tapering to the base, inamyloid (in IKI or MLZ , without or with KOH-pretreatment), no clear ring or pore structure visible, apical wall thin; ascus base non-furcate, arising from repeating croziers with two septa, croziers non-perforated, some arising from simple-septa; Ascus wall thin, gelatinous, inamyloid; measurements (101,6–) 109,9 – 123,4 (–131,4) x (6,8–) 8,4 – 11,4 (–11,8) µm. ***Ascospores*** (sub-)cylindrical, ellipsoid-fusiform, rhomboid-fusiform, slightly curved to inequilateral, without ornamentation, multi-guttulate with two to five large LBs confluent to one ellipsoid body or seperate (appearing almost septate but with septa hard to define), several smaller LBs (lipid content very high) , hyaline, unpigmented; spore apex subacute; spore base medium tapered/attenuated; measurements (15,1–) 16,7 – 20,6 (–23,1) x (4,0–) 4,2 – 5,6 (–6,7) µm. ***Paraphyses*** filiform, slender, thin, cylindrical, straight to slightly curved at apex; apex slightly capitate (knob-like), measurements (0,8–) 1,1 – 1,8 (–1,9) µm; generally dichotomous branching (mostly at base), multiseptate, hyaline, inamyloid. ***Ectal excipulum*** loosely interwoven hyphae resembling a *textura intricata*, thin, cylindrical, gelatinous, multiseptated, usually with distinct interhyphal spaces, separated from medullary excipulum by a vague transition between *t. globulosa* and *t. intricata*. ***Medullary excipulum*** densely packed, globose cells resembling a *textura globulosa,* between hymenium and ectal excipulum, non-gelatinous.

Habitat, distribution and ecology:
Widespread (recorded in Europe, North America, Asia and Australasia), hygrophilous, usually found in small groups in coniferous or deciduous woodland and along forest edges, in summer and autumn (July–December),

on the ground on bare, loamy soil, among mosses and grasses, at the foot of trees on rotting wood. It varies in colour and shape depending on age and growing conditions (Grelet 1947, Kuo, .2012). Conventionally regarded as a saprotroph but evidence of Kühdorf et al. (2015) have suggested that Leotia lubrica forms ectomycorrhizae with *Comarostaphylis arbutoides* (Ericaceae).

Leotia baculum Lauwers & Haelew., nom. prov. 1

Etymology

The literal translation of *baculum* is walking stick or cane. This refers to the peculiar shape of the paraphyses. These form slender sticks with the upper part curled inwards and upper cells slightly enlarged and constricted.

Holotype

BÉNIN. Département Donga, Bassila 9.0012222 N 1.6553056 E, 376 m a.s.l., ascomata in dense groups on bare soil of a termite mound in Gallery forest, northernmost part of Forêt Classée de Bassila (Unité d'aménagement de Bassila), dominated by *Berlinia grandiflora* (Caesalbiniaceae), *Uapaca somon* (Euphorbiaceae), *Lonchocarpus sericeus*, *Pterocarpus santalinoides* (Fabaceae) alongside the waterline *Elais guineensis* (Oil palm), 17 June 2004, coll. A. De Kesel, ADK3669, D. Haelew. F-1783 (BR-MYCO 157073-30). Ex-holotype sequences: ITS.

Additional specimens examined

BÉNIN. Département Atacora, 12 km SE of Natitingou, Kota Falls, 10.2113333 N 1.4453833 E, 523 m a.s.l., on soil in gallery forest with *Isoberlinia doka* and *Uapaca guineensis*, 29 August 1997, coll. A. De Kesel, ADK2010, D. Haelew. F-1775 (BR-MYCO 74856-69); Boukoumbé, Kossoucoingou, 24 km WSW of Natitingou, 10.1797333 N 1.1996667 E, 556 m a.s.l., on soil in forêt claire with *Isoberlinia* sp., 5 September 1997, coll. A. De Kesel, ADK2051, D. Haelew. F-1776 (BR-MYCO 74884-00); Département Borgou, 55 km SW of Parakou, Forêt Classée de Wari-Maro, 9.1551667 N 2.143 E, 342 m a.s.l., on soil in forêt claire dominated by *Isoberlinia doka*, attached to the base of grasses, 29 September 1999, coll. A. De Kesel, ADK2715, D. Haelew. F-1782 (BR-MYCO 116724-33).
ZIMBABWE. Département Manicaland, Chimanimani District, 13 km S of Chimanimani, Mguzu, 19.9207833 S 32.8614 E, 1200 m a.s.l., on soil in Miombo forest on SW slope, forest dominated by *Uapaca kirkiana* (Phyllanthaceae), 6 January 1999, coll. A. De Kesel, ADK2419, D. Haelew. F-1780 (BR-MYCO 112589-69).

Description

Ascocarps scattered individually or gregarious in small groups, total length 20 – 41 mm. ***Apothecia*** stipitate-capitate, diameter 3 – 12 mm, usually one cap per stipe, sometimes fasciculate with two to three apothecia originating from a common and branching stipe, bifurcate branching with two to three splitting caps, sometimes separated at root by a wide but shallow groove along the entire length; convex, brain-like, lobed with a overhanging, enrolled, undulating margin, somewhat irregular, dark brown to black-brown to ochraceous, when fresh jelly-like, smooth, matte, velvet-like surface, when wet glossy, somewhat viscid. ***Stipe*** distinct and long, diameter 2 – 8 mm, cylindrical or tapering, sometimes with undeep furrow along the entire length, bright yellowish-ochraceous to dark, furfuraceous, slightly granulate to distinctly floccose. ***Asci*** eight-spored, elongate, cylindrical to clavate, gradually tapering towards the base, apex rounded, blunt, inamyloid (in IKI or MLZ , without or with KOH-pretreatment), no clear ring or pore structure visible; ascus base non-furcate, arising from repeating croziers with two septa, some arising from simple-septa; ascus wall thin, gelatinous, inamyloid, sometimes small LBs present (especially at base); measurements (119,18–) 122,5 – 136,6 (–147,3) x (8,4–) 9,4 – 13,0 (–14,0) µm. ***Ascospores*** (sub-)cylindrical, ellipsoid, rhomboid-fusiform, fusoid, without ornamentation; curvature inequilateral to slightly curved, sometimes slightly helicoid; often multi-guttulate with two to five large LBs confluent to one ellipsoid body or separate (appearing almost septate but with septa hard to define), several smaller LBs (lipid content very high), hyaline, unpigmented; spore-apex obtuse to subacute; spore base slightly to medium tapered/attenuated; measurements (15,9–) 16,8 – 23,4 (–27,2) x (4,8–) 5,5 – 7,4 (–8,3) µm;

Paraphyses slender, exceeding the asci , upper part very strongly curved inwards; apex enlarged, swollen, tapering, medium capitate, strongly clavate, sometimes moniliform with irregular clavate thickenings and more or less constricted at the septa, strongly reddish-brown pigmented in Congo Red SDS, measurements (4,2–) 4,7 – 6,6 (–8,2) µm; dichotomous branching (bifurcation) only at base, multiseptated, sometimes anastomoses; hyaline, inamyloid, cell contents sometimes visible (LBs). ***Ectal Excipulum*** loosely interwoven hyphae resembling a *textura intricata,* thin, multiseptated, bifurcate, sometimes abundant presence of cell contents (LBs), hyaline, gelatinous, usually with distinct interhyphal spaces. ***Medullary excipulum*** thin layer just under hymenium with densely packed, globose cells resembling a *textura globulosa* of different size, pigmented, non-gelatinous.

Habitat and distribution
Growing in small groups, mostly on soil in tropical woodland (Gallery forests, Miombo forests, open forests); during winter and summer; attached to the base of grasses or on bare soil; soil ferruginous, weakly hydromorphic, finely alluvial, clay-rich; sometimes close to or on large termite mounds, often nearby watercourse.

Notes

The ex-holotype ITS sequence of *Leotia baculum* (DHF-1783a) shares 88,91% identity with that of *Leotia virgula* (DHF-1778), 87,62% with *Leotia sp. nov*. 4 (DHF-1786a), 86,30% with *Leotia rufa* (DHF-1777a), and 80,97% with the previously described *Leotia atrovirens (*strain 1Chi, GenBank accession AY144567). Next, a brief discussion of key morphological features/differences of *Leotia baculum* and its two phylogenetic relatives that are descripted in this paper is given.
The examined species can be distinguished in their colour of the ascomata. *Leotia baculum* exhibits a rather yellowish-brown colour, sometimes with an olivaceous hue, and turning dark brown to black with age. Young specimens typically have a pale yellow stem. *Leotia rufa* on the other hand, show rather yellowish- to brown-ochre colours with a pale yellow-ochraceous stipe. They somewhat differ morphologically in ascus size, respectively (119,18–) 122,5 – 136,6 (–147,3) x (8,4–) 9,4 – 13,0 (–14,0) µm for *Leotia baculum,* (95,1–) 107,9 – 126,3 (–135,7) x (7,4–) 8,1 – 10,0 (–10,9) µm for *Leotia sp. nov. 2*, and (102,6–) 116,4 – 135,9 (–139,9) x (8,0–) 10,1 – 14,3 (–15,0) µm for *Leotia rufa.* The asci are typically elongated, cylindrical-clavate and gradually tapering towards the base. All species show inamyloid asci with no clear ring or pore structure visible in various medium (IKI, CR SDS, MLZ). Ascospores are typically (sub-)cylindrical, ellipsoid and fusiform with often containing LBs. They somewhat differ in length, respectively (15,9–) 16,8 – 23,4 (–27,2) x (4,8–) 5,5 – 7,4 (–8,3) µm vs (11,1–) 13,8 – 18,6 (–21,8) x (1,6–) 4,1 – 6,1 (–6,2) µm vs (14,6–) 16,1 –18,9 (–20,0) x (5,9–) 6,3 – 7,6 (–8,3) µm. No septate ascospores were observed in none of the collections. All species show a variety of shapes and sizes of paraphyses depending on maturity. They are generally characterised by a slender, filiform appearance with a swollen stipitate-clavate apex composed of constricted cells. *Leotia baculum* shows distinctly stronger arched and more pigmented apices of the paraphyses, measuring (4,2–) 4,7 – 6,6 (–8,2) µm. compared to the two other new species: (2,5–) 2,9 – 4,8 (–5,9) µm for *Leotia sp. nov. 2* and (2,3–) 3,9 – 6,2 (–6,6) µm for *Leotia rufa.*

Figure 2: ascomata of DHF1782 and DHF1783 (5 little squares on graph paper stands for 5 mm)

Leotia virgula Lauwers & Haelew., nom. prov. 2

Remarks

After confirmation of the collector A. De Kesel, no photographs were provided for this species. As a consequence, no macromorphological description of the ascomata was carried out.

Etymology

The literal translation of *virgula* is stick, wand or comma. This refers to the peculiar shape of the paraphyses. These form slender sticks with the upper part curled inwards and upper cells slightly enlarged and constricted.

Holotype

ZIMBABWE. Manicaland Province, Chimanimani District, 13 km S of Chimanimani, Mguzu, 19.9207833 S 32.8614 E, 1200 m a.s.l., on soil in Miombo forest on SW slope, forest dominated by *Uapaca kirkiana* (Phyllanthaceae), 6 February 1999, coll. A. De Kesel, ADK2417, D. Haelew. F-1778 (BR-MYCO 112587-67). Ex-holotype sequences: ITS.

Additional specimens examined

ZIMBABWE. Manicaland Province, Chimanimani District, 13 km S of Chimanimani, Mguzu, 19.9207833 S 32.8614 E, 1200 m a.s.l., on soil in Miombo forest on SW slope, forest dominated by *Uapaca kirkiana* (Phyllanthaceae), 6 February 1999, coll. A. De Kesel, ADK2418, D. Haelew. F-1779 (BR-MYCO 112588-68).
DRCONGO. Haut-Katanga Province, Kipushi, 29 km NE of Lubumbashi, 11.4845 S 27.6731667 E, 1232 m a.s.l., on soil in Forêt claire dominated by *Marquesia macroura*, some young samples of *Julbernardia globiflora* are present, 21 January 2013, coll. A. De Kesel, ADK5452, D. Haelew. F-1785 (BR5020212171270V).

Description

Asci eight-spored, elongate, cylindrical, strongly clavate, gradually tapering towards the base; apex rounded, blunt, inamyloid (in IKI or MLZ , without or with KOH-pretreatment), no clear ring or pore structure visible, sometimes apical wall thickening visible; ascus base non-furcate, arising from repeating croziers with two septa, croziers non-perforated; ascus wall thin, gelatinous, inamyloid; measurements (95,1–) 107,9 – 126,3 (–135,7) x (7,4–) 8,1 – 10,0 (–10,9) µm. ***Ascospores*** (sub-)cylindrical, ellipsoid, rhomboid-fusiform, curvature inequilateral to slightly curved, without ornamentation, often multi-guttulate with two to five large LBs confluent to one ellipsoid body or separate (appearing almost septate but with septa hard to define), several smaller LBs (lipid content very high), hyaline, unpigmented; spore-apex obtuse to subacute; spore base slightly tapered/attenuated; measurements (11,1–) 13,8 – 18,6 (–21,8) x (1,6–) 4,1 – 6,1 (–6,2) µm. ***Paraphyses*** slender, slightly exceeding asci, medium to strongly curved at apex; apex slightly swollen, tapering, slightly capitate, medium to strongly clavate, sometimes moniliform with irregular clavate thickenings and more or less constricted at the septa, reddish-brown pigmented in Congo Red SDS, measurements (2,5–) 2,9 – 4,8 (–5,9) µm; dichotomous branching (bifurcation) only at base, multiseptated, sometimes anastomoses; hyaline, inamyloid, cell contents sometimes visible (LBs). ***Ectal Excipulum*** loosely interwoven hyphae resembling a textura intricata, thin, multiseptated, bifurcate, hyaline, gelatinous, usually with distinct interhyphal spaces. ***Medullary excipulum*** thin layer just under hymenium with densely packed, globose cells of different size, pigmented, non-gelatinous.

Habitat and distribution

Growing on soil in (sub-)tropical woodland (Miombo forests, open forests). Collected and recorded in Zimbabwe in winter.

Leotia rufa Lauwers & Haelew., nom. prov. 3

Etymology

The literal translation of *rufa* is ochre or ginger. This refers to the ochraceous or rusty coloured apothecia. This colour was not yet observed in other *Leotia* members.

Holotype

ZIMBABWE. Manicaland Province, Chimanimani District, Eastern Highlands mountain range, 8 km NE of Stapleford, 18.70425 S 32.8755 E, 1600 m a.s.l., on soil in Miombo forest on a steep slope, forest dominated by *Brachystegia spiciformis,* 2 February 1999, coll. A. De Kesel ADK2376, D. Haelew. F-1777 *(*BR-MYCO 112546-26). Ex-holotype sequences: ITS.

Description

Ascocarps scattered individually or gregarious in small groups, total length 31 – 50 mm. ***apothecia*** stipitate-capitate, diameter 7 – 20 mm, mostly multiple caps (apothecia) per stipe, fasciculate with two to three apothecia originating from a common and branching stipe, tree-like or rosette-shaped branching with two to three splitting caps, sometimes bifurcation at root but the two merging stipes stay connected by a groove along the entire length, convex, brain-like, rather nodular or knobbly, with a lobed overhanging, enrolled margin, somewhat irregular division into spherical sections, bright ochraceous to brownish-beige, when fresh jelly-like, smooth, matte, velvet-like surface, when wet glossy, somewhat viscid. ***stipe*** distinct and long, cylindrical or tapering, diameter 3 – 7 mm, subequal or slightly swollen at the base, sometimes slightly compressed, white-yellowish to ochraceous, viscid, smooth, furfuraceous, densely granulated with large darker coloured granules; flesh gelatinous, bright yellowish to ochraceous, with a slight cavity. ***Asci*** eight-spored, elongate, cylindrical to clavate; apex rounded, blunt, gradually tapering to the base, inamyloid (no occurrence of blue iodine-reaction after KOH and Lugol treatment), no clear ring or pore structure visible; ascus base non-furcate, arising from repeating croziers with two septa, croziers non-perforated, sometimes simple-septa; Ascus wall thin, gelatinous, inamyloid; measurements (102,6–) 116,4 – 135,9 (–139,9) x (8,0–) 10,1 – 14,3 (–15,0) µm. ***Ascospores*** subcylindrical, ellipsoid, fusiform, straight or slightly curved to inequilateral, without ornamentation, 13-17 x 5-6 µm; spore apex subacute; spore base slightly tapered/attenuated, sometimes multi-guttulate with two to five small oil drops, some are fused, hyaline; measurements (14,6–) 16,1 –18,9 (–20,0) x (5,9–) 6,3 – 7,6 (–8,3) µm. ***Paraphyses*** slender, straight, sometimes slightly curved at apex, thin, cylindrical, filiform; apex, slightly to medium capitate (knob-like), slightly to medium clavate, sometimes moniliform, measurements (2,3–) 3,9 – 6,2 (–6,6) µm; generally dichotomous branching, multiseptated, hyaline, inamyloid. ***Ectal excipulum*** loosely interwoven hyphae resembling a *textura intricata,* thin, cylindrical hyphae, gelatinous, multiseptated. ***Medullary excipulum*** densely packed, globose cells resembling a *textura globulosa* between hymenium and ectal excipulum, non-gelatinous

Habitat and distribution
Growing on soil in (sub-)tropical woodland (Miombo forest). Collected and recorded in Zimbabwe in winter.

Figure 3: Ascomata of DHF1777 (5 little squares on graph paper stands for 5 mm)

Discussion and conclusion

In this study, an integrated morphological and phylogenetic approach is done for new collections of *Leotia*-species collected in Zimbabwe, Bénin and D.R. Congo. The important elements such as the size and shape of spores, asci, paraphyses and excipulum were recorded, studied and compared to the previously described *Leotia lubrica*. Based on our observations of different collections we can take some provisional conclusions about species delimitation within this genus. Even though minor variations occurred in the size and shape of different microscopic structures. The variation was continuous and no disjunct patterns were found.

Leotia members show high variability in ascomatal colour, size and form (Zhong & Pfister, 2004). The ascocarp is generally capitate-stipitate and gelatinous, with the hymenium covering the upper surface of the convex apothecia (Mains, 1956). *Leotia baculum* displays rather dark-brown to brownish-olivaceous colours, while *Leotia rufa* shows yellowish-beige and ochraceous tints. Generally the structure of the ascocarp is distinctively constructed of interwoven hyphae in a gelatinous matrix. The excipulum is positioned underneath the hymenium and typically consists of non-gelatinous medullary excipulum and gelatinous ectal excipulum. The ectal excipulum resembles a *textura intricata,* consisting of thin, interwoven hyphae with distinct interhyphael spaces. The medullary excipulum is rather thin and resembles a cortical layer of non-gelatinous *textura globulosa* underneath the hymenium.

With regard to the microscopic features, most variability among the new species was remarked in the form and size of the paraphyses. *Leotia lubrica* showed little abundance of paraphyses in the hymenium and the paraphyses almost never exceeded the asci. They were filiform and very thin with a subtle and not predominant capitate apex ((0,8–) 1,1 – 1,8 (–1,9) µm). The paraphyses of the newly described species on the other hand all showed distinctly inflated, and pigmented capitate-clavate apices with constrictions at the septa. What really stood out here was that these apices all curled up in an arch, almost like a sprouting fern. The apex of the paraphyses of *Leotia baculum* measured (4,2–) 4,7 – 6,6 (–8,2) µm and exceeded a few µm above the asci. However, variability in size and form among the paraphyses occurred between different collections in the same species. According to Baral et al. (2020) apically narrower and shorter paraphyses are young paraphyses that have not yet developed their typical shape. These were, as a consequence, disregarded in the descriptions.

The asci of all species bear 8 ascospores and are clavate and slightly or strongly narrowing towards the base depending on maturity. The apex is typically rounded and blunt. Conforming to Jaklitsch et al. (2016), *Leotia* is the only genus within the family Leotiaceae that includes inamyloid asci as opposed to its sister taxa. This runs parallel with the observations of Verkley (1994) in his study of the ultrastructure of the ascus apical apparatus in *Leotia lubrica*. He states that no blueing is observed with iodine solutions in the apex of *Leotia lubrica*. In this study both *Leotia lubrica* and all other collections of the new species showed no blue reaction after IKI or MLZ without or with KOH-pretreatment. Moreover, no clear ring or pore structure was visible. From this, we concluded that the genus is inamyloid. However, when encountered with an inamyloid reaction of the apical asci, supplementary issues needed to be addressed. This is because variability actually occurs in some species in the

case of iodine reactions (Kohn & Korf, 1975; Nannfeldt, 1976; Baral, 2009). In order to truly confirm the inamyloid outcome, a negative amyloid and hemiamyloid reaction is required. These problems were forwarded by Candice Perrotta in her research of *Hymenoscyphus*. Firstly, an inamyloid type of reaction could indicate the reagent is old or degraded. switching to a new bottle was required. Secondly, fungal material rehydrated with diH2O prior to KOH pre-treatment and IKI application could only provoke a faint blue reaction. Rehydrating directly with KOH could yield a more intense blue reaction of the apical ring. Above-mentioned issues had been practically raised and a non-amyloid reaction came out clearly. MLZ was used afterwards for a final additional confirmation that the asci are inamyloid.
Further, asci arising from repeating croziers were observed in all collections. Some collections on the other hand showed a simple-septate ascus-base, or a combination of both simple-septate and croziers. Overall, this was hard to observe and difficult to clearly distinguish one from the other.

The ascospores investigated in all collections showed high variability. The shape in general amount to (sub-)cylindrical, ellipsoid-fusiform and usually asymmetric. Deviations in both size and shape were noticeable within and between collections. Baral et al. (2020) suggests that they might in some cases be the result of external conditions rather than having a genetical basis. The ascospores in *Leotia lubrica* measured (15,1–) 16,7 – 20,6 (–23,1) x (4,0–) 4,2 – 5,6 (–6,7) µm. The measurements of the new species did not deviate particularly far from this but *Leotia sp*. 1 measured approximately the largest spores, (15,9–) 16,8 – 23,4 (–27,2) x (4,8–) 5,5 – 7,4 (–8,3) µm respectively.
Also noteworthy is the multi-guttulate appearance due to the presence of multiple LBs inside the spores of all observed collections. This was most notably in *Leotia lubrica* and *Leotia rufa* where mainly two to five LBs occurred almost confluent to one ellipsoid body or separate. As a result the prevailing question arose whether the spores are multi-septate. Many authors state the multi-septate character of the spores of *Leotia lubrica (*especially becoming multi-septate with age) in their descriptions (Grelet, 1947; Dennis, 1978; Breitenbach, 1981; Otani, 1982; Hanlin, 1990; Hansen & Knudsen, 2000). However, septate spores were not seen in neither *Leotia lubrica* nor in the collections of the new *Leotia* species in this study.

The phylogenetic tree (Fig. 3) as provided by Dr. Haelewaters reveals the monophyletic character of the clade that comprises the presumably new African species. The subclades are supported by bootstrap values ranging from 71% to 100 %. After performing ITS-sequences in BLAST the following results came about: *Leotia baculum* overlaps 88,91% with *Leotia virgula*, 87,62% with *Leotia sp. nov. 4* and 86,30% with *Leotia rufa*. The previously described *Leotia atrovirens* (strain 1Chi, GenBank accession AY144567) overlaps 80,97% with *Leotia baculum.* These results provided evidence for the emergence of four or five new species within the genus *Leotia.* The emphasis here lies on the number of species, because the question arose whether the collection DHF1785a from D.R. Congo should or should not be included in the clade with DHF1778a and DHF1779a (*Leotia sp. nov. 2)*. It had emerged that *Leotia virgula* is substantially closely related to the collection from DR Congo, DHF1785a. In BLAST, both ITS sequences of DHF1778 and DHF1779 are 98,10% identical to the ITS sequence of DH1785. What this means is currently not very certain. Based on the phylogenetic tree and the differences within Leotia atrovirens and Leotia lubrica, this subclade can be considered as one species. Although this is still uncertain. Before we actually included DHF1785a with the other two collections, a microscopic comparison between them was carried out. This brought forth only minimal morphological differences. For example, less pigmentation in the upper part and less swollen and constricted cells at the apex of the paraphyses were detected in the strain DHF1785a. In addition, the asci were slightly more tapering towards the base and thinner in DHF1787a. The difficulty was whether these minimal differences were reliable enough to ultimately speak of one species. Potentially those differences could be assigned to phenotypic plasticity as a consequence of different habitat and climate of the three collections (Slepecky & Starmer, 2009). In that case we can, indeed consider the species as one. Alternately, it could be that the rate of evolution in the African clade is different from the other clade *(leotia lubrica, Leotia atrovirens, Leotia viscosa) (Maharachchikumbura et al., 2021).* In such case, it could still be that the collections from Zimbabwe and the collection from D.R. Congo can be considered two species.

It might even be that the subclade that we behold here as a clade within the genus *Leotia,* represents a different genus altogether. Although, to make this taxonomic decision, more genetic data and more sampling in poorly

studied geographical regions is needed. We definitely suspect more specimens and higher diversity of the genus being present in Africa. But the taxonomy has been based primarily on the diversity in the temperate Northern Hemisphere, especially in Western Europe and the United States (Quandt & Haelewaters, 2021). In this way, I advocate for further molecular investigation on *Leotia* by sampling more taxa (especially in underrepresented tropical locations) and to uncover the still unsettled taxonomic diversity of this species.

Summary

In this bachelor thesis three presumably new species, named *Leotia baculum, Leotia virgula,* and *Leotia rufa,* belonging to the genus *Leotia* is described, photographed and drawn. The genus is found within the Leociomycetes, a class that is poorly understood and from which the classification is not yet fully resolved. . The described species were collected in Bénin, Mozambique and D.R. Congo. The fruiting bodies of all collections were found on bare soil in in (sub-)tropical Miombo forests, Gallery forests and open forests. *Leotia* represents stipitate-capitate ascomata that includes a convex, brain-like apothecia on a distinctive stipe. Further distinguishment among other taxa is the excipulum, that consist of hyphae in a gelatinous matrix. In comparison to the already described *Leotia lubrica,* only minor variations occurred in the size and shape of different microscopic structures. Most variability between all examined species was found in the form and size of the multi-septate paraphyses. The paraphyses features arched apices that are typically capitate-clavate and constricted at the septa. In general, all species were found to have inamyloid, cylindrical to clavate asci that contain 8 ascospores and arise from croziers and/or simple-septa. The ascospores take on various shapes depending on maturity but mostly are fusiform-cylindrical-ellipsoid, with a slight curvature, no ornamentation and often multi-guttulate. Dr. Haelewaters' phylogenetic tree based on ITS sequences shows that the new African species belong to a monophyletic clade. This clade is divided into four subclades, supported by relatively strong bootstrap values ranging from 71% to 100%. After comparing ITS-sequences using BLAST, it was discovered that *Leotia baculum* has genetic similarities of 88.91%, and 86.30% with *Leotia virgula* and *Leota rufa* respectively. Overall, further molecular investigation and sampling in understudied regions needs to be executed in order to understand and map the genus.

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Tables and figures

Measurements of asci, ascospores and paraphyses

Table 1: measurements of 20 asci (length and width), 20 ascospores (length and width) and 20 apices of paraphyses (width) of Leotia lubrica

Ascus length (µm)	Ascus apex width (µm)	Spore length (µm)	Spore width (µm)	Paraphyse apex width (µm)
131,4	10,8	20,3	6,7	1,4
109,1	9,3	18,5	6,1	1,5
120,0	8,9	19,5	5,3	1,5
105,3	9,0	21,7	4,6	1,3
105,4	9,7	23,1	5,8	1,4
122,6	10,9	20,3	4,7	1,3
117,2	6,8	19,2	5,2	1,9
120,9	11,8	18,1	4,0	1,3
116,1	11,3	18,0	4,0	1,7
107,6	9,1	17,9	4,2	1,9
115,9	11,7	18,8	5,0	1,7
113,6	7,3	18,9	4,9	1,2
118,1	10,7	16,7	4,4	1,7
109,4	7,3	20,6	5,8	1,0
125,4	11,8	16,3	4,7	0,8
119,7	9,5	17,2	4,3	1,2
101,6	11,0	16,6	4,6	1,6
125,8	10,4	15,1	4,5	1,8
113,6	10,6	18,9	4,9	1,9
114,4	9,6	17,4	4,3	1,3

Table 2: measurements of 10 asci (length and width), 10 ascospores (length and width) and 10 apices of paraphyses (width) of both isolate DHF1775 (isotype) and isolate DHF1783 (holotype) of Leotia baculum (measurements of different isolates are separated by a bold crossline margin)

Ascus length (µm)	Ascus apex width (µm)	Spore length (µm)	Spore width (µm)	Paraphyse apex width (µm)
119,2	12,8	16,3	7,9	6,5
128,5	14,0	17,7	7,4	5,6
130,7	11,3	18,0	6,9	5,4
121,2	11,3	19,0	6,7	5,7
132,3	9,6	20,4	6,3	8,2
132,6	11,7	19,6	7,3	6,4
121,9	13,6	18,3	6,2	5,5
120,9	12,6	19,0	8,1	4,8

130,0	14,0	20,4	7,1	5,5
125,6	12,8	18,0	8,3	7,3
137,0	10,8	19,1	6,1	5,5
131,7	9,1	26,2	5,8	6,0
138,4	11,4	15,9	4,8	5,4
147,3	9,1	26,0	6,0	4,6
124,7	11,4	27,2	5,9	4,2
123,2	8,6	21,8	6,1	5,0
131,5	8,8	17,3	5,4	4,9
136,6	10,5	20,1	6,5	6,0
125,8	12,0	24,3	4,9	4,9
131,8	8,4	18,0	5,8	5,2

Table 3: measurements of 10 asci (length and width), 10 ascospores (length and width) and 10 apices of paraphyses (width) of both isolate DHF1778 (holotype) and isolate DHF1779 (isotype) of Leotia virgula (measurements of different isolates are separated by a bold crossline margin)

Ascus length (µm)	**Ascus apex width (µm)**	**Spore length (µm)**	**Spore width (µm)**	**Paraphyse apex width (µm)**
119,1	8,6	15,9	5,9	5,7
119,5	7,4	17,6	5,6	5,6
119,3	7,7	15,7	5,3	4,1
97,7	9,7	21,8	6,1	5,9
119,4	9,0	11,7	5,7	4,4
124,0	8,0	17,2	5,2	3,5
122,8	9,5	17,1	6,1	2,5
117,2	10,6	14,5	4,1	4,1
135,7	10,9	16,7	5,9	3,6
107,5	8,8	15,5	6,2	3,4
120,1	9,0	14,8	1,6	3,8
121,0	8,4	16,8	4,8	3,7
113,0	9,9	15,5	4,9	3,6
95,1	8,6	15,0	4,7	3,2
112,7	8,7	16,7	4,9	3,2
123,7	8,6	20,0	4,8	3,2
114,2	9,6	11,1	4,9	3,5
122,9	8,0	16,3	4,7	3,3
123,5	10,1	16,2	6,0	4,0
113,7	9,9	18,5	4,9	2,9

Table 4: measurements of 10 asci (length and width), 10 ascospores (length and width) and 10 apices of paraphyses (width) of isolate DHF1783 from D.R. Congo of Leotia virgula

Ascus length (µm)	Ascus apex width (µm)	Spore length (µm)	Spore width (µm)	Paraphyse apex width (µm)
123,55	9,29	18,49	7,35	4,19
126,92	10,3	19,06	6,43	4,29
144,43	10,64	17,42	6,94	4,02
120,55	11,2	17,42	6,94	3,82
137,94	10,71	15,22	6,63	4,57
123,41	11,9	17,58	6,29	4,83
141,35	10,85	14,24	5,1	4,82
118,3	10,52	18,86	7,55	4,39
116,82	9,13	19,16	7,74	5,76
127,84	8,38	16,86	5,58	4,32

Table 5: measurements of 20 asci (length and width), 20 ascospores (length and width) and 20 apices of paraphyses (width) of isolate DHF1777 of Leotia rufa

Ascus length (µm)	Ascus apex width (µm)	Spore length (µm)	Spore width (µm)	Paraphyse apex width (µm)
130,9	15,0	18,7	7,0	5,1
135,0	13,1	15,5	7,3	4,6
124,9	11,7	17,4	7,4	5,5
139,9	14,7	17,4	5,9	5,9
135,6	11,6	19,3	6,1	6,6
102,6	8,9	15,8	6,6	6,6
120,4	11,9	14,6	6,8	4,1
118,6	9,2	16,0	8,3	6,3
135,0	15,0	17,0	7,1	5,9
133,3	15,0	17,5	7,7	4,6
126,4	11,1	20,0	6,9	5,3
115,3	11,5	18,1	6,8	4,8
122,8	12,7	17,9	6,6	5,7
127,7	12,4	17,2	6,4	6,3
134,4	13,7	16,4	6,9	2,3
113,5	8,0	18,3	6,8	3,3
114,3	12,1	19,5	7,4	4,3
134,6	11,5	16,8	8,1	4,3
123,0	10,3	18,2	6,4	5,9
134,2	15,0	18,1	6,9	3,9

Photographs of *Leotia lubrica*

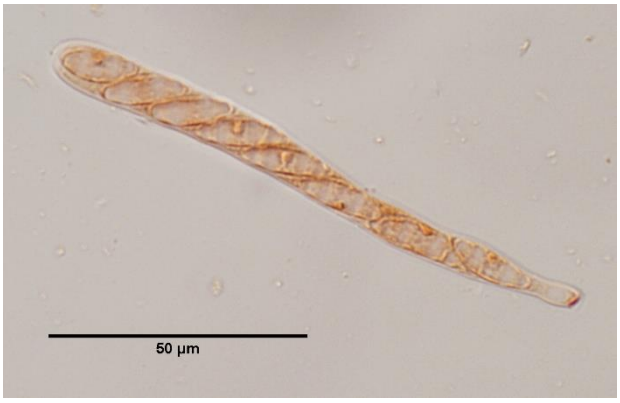

Figure 1: Ascus of Leotia lubrica, stained with CR SDS (scale bar = 50 µm)

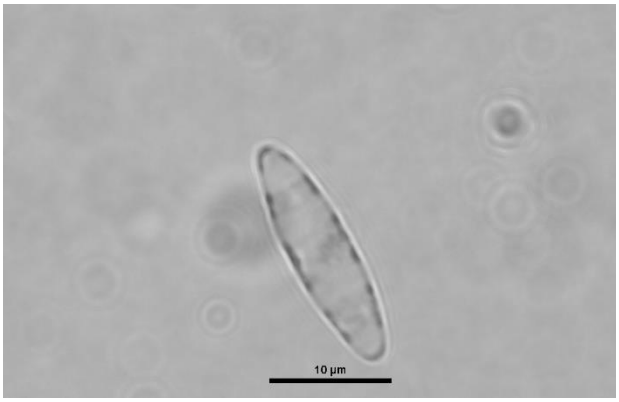

Figure 1: Ascospore of Leotia lubrica, stained with IKI (scale bar = 10 µm)

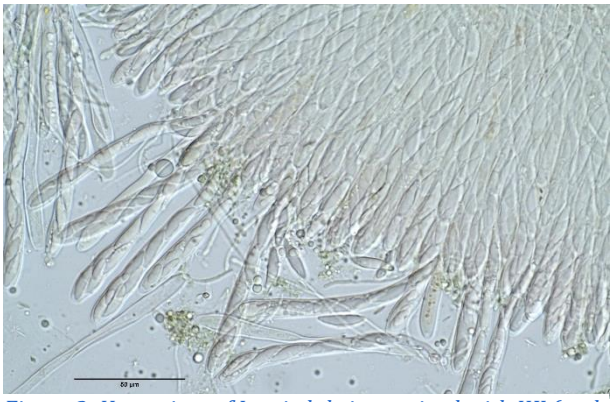

Figure 3: Hymenium of Leotia lubrica, stained with IKI (scale bar = 50 µm)

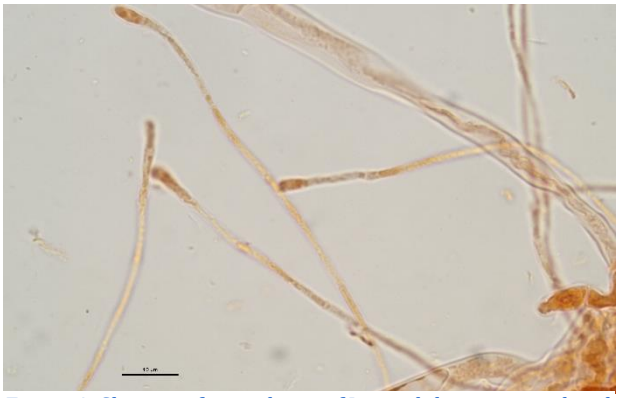

Figure 4: Close up of paraphyses of Leotia lubrica, stained with CR SDS (scale bar = 10 µm)

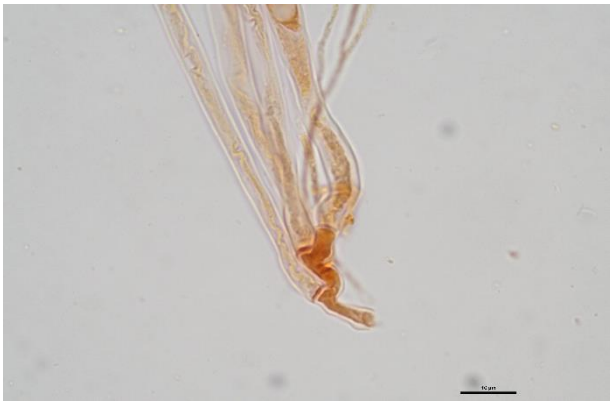

Figure 5: Ascus base arising from croziers of Leotia lubrica, stained with CR SDS (scale bar = 10 µm)

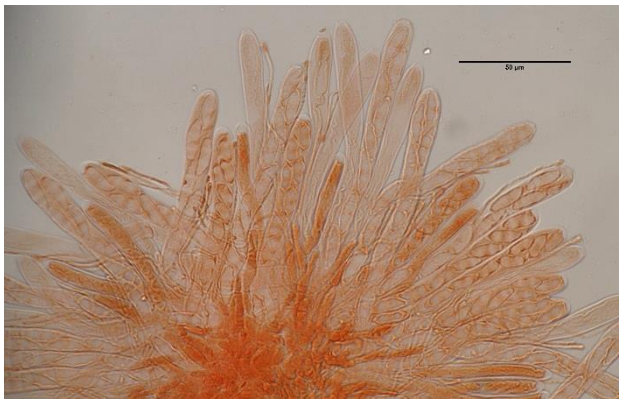

Figure 6: Hymenium of Leotia lubrica, stained with CR SDS (scale bar = 50 µm)

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Photographs of *Leotia baculum*

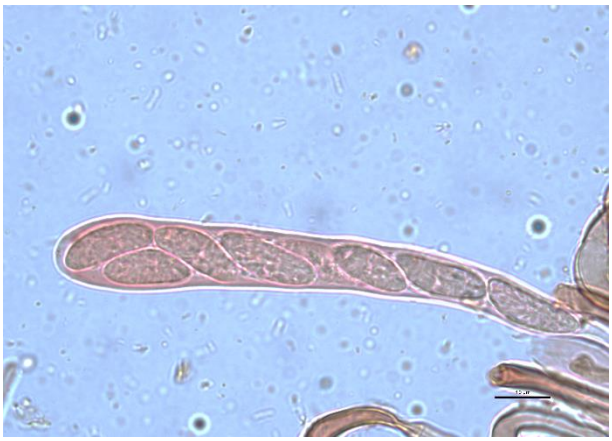

Figure 1: Asus of DHF1783 (holotype), stained with CR SDS (scale bar = 10 µm)

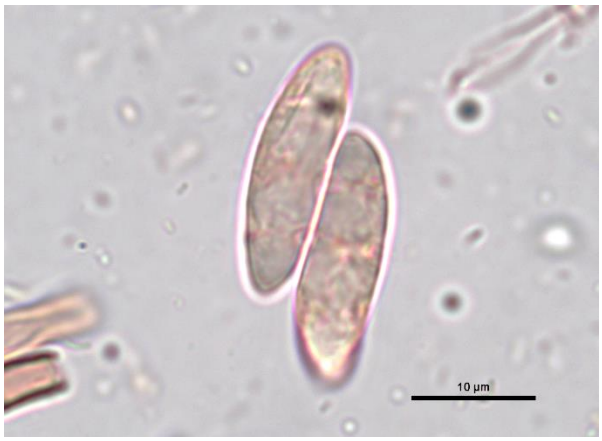

Figure 2: Ascospores of DHF1783 (holotype), stained with CR SDS (scale bar = 10 µm)

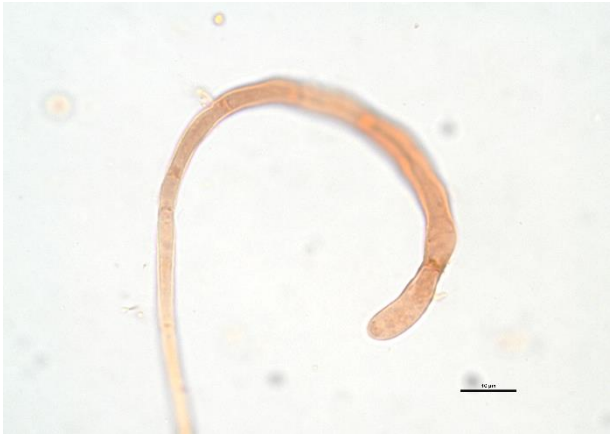

Figure 3: Paraphyse of DHF1776 (isotype), stained with CR SDS (scale bar = 10 µm)

Figure 4: close up of hymenium of DHF1783 (holotype), stained with CR SDS (scale bar = 10 µm)

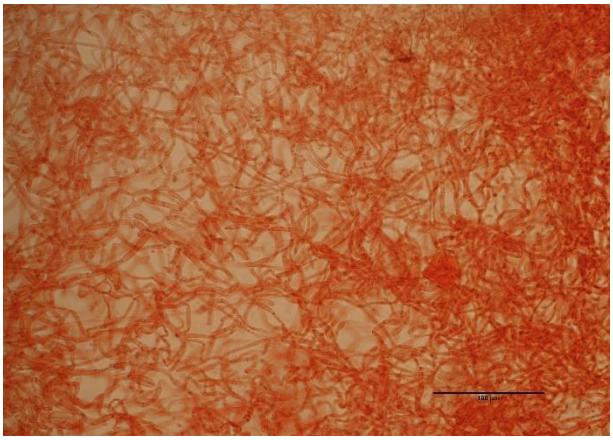

Figure 5: Ectal excipulum DHF1780(isotype), stained with CR SDS (scale bar = 100 µm)

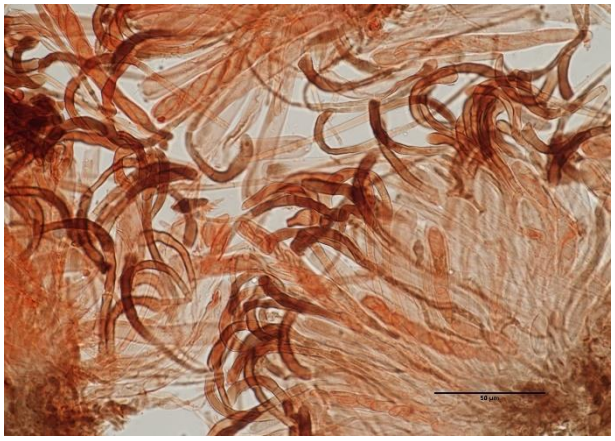

Figure 6: Paraphyses DHF1780 (isotype), stained with CR SDS (scale bar = 50 µm)

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Photographs of *Leotia virgula*

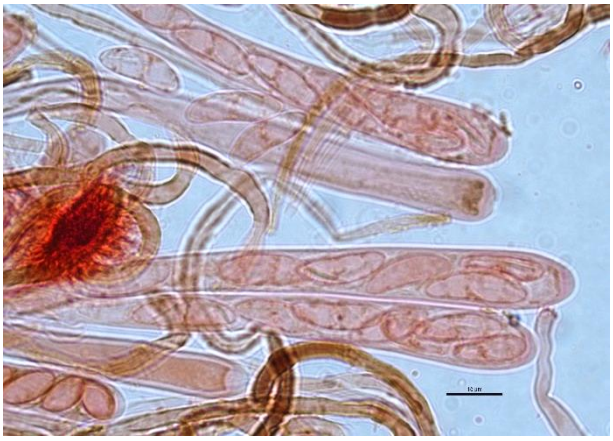

Figure 1: Asci of DHF1779 (isotype), stained with CR SDS (scale bar = 10 µm)

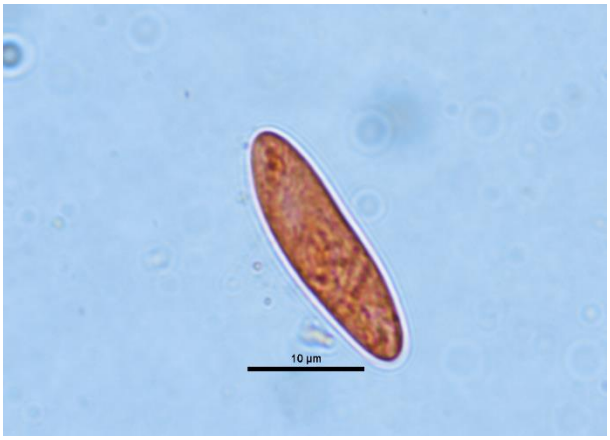

Figure 2: Ascospore of DHF1778 (holotype), stained with CR SDS (scale bar = 10 µm)

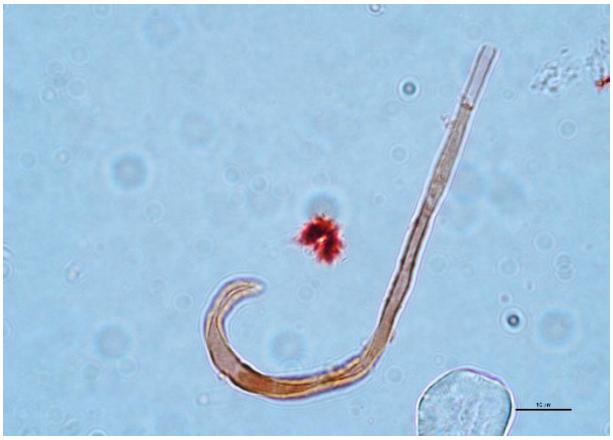

Figure 3: Paraphyse of DHF1778 (holotype), stained with CR SDS (scale bar = 10 µm)

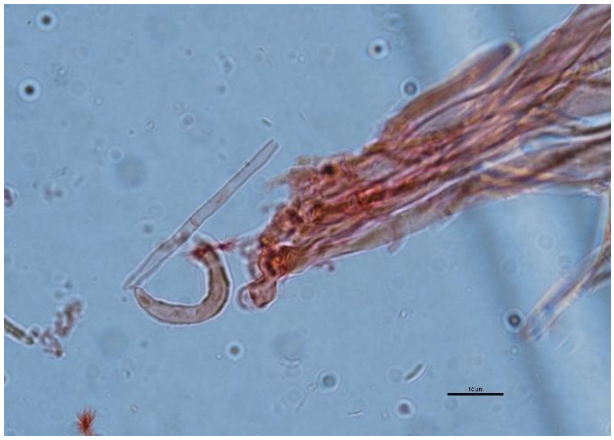

Figure 4: Asci arising from repeating croziers of DHF1778 (holotype), stained with CR SDS (scale bar = 10 µm)

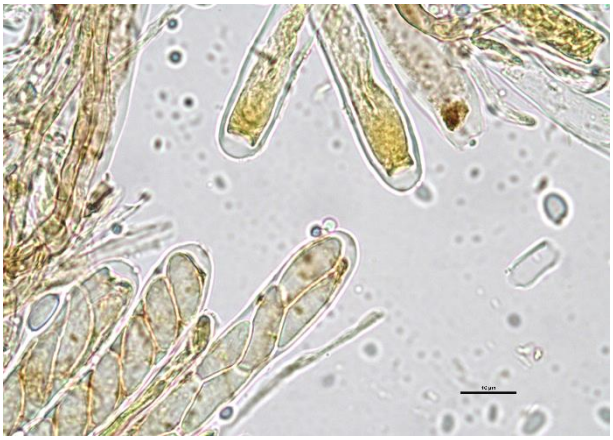

Figure 5: Asci of DHF1779 (isotype), stained with IKI (scale bar = 10 µm)

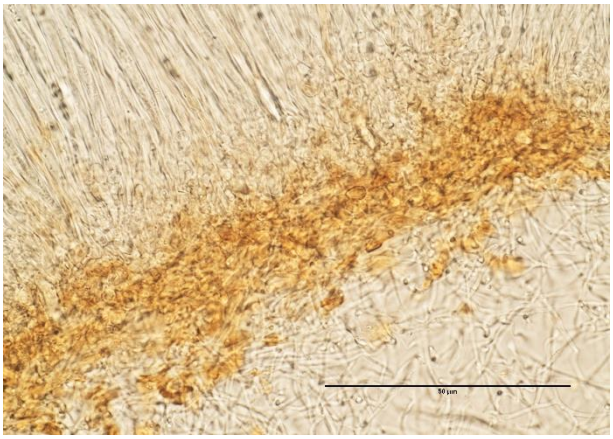

Figure 6: close up of hymenium and excipulum of DHF1779 (isotype), stained with IKI (scale bar not representative to true size due to defect or mistake while taking photographs)

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Photographs of *Leotia rufa*

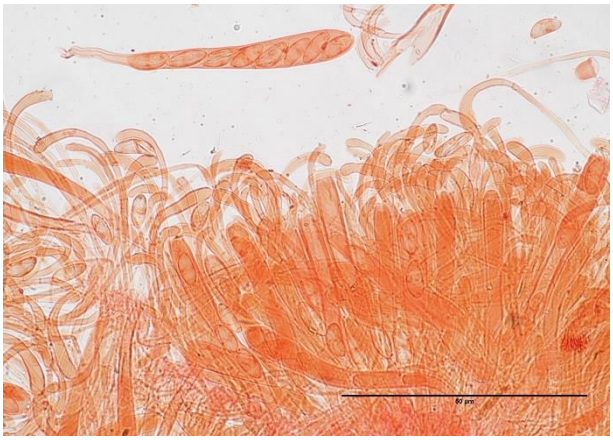

Figure 1: close up of hymenium and free ascus of DHF1777 (holotype), stained with CR SDS IKI (scale bar not representative to true size due to defect or mistake)

Figure 2: Close up of upper part of inamyloid asci of DHF1777 (holotype), stained with IKI (scale bar = 10 µm)

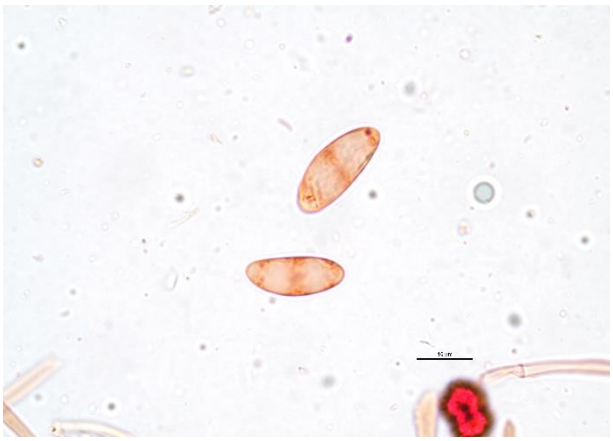

Figure 3: Ascospores of DHF1777 (holotype), stained with CR SDS (scale bar = 10 µm)

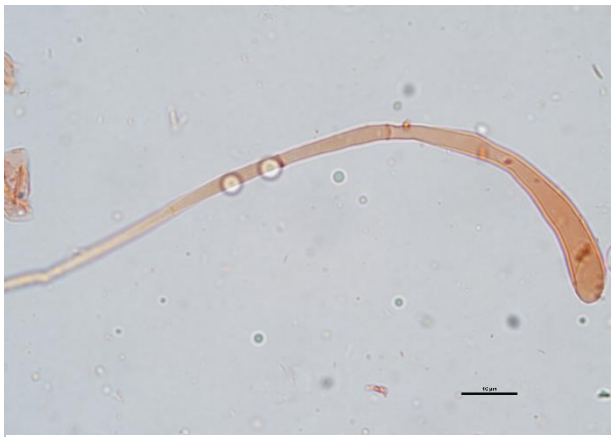

Figure 4: Paraphyse of DHF1777 (holotype), stained with CR SDS (scale bar = 10 µm)

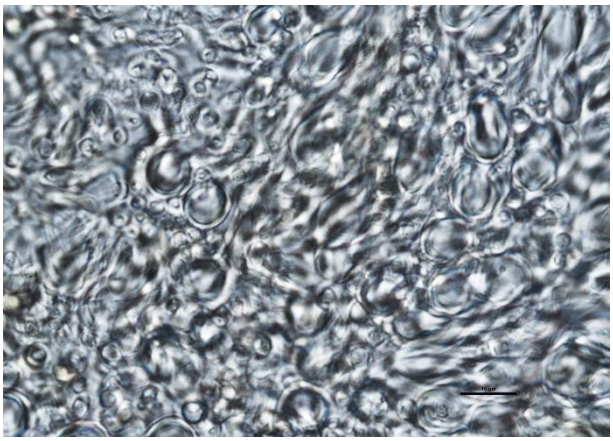

Figure 5: Close up of medullary excipulum (textura globulosa) of DHF177 (holotype), stained with IKI (scale bar = 10 µm)

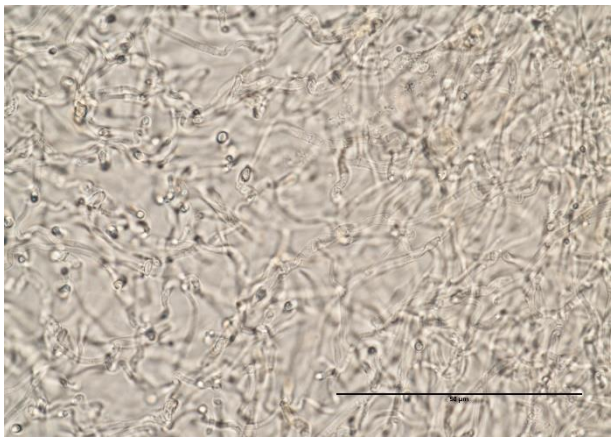

Figure 6: Close up of ectal excipulum (textura intricata) of DHF1777 (holotype), stained with IKI (scale bar is not representative to true size due to defect or mistake)

Drawings of *Leotia lubrica*

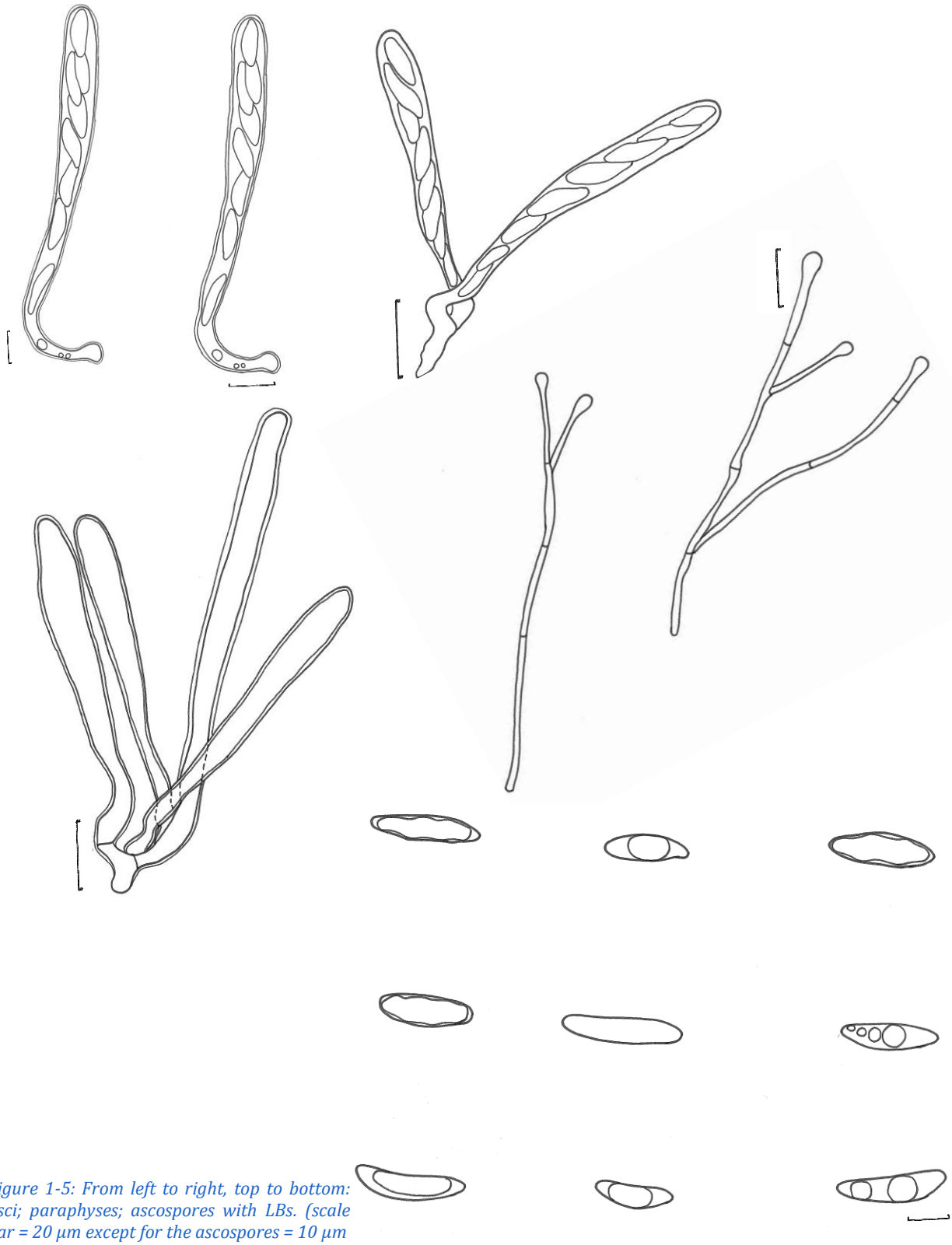

Figure 1-5: From left to right, top to bottom: asci; paraphyses; ascospores with LBs. (scale bar = 20 µm except for the ascospores = 10 µm

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Drawings of *Leotia baculum*

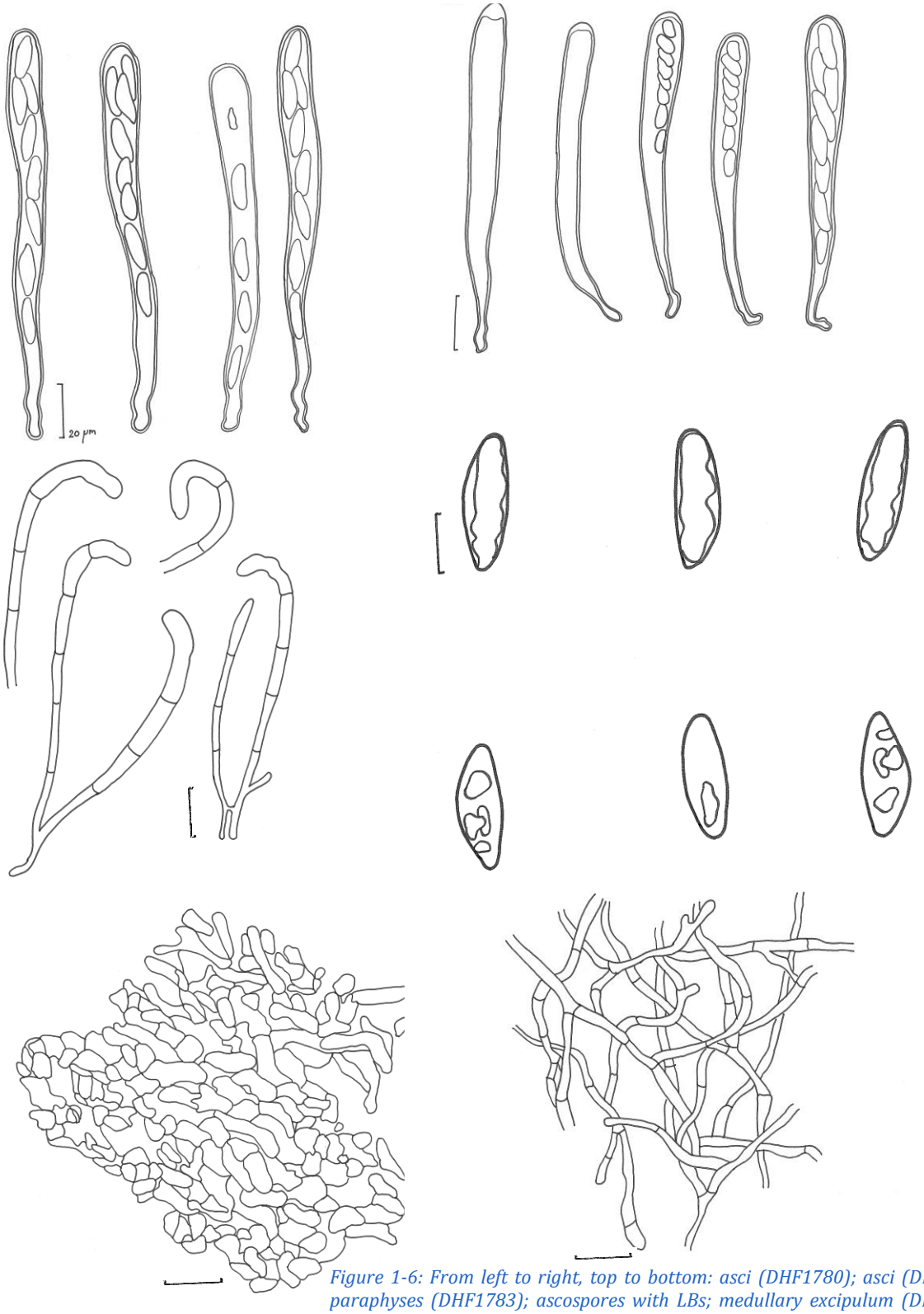

Figure 1-6: From left to right, top to bottom: asci (DHF1780); asci (DHF1783); paraphyses (DHF1783); ascospores with LBs; medullary excipulum (DHF1775); ectal excipulum (DHF1775). (scale bar = 20 µm except for the ascospores = 10 µm

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Drawings of *Leotia virgula*

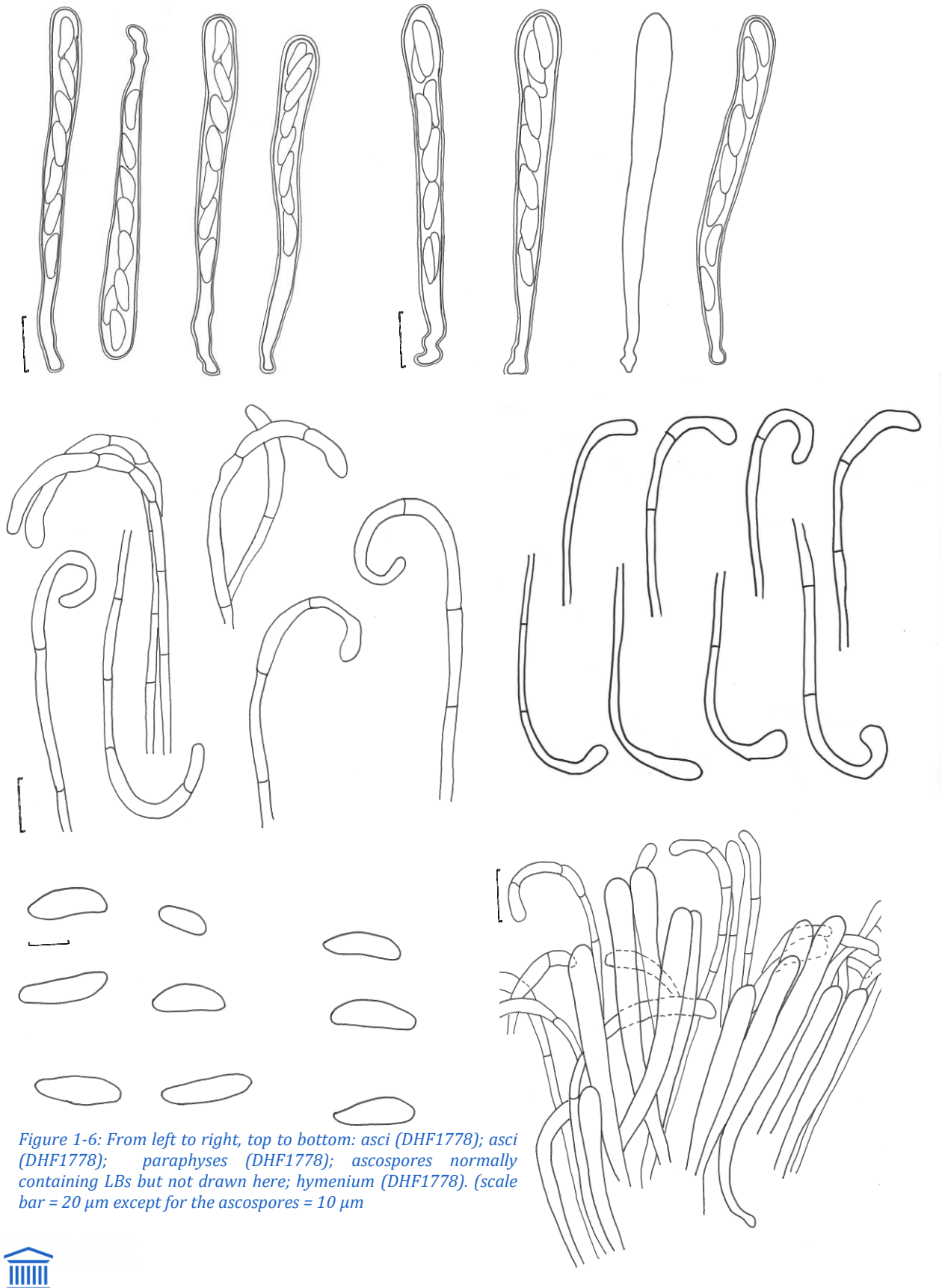

Figure 1-6: From left to right, top to bottom: asci (DHF1778); asci (DHF1778); paraphyses (DHF1778); ascospores normally containing LBs but not drawn here; hymenium (DHF1778). (scale bar = 20 µm except for the ascospores = 10 µm

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Drawings of *Leotia rufa*

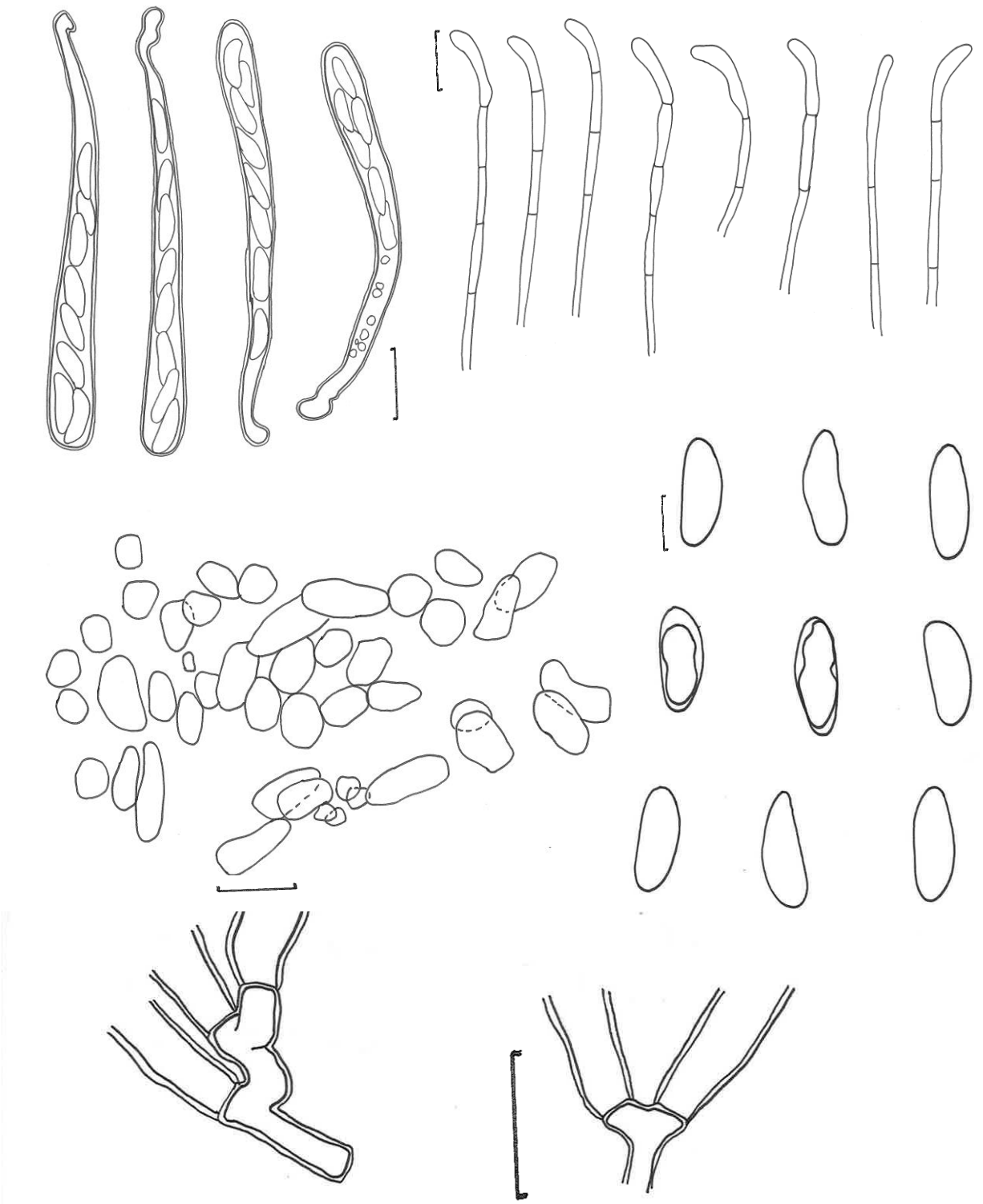

Figure 1-6: From left to right, top to bottom: asci (DHF1777); paraphyses (DHF1777); medullary excipulum (DHF177); ascospores (DHF177); ascus base arising from crozier; ascus base arising from qimple-septa. (scale bar = 20 µm except for the ascospores = 10 µm

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