Sclerotinia cirsii-spinosissimi, a new species from the Alps

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Abstract: *Sclerotinia cirsii-spinosissimi* is described as a new species from bracts of *Cirsium spinosissimum* based on morphological, cultural and molecular studies. Ascospore size and absence of small lipid bodies inside the spores are distinctive, medium sized sclerotia are typically produced on solid media in cultures. This description includes earlier molecular studies showing a close relationship to *S. borealis*. A key is added to *Sclerotinia* species from arctic-alpine habitats.
Keywords: Alpine ecosystem, ascomycetes, DNA sequences, taxonomy.

Zusammenfassung: *Sclerotinia cirsii-spinosissimi* wird als neue Art von den Brakteen der Alpen-Kratzdistel *Cirsium spinosissimum* beschrieben basierend auf morphologischen, und molekularen Studien zusammen mit Merkmalen aus Kulturen. Charakteristisch sind die Grösse der Ascosporen und das Fehlen von kleinen Oeltröpfchen in den Ascosporen sowie mittelgrosse Sklerotien auf festen Nährmedien. Die neue Art entspricht Kollektionen deren molekulargenetische Analyse eine nahe Verwandtschaft zu *S. borealis* zeigten. Ein Schlüssel zu *Sclerotinia*-Arten aus arktisch-alpinen Standorten wird beigefügt.
Schlüsselwörter: xxxx

Introduction

Sclerotinia Fuckel and related genera such as *Dumontinia* Kohn, *Monilinia* Honey, *Botryotinia* Whetzel and others comprise plant pathogens as well as necrotrophs.

The evolution in the taxonomical concepts of these genera and their species reflect exemplary advancements in taxonomy. As in many genera of phytopathogenic fungi the taxonomical delimitation of species based on morphological diversity was given little attention in the decades around the year 1900, when emphasis was placed on host specificity. This resulted in a large number of described taxa. Whetzel (1945) gave a first synopsis of genera and species hitherto described. With the development of new techniques reassessments became possible. Kohn (1979) gave the genus *Sclerotinia* a clear circumscription with very few remaining species based both on morphological and cultural characteristics. Kohn *et al.* (1988), using molecular techniques, suggested the presence of a putative further species in Japan, later described as *Sclerotinia nivalis* from various plant hosts by Saito (1997). Holst-Jensen *et al.* (1998) provided a phylogenetic analysis based on nuclear ribosomal sequences of *Sclerotinia* and related genera, which included two reputedly new species closely related to *S. minor*. One of them, *Sclerotinia* sp. 2, i.e. genotype G, according to Holst-Jensen *et al.* (1998) is described here as a new taxon.

Materials and methods

Specimens were found in different years at various localities in the Swiss Alps always on fallen bracts of *Cirsium spinosissimum* Scop. (*Asteraceae*), an endemic plant of the European Alps. Fresh material was described and used for ascospore isolation. Mass ascospore discharges were allowed directly on petri-dishes containing Difco potato dextrose agar (PDA) or 2.5% malt agar (MA). The mycelium was maintained on PDA or MA at room temperatures. Microscopic investigations were performed on fresh and dried specimens rehydrated in water, using squash mounts or thin sections cut freehand. Observations on living cells are marked by (*), those on dead cells by (†). Measurements are separated according to their state. Those of the dead spores are obtained in Congo red or 5% KOH (20 spores per collection). Indicated are the calculated 95% confidence intervals and in brackets the observed minimum and maximum values. The apical apparatus of the asci was checked in Melzer's reagent (MLZ) and in Lugol (IKI) without and with pretreatment with KOH according to Baral (1987). The sclerotia found close to the plant material and those produced on agar in petri dishes were studied from 3–8 µm thick sections cut by a tungsten carbide knife on an ordinary rotary microtome mounted in a methacrylate-mixture. Nuclei were stained following the protocol for siderophilous reactions by Clémençon (1978). Herbaria are abbreviated according to Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp).

Molecular analyses

DNA extraction and sequencing — DNA was extracted from a piece of dried apothecium using the DNeasy Plant Mini Kit of Qiagen. DNA was extracted from three isolates of *Sclerotinia cirsii-spinosissimi* from different locations in Switzerland and from the type isolate of *Sclerotinia nivalis* from Japan kindly provided by I. Saito (cf. Saito, 1997). PCR amplification of the ITS region of rDNA was performed according to the PCR protocol of Gardes & Bruns (1996) using the primers ITS1 and ITS4 developed by White *et al.* (1990). We verified the amplification success by running the products on a 1.5% agarose gel and visualizing them with ethidium bromide under UV-light. PCR products were purified with the PCR purification Kit (Qiagen). The sequencing reaction was performed with the BigDye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems) and cleaned by an ethanol/sodium acetate precipitation according to the manufacturer's instructions. The products were electrophoresed using an ABI 3100-avant automated DNA sequencer (Applied Biosystems). Both strands were sequenced using the same primers as in the PCR reaction and aligned using the software DNADynamo (Blue Tractor Software). The new sequences have been submitted to GenBank of NCBI under accession numbers EU330398 – EU330400 and GQ848548.

Phylogenetic analyses — Phylogenetic analyses of the DNA sequences were performed using the dataset described in Holst-Jensen *et al.* (1998) and applying maximum parsimony (PAUP version 4.0, Swafford, 2002). We used the executable nexus file of these authors and introduced the additional 4 *Sclerotinia* sequences by manually aligning them. This nexus file contained 50 isolates representing 24 taxa of the discomycete family *Sclerotiniaceae* and an outgroup consisting of five related taxa of the same family (Holst-Jensen *et al.*, 1998). It already contained two different isolates of *S. cirsii-spinosissimi*, named *Sclerotinia* sp. 2, also found in Switzerland. Phylogenetic analyses were performed on a subset of isolates representing all the observed genotypes (pairwise distances among all included isolates ≠ 0; cf. Holst-Jensen *et al.*, 1998). We used the same parameters for the analysis as described by these authors. In short, a heuristic search in PAUP was performed employing the random, stepwise addition sequence option with 100 replicates, collapse zero branch lengths, TBR branch swapping and the gapmode "newstate". Suc-

cessive weighting based on the maximum rescaled consistency index (RC) for each character over all most parsimonious trees was used to identify and downweight homoplastic characters, and to reduce the number of competing trees. This procedure proved to provide a sound basis for selecting characters of importance in the molecular dataset from a taxonomic point of view (Holst-Jensen *et al.*, 1998). The support for each clade was examined by bootstrapping (Felsenstein, 1985) with 500 replicates as described by Holst-Jensen *et al.* (1998).

Results

Sclerotinia cirsii-spinosissimi Senn-Irlet *sp. nov.* — MycoBank 516616, Plate 1-3.

Apothecia solitaria, discus acetabuliformis, 5-12 *mm diam, stipitatus, hymenio brunneo, stipes cylindraceus, colore simile disco,* 5–20 × 0.8–1.5 *mm. Asci cylindracei,* † (120–)130–170 × 7–9 *µm, octospori, poro jodo caerulescenti. Ascosporae uniseriatae, hyalinae, elipsoideae, inaequilaterales,* † 9.5–11.8 × 4.7–6.6 *µm. Microconidia* 2–4 *µm diam., globosae, in sporodochiis in hymenio vel in vitro. Sclerotia tuberoidea,* 4–8 × 1.5–4 *mm, extus nigra, intus albida.*

Habitat in bracteas Cirsii spinosissimi in regio alpina, Helvetia.

Holotypus: Senn-Irlet 96/32, Uri, Attinghausen, 31 Augusto 1996, *in herbario* Z-ZT *conservatur.*

Etymology — Refers to the species name of the plant host.

Apothecia arising singly or rarely in pairs or in triplet from a true sclerotium, embedded in fallen old, last-year bracts of *Cirsium spinosissimum* lying on the soil (Plate 1a); receptacle 5–12 mm diam., 2–4 mm high, cupulate or discoid with a central depression; hymenium ochraceous to burnt Sienna, smooth; stipe cylindrical, often flexuous, 5–20 × 0.8–1.5 mm, tapering towards base, concolorous or paler than hymenium, glabrous to felted under hand lens.

Asci arising from croziers, cylindric–clavate, (*) 140–180 × 9–10.5 µm, (†) (120–) 130–140 × 7–9 µm, with a tapering, blunt–end base, regularly 8–spored, all spores of about equal size, apex truncated-rounded, apical pore MLZ+ blue, IKI blueish, deep blue in MLZ after KOH (Plate 2d). **Ascospores** (*) 11.3–15.3 (17) × 5.7–7.1 µm (fresh in H_2O), Q = 1.6–2.4, mean Q = 2.04, (†) (8) 9.5–11.8 (14.5) × 4.7–6.6 µm, Q = 1.5–2.2 (in Congo red and 5% KOH) mean Q = 1.85, (†) uniseriate, young in ascus biseriate, hyaline, unicellular, ellipsoid and slightly inequilateral, eguttulate, with two (to four?) nuclei, content (†) cyanophilous (in Cotton blue), IKI wall hyaline, content yellow. **Microconidial state** with microconidia globose, hyaline, 2–4 µm diam., produced from phialides in sporodochia (Plate 1f), superficial in hymenium of older apothecia from germinating ascospores, especially towards margin. **Paraphyses** filiform, septate, simple or sparsely branched, hyaline, in upper part 3–4 µm wide, fresh with large pale brown guttules in the upper cells, without gelatinous covering, even in living state. **Subhymenium** 45–60 µm, of densely septate, prismatic cells, brown-walled, reddish brown. **Medullary excipulum** of loosely interwoven, filiform, 2–5 µm wide, colourless hyphae, forming a textura intricata, 40–250 µm thick, without crystals, no blueing in IKI. **Ectal excipulum** 50–150 µm, 3–5 cells thick, of pale brown angular to prismatic cells, 20–35 × 10–16 µm in size, orientated perpendicularly to apothecial surface. No gelatinous matrix observed.

Stipe composed of a medullary excipulum forming a *textura porrecta*, composed of thin-walled, elongated, 5–8 µm wide, hyaline cells, and an ectal excipulum, 8–20 µm thick, of 2–3 rows of elongated, light brown, thin-walled cells, forming a *textura angularis*, outermost cells often with short outgrowths. Both textures arranged parallel to the stipe axis.

Pigment brownish, membranaceous, in subhymenium, and outer cells of the stipe, intracellular in paraphyses.

Stroma an irregular tuberoid sclerotium (Plate 1b), globose to cylindrical, constricted and often furrowed, variable in shape, 4–8 × 1.5–4 mm, with scrobiculate, black outer rind and white inner context, developing within fallen, straw-like involucral bracts of *Cirsium spinosissimum*, but never incorporating remnants of plant material. Sclerotial rind two to four cells wide, of dark brown-walled, angular to prismatic cells, outermost cells heavily melanized, carbonaceous; uppermost cells pale brown, cells compact, (†) 12–20 × 6–10 µm, forming a *textura angularis* to *textura oblita*, sclerotial medulla of interwoven, hyaline hyphae, 4–6 µm wide, forming a textura oblita-intricata, walls gelatinized, without apparent remnants of host tissue.

Ascospores germinate readily on PDA and MA, mycelium whitish, adhering to the agar surface, after 4–6 weeks applanate sclerotia are produced regularly spread over the whole surface of the petridish on PDA (Plate 1c), half to three quarter immerged, irregularly spread and larger (up to 12 × 7 mm) on MA.

18 days after inoculation scattered flocculose tufts produced small globular sporodochia, with conidia born on phialides on aerial mycelium, phialides with no obvious collarettes, conidia globose to slightly ovate, 2.5–4 µm in diam., with one internal guttule.

The ITS sequences of the five isolates of *Sclerotinia cirsii-spinosissimi* collected from different locations in Switzerland were identical. Parsimony analysis revealed 44 of the 514 characters to be parsimony informative. The analysis yielded 530 most parsimonious trees (MPTs) which were reduced to 48 after reweighting (cf. Holst-Jensen *et al*., 1998). *S. cirsii-spinosissimi* was consistently placed in a cluster with *S. borealis*, which was supported by the strict consensus tree and a bootstrap value of 64% (Fig. 6). In 32 of the 48 MPTs, *S. nivalis* clustered with *S. glacialis* (52% bootstrap).

Substratum — *Sclerotinia cirsii-spinosissimi*, always on *Cirsium spinosissimum*.

Distribution — Europe, Alps.

Specimens examined. Switzerland – Uri, Attinghausen-Geissberg, 1740 m alt., 31 August 1996, B. Senn-Irlet & R. Mürner (ZT, holotype, BSI 96/32, culture isolate, NCBI GQ848548); Spirigen-Kinzigpass, 2070 m alt., 19 August 2001, B. Senn-Irlet & R. Mürner (ZT, BSI 01/194); – Bern, Guttannen-Oberaar, 2330 m alt., 30 August 1994, B. Senn-Irlet (ZT, BSI 94/43); 15 August 1995, H.U. Aeberhard & B. Senn-Irlet (ZT, BSI 95/150, culture isolate, *Sclerotinia* spec 2 *sensu* Holst-Jensen *et al.*, 1997), 8 August 1998, H.U. Aeberhard (ZT, 024.98), 2 September 2004 (ZT, BSI 04/130, NCBI EU330398), 17 August 2010, B. Senn-Irlet (ZT, BSI 10/63), 22 August 2010, H. Woltsche (ZT, BSI 10/93); – Graubünden, Bivio, near Leg Grevasalvas, 2460 m alt., B. Senn-Irlet (ZT, BSI 03/80, NCBI EU330399); – Ticino, Bedretto, Ponte di Paltano, 1840 m alt., 20 August 2002, H. Woltsche & B. Senn-Irlet (ZT, BSI 02/79); – Valais, Val d'Anniviers, Moiry, 21 August 2010, B. Senn-Irlet (ZT, BSI 10/83).

Sclerotinia nivalis Saito: Japan – Hokkaido, Makubedtsu-cho, on *Arctium lappa*, 15 May 1982, I. Saito (HAK, Holotype, 24055, NCBI EU 330400).

Habitat and ecology

Sclerotinia cirsii-spinosissimi was exclusively found in association with *Cirsium spinosissimum*, a widespread, abundant forb of the lower alpine zone of the Alps, forming clones (Urbanska, 1992) on pastures and near rivulets, especially at nutrient rich sites with forb vegetation and in scree vegetation, often at places indirectly favoured by cattle. The apothecia were always found on the involucral bracts from the almost intact, fallen inflorescenses from the previous year below the forb plant, profiting thus from a favourable humid microclimate. The accompanying macromycetes found were *Peziza granularis* Donadini, *Ombrophila* spec., *Scutellinia* spec., *Tarzetta cu-*

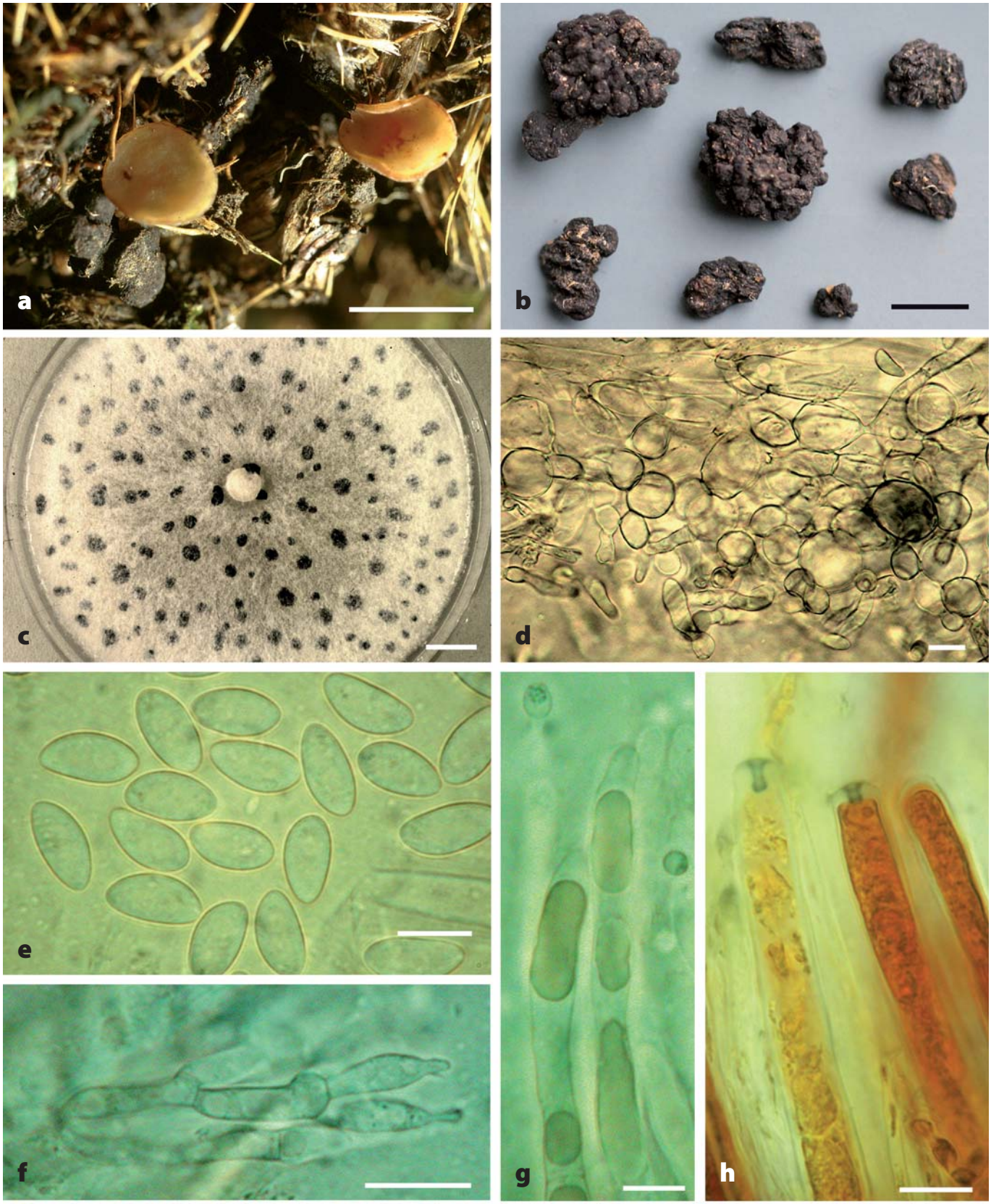

Plate 1 – *Sclerotinia cirsii-spinosissimi*

a) Fresh fruit-bodies from holotype (coll. BSI 96/32); b) Sclerotia (coll. 024.98); c) Sclerotia on PDA in culture after 2 months (isolate from BSI 96/32); d) Ectal excipulum, fresh in water (coll. BSI 10/93); e) Ascospores, fresh in water (coll. BSI 10/63); f) Microconidia in hymenium, in water (coll. BSI 10/83); g) Paraphyses in water (coll. BSI 10/83); h) Ascus apex in Lugol (coll. BSI 10/93). Bars = 10 µm, 1 cm in a), b) and c).

pularis (L.) Svrček, and *Hemimycena ochrogaleata* (J. Favre) M.M. Moser.

Involucral bracts of *Cirsium spinosissimum* lying beyond the forb plant on open grassland are often infected by *Crocicreas calathicola* (Rehm) S.E. Carpenter, a saprotrophic ascomycete with orange coloured apothecia. *Sclerotinia cirsii-spinosissimi* and *Crocicreas calathicola* were never found together suggesting that microclimatic conditions were required for germination and establishment, and humid conditions favoured *Sclerotinia*. A necrotrophic life-form is suggested as no signs of reduced plant growth or flowering has been observed.

Discussion

Sclerotinia cirsii-spinosissimi shares the typical features of the genus, i.e. cup-shaped, brownish, apothecia arising from a stipe and from free, distinct tuberoid sclerotia with a carbonaceous rind and a white medulla without incorporated remains of host tissue, an outer excipulum of globose to angular cells, asci with IKI deep blue apical ring, and hyaline ellipsoid unicellular 2–4 nucleate ascospores (Baral, *in* Baral & Marson, 2005), budding into microconidia with age, and lacking a macroconidial anamorph. The living ascospores show hardly any lipid guttules in contrast to most other *Sclerotinia* species (Baral, *in* Baral & Marson, 2005). Within the genus it falls in a group of similar species with eight homomorphic ascospores, which can be separated on ascospore size and shape, lipid guttules, and cultural characteristics such as size of sclerotia produced.

The key on page 240 highlights the distinctive morphological differences in spore size and anatomy of *Sclerotinia* species found in arctic-alpine environments. Spore size refers to the dead state.

Molecular analysis confirmed *S. cirsii-spinosissimi* to be a new species with a unique ITS sequence and its inclusion in *Sclerotinia* since it was consistently placed as a sister taxon of *S. borealis*. Based on the detailed description of *S. nivalis* (Saito, 1997), a snow-mould described from a composite and other dicots, a close relationship, if not identity, could be suggested. Molecular analysis now reveals a closer relationship of the eight-spored *S. nivalis* with the four-spored *S. gla-*

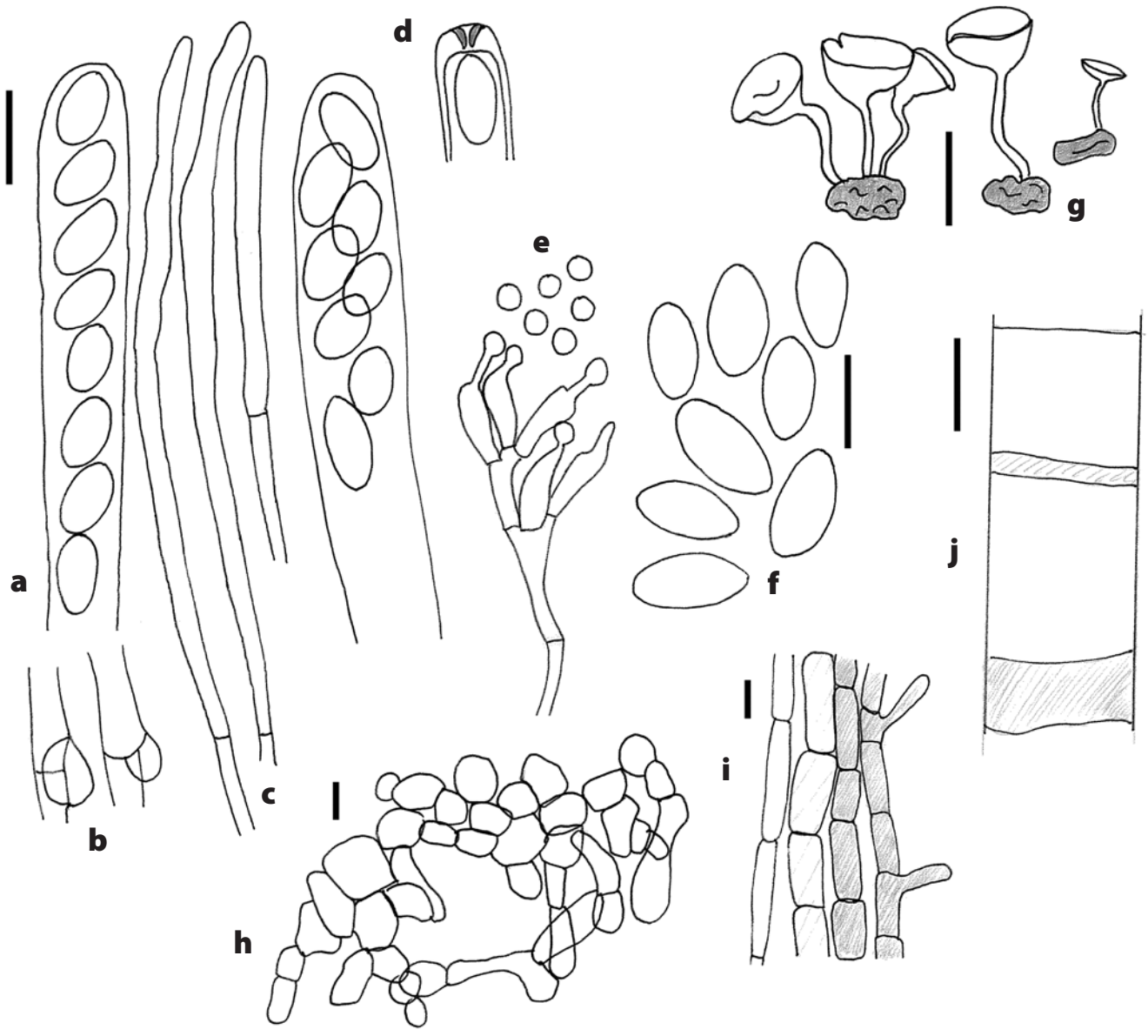

Plate 2 – *Sclerotinia cirsii-spinosissimi*

a) Asci with uniseriate ascospores in early stage of maturation and biseriate ascospores in late stage of maturation; b) croziers; c) paraphyses; d) apical apparatus in Lugol; e) Microconidia; f) Ejected ascospores; g) Fruit-bodies with sclerotia; h) Ectal excipulum in section (underside of cup); h) Excipulum of stipe; j) Section through apothecium. Bars = 10 µm, 1 cm in g), 100 µm in j).

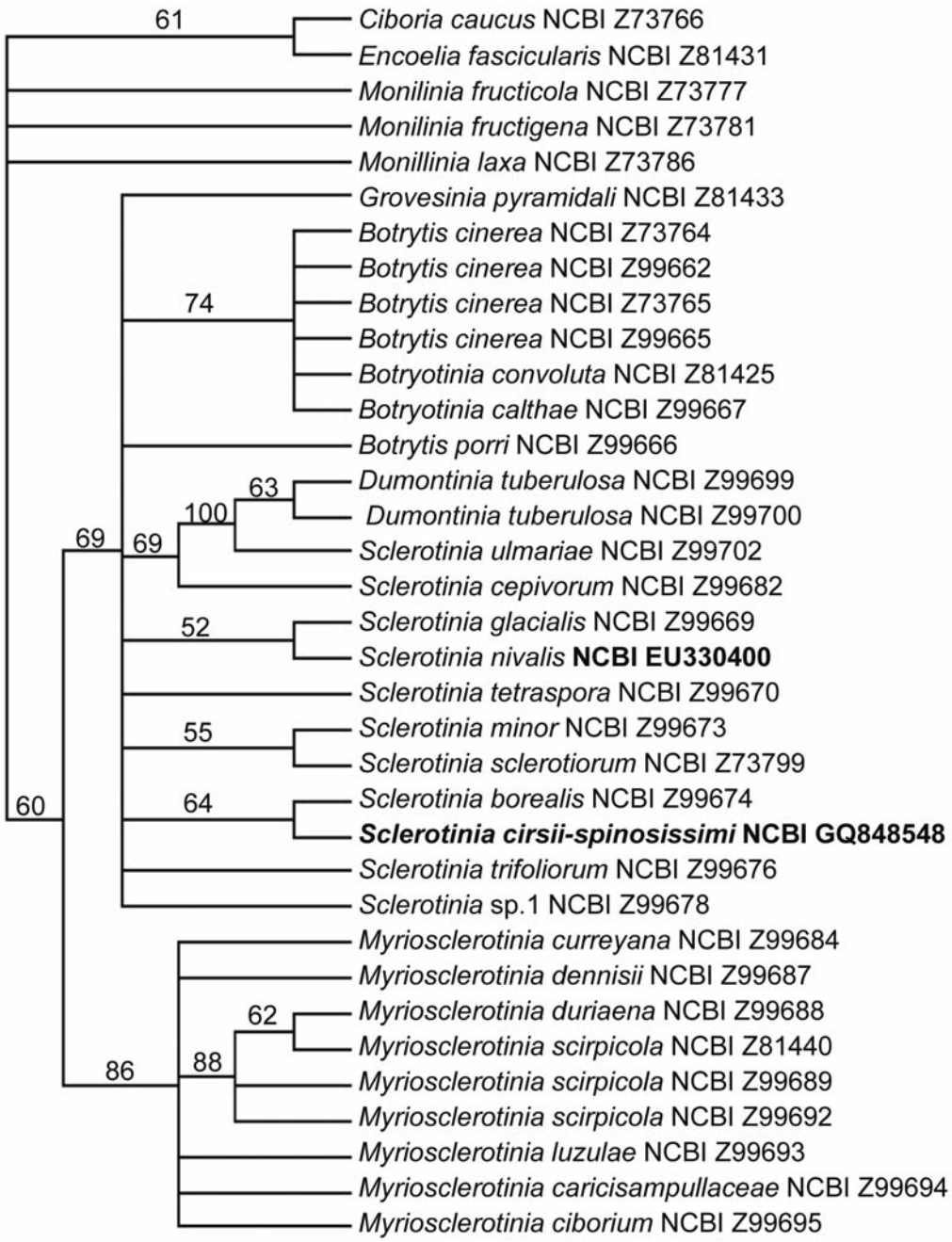

Plate 3 – Phylogenetic placement of *Sclerotinia cirsii-spinosissimi* within members of the *Sclerotiniaceae*, obtained by sequence comparison of the ITS region. The phylogram is based on maximum parsimony. The strict consensus tree of 48 most parsimonious trees is shown, numbers percentages of 500 bootstrap replicates that support the indicated branches (only values above 50% are shown). Sequences other than *S. cirsii-spinosissimi* and *S. nivalis* were retrieved from the executable nexus file of Holst-Jensen *et al.* (1998).

cialis rather than with our new species. *S. borealis*, known as a snow-mould attacking winter cereals and grasses in boreal and sub-arctic zones, such as Finland, Norway and Alaska (Gaudet *et al.*, 1999) came out as closely related. Yet, this species is clearly separated by spore size from our new species.

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Key of *Sclerotinia* species from arctic-alpine habitats

1a Asci with four ascospores **2**
1b Asci with eight ascospores **3**

2a Ascospores 22–27 × 8–10 µm; on leaf base of *Ranunculus glacialis*, fruiting shortly after snow melting ..... ***S. glacialis*** F. Graf & T. Schumach.
2b Ascospores 10–15 × 5.6–8 µm, on previous year stems of *Rubus chamaemorus* ..... ***S. tetraspora*** Holst-Jensen & T. Schumach.

3a Ascospores in size dimorphic, (10) 12–18 × 6–10 µm including small spores; frequently on leguminous hosts, fruiting in summer in alpine environments ..... ***S. trifoliorum*** Erikss.
3b Ascospores not dimorphic **4**

4a Ascospores 14–21 × 6–9 µm, subfusiform; on a variety of hosts, most frequently on *Poa* and *Festuca* (*Poaceae*) ..... ***S. borealis*** Bubak & Vleugel
4b Ascospores elliptic, oval, not subfusiform **5**

5a Ectal excipulum at margin of apothecium composed of globose cells, asci arising from croziers, ascospores 8–17 (–20) × 5–7 (9) µm, with four nuclei, apothecia arising singly from each sclerotium ..... ***S. minor*** Jagger
5b Ectal excipulum at margin of apothecium composed of prosenchymatic cells oriented perpendicular to the apothecial surface, asci with or without croziers, ascospores with 2(–4) nuclei, apothecia arising singly or multiply from a sclerotium **6**

6a Ascospores up to 5 µm wide, asci without croziers, ascospores 9.2–11.7 × 3.8–5.0 µm, medullary excipulum 150–300 µm thick; sclerotia in axenic culture 0.6–4 mm diam.; on various dicots ..... ***S. nivalis*** Saito
6b Ascospores up to 7 µm wide, asci with croziers, sclerotia in axenic culture 2–15 (–30) mm diam. apothecia 2–12 mm diam. **7**

7a Ascospores 9–13.5 (–15) × 4–6.5 (–7) µm, mean Q > 2, with usually 2 small (ca 1 µm diam) lipid bodies, with two nuclei, medullary excipulum 150–700 µm thick, faintly to distinctly blueing in IKI; sclerotia in the soil without evident connection to the host plant, small to large, 4–25 × 2–10 mm, various host plants..... ***S. sclerotiorum*** (Lib.) De Bary
7b Ascospores 9.5–12 × 5–6.5 µm, mean Q < 2, without lipid bodies, with 2(–4?) nuclei, sclerotia on and within host plant bracts, small, 4–8 × 1.5–4 mm, warty, medullary excipulum 40–250 µm thick, not blueing in IKI, on *Cirsium spinosissimum* ..... ***S. cirsii-spinosissimi***

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