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Comparison of Two *Coniochaeta* Species (*C. ligniaria* and *C. malacotricha*) with a New Pathogen of Black Pine Needles – *Sordaria macrospora*

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Abstract

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A new pathogen, *Sordaria macrospora,* isolated from damaged needles of black pine (*Pinus nigra*) causes discolouration, brown spots, blight symptoms, and necroses spoiling aesthetic value. Two species, *C. ligniaria* and *C. malacotricha*, the most common anamorphs attributed to *Coniochaeta* species occurring on selected conifers, and a new pathogen, *Sordaria macrospora,* occurring on *Pinus nigra,* are compared. Specific differences in spore size and anamorph morphology between the similar species *C. malacotricha* and *C. ligniaria* could be confirmed.

Keywords: Ascomycota; morphological characteristics; phylogenetic analysis; *Pinus nigra*

Sordariomycetes is a heterogeneous group of unitunicate pyrenomycetes with globose or flask-shaped solitary perithecial large ascomata, with large-celled membraneous or coriaceous walls. These fungi are worldwide distributed, commonly in dung or decaying plant matter, rarely on coniferous needles. These species colonise whole stems and xylem of trees at dry sites (Fisher *et al*. 1992, 1993; Petrini & Fisher 1988, 1990). Members of the order Sordariales can be coprophiliac, fungicolous, herbicolous, lignicolous, soil-inhabiting or grow on plant debris. Species of the family Sordariaceae have persistent cylindrical, basally clustered asci in perithecial forms and clavate asci in cleistothecial forms. Asci are commonly cylindrical with an apical ring. The ascus apex usually has one or several germ pores and a refractile ring through which the ascospores are discharged. *Sordaria* species has smooth-walled, dark brown ascospores, generally aseptate, with the surface smooth, pitted, reticulate or striate, sheathed or unsheathed. Spores are surrounded by gelatinous sheath which is sometimes thick and conspicuous to even difficult to detect. Darkly pigmented ascospores show wide variation in the kinds of appendages or sheaths (Alexopoulos *et al*. 1996; García *et al*. 2004).

In the recent literature the family Coniochaetaceae is placed under Sordariales, but differs from them in ellipsoidal to fusoid, nearly globose ascospores with longitudinal germ slits extending over the narrow side (Mahoney & La Favre 1981; Hanlin 1990; Lee & Hanlin 1999; Kirk *et al.* 2001; Weber 2002). A distinguishing character of the genus *Coniochaeta* is the presence of brown, usually straight and pointed setae (Asgari & Zare 2006). *Coniochaeta* species have been reported from different substrates and in many cases were isolated from wood samples that rarely showed necrosis, and were always found in combination with other fungi and basidiomycetes (Asgari & Zare 2006;

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Damm *et al*. 2010). *Coniochaeta* species are of low virulence on most hosts, usually appearing on dead tissue or as opportunistic invaders of previously infected, wounded or senescent tissue (Damm *et al*. 2010).

During the investigation of mycoflora on a pine tree (*Pinus nigra*) a fungus with distinctive characteristics was isolated. The aim of this work was to compare, on the basis of light-microscopic morphological studies, two *Coniochaeta* species (*C. ligniaria* and *C. malacotricha*) commonly occurring on conifer needles, with a newly detected fungus *Sordaria macrospora* Auersw., that was confirmed by phylogenetic analysis. The similarity and the differences of these fungi are discussed.

MATERIAL AND METHODS

From spring to autumn 2013 and in spring 2014, needles of *Pinus nigra* with blight symptoms were collected from plants growing in private gardens and public greenery at several locations of Nitra, Slovakia (Nitra-Zobor, Nitra-Chrenová, Nitra-Sihoť, Nitra-Kynek). Altogether 20 trees were studied. The age of the evaluated trees was 35–40 years. Samples were taken at each of these localities from some sections of trees with damaged needles. Each sample was cultivated on 30 Petri dishes (PD) with 3% PDA medium. Occurrence of the new fungus *S. macrospora* was enumerated in % and identified by microscopical analysis based on the appearance of the fruiting bodies, spore bearing organs (asci), reproduction organs (conidia and ascospores) and on the appearance of the fungus in pure cultures. Morphometric measurements of ascomata, setae, asci, and ascospores were made for each sample PD with the occurrence of the fungus *S. macrospora*. The main value of length and width was assessed for 30 conidia of each isolate. The samples of biological material were deposited in herbarium at the Institute of Forest Ecology of the Slovak Academy of Sciences, Branch for Woody Plant Biology in Nitra, Slovakia.

To isolate and obtain pure cultures, cultivation was performed on nutritive 3% PDA medium in a versatile environmental test chamber MLR-351H (Sanyo, Osaka, Japan) with constant temperature (24 ± 1°C), humidity (45%), under dark conditions. The needles parts cut from the diseased plants were surface-sterilized for 20 min. Fungal structures were investigated using a light clinical microscope BX41 (Olympus, Tokyo, Japan) under a 400× and 1000× magnification. Measurements were performed using the QuickPhotomicro 2.2 programme and the morphometric values were compared with previously published data for the taxa (Petrini & Fischer 1988; Fischer *et al*. 1992, 1993; Alexopoulos *et al*. 1996; Crous *et al*. 2009).

DNA extraction, PCR amplification and sequencing. For DNA extraction, 7–8-day-old fungal mycelia were used. Mycelium was scraped from MEA, ground and homogenised in liquid nitrogen by mortar and pestle. Total DNA was extracted according to DNeasy Plant Mini Kit (Qiagen, Germany) protocol and stored in –20°C. Primer pairs ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes & Burns 1993; White *et al*. 1990), and LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR7 (5'-TACTACCACCAAGATCT-3') (Vilgalys & Hester 1990) were used for amplification of ITS or the partial large subunit ribosomal RNA (LSU) region, respectively. PCR amplifications were performed using a thermocycler (Biometra T Professional, Göttingen, Germany) under the following conditions: 95°C/5 min, 95°C/15 s, 50°C/30 s, 72°C/80 s (Husson *et al*. 2011).

PCR reaction mixture (20 µl in total) contained 50 ng of DNA, 20 pmol of each primer, 0.2 mM dNTPs, and 1U Hot Firepol Blend Master mix (Solis BioDyne, Estonia). The PCR products were purified with NucleoSpin Gel and PCR Clean up (Macherey-Nagel, Germany) prior to sequencing. The PCR products were sequenced with each primer used in the PCR (Sigma Aldrich, Germany). DNA sequences were assembled using DNA Baser software (Heracle BioSoft SRL, Romania) and submitted to the GenBank database under the accession No. KT013302 for *S. macrospora* Iv 1 partial LSU sequence and No. KT013303 for *S. macrospora* Iv 1 ITS sequence. The DNA sequences were compared against GenBank database using BLASTn and BLASTx algorithms (Altschul *et al*. 1990).

For analysis of phylogenetic relatedness DNA sequences were aligned using Muscle algorithm (Edgar 2004) and phylogenetic relationships were constructed using the Neighbor Joining method available in the MEGA software version 5 (Tamura *et al*. 2011). Phylogenetic robustness of trees obtained was tested by bootstrap analysis after 1000 replications.

RESULTS

Culture characteristics of the fungus isolated in our experiments from needles of *Pinus nigra* cul-

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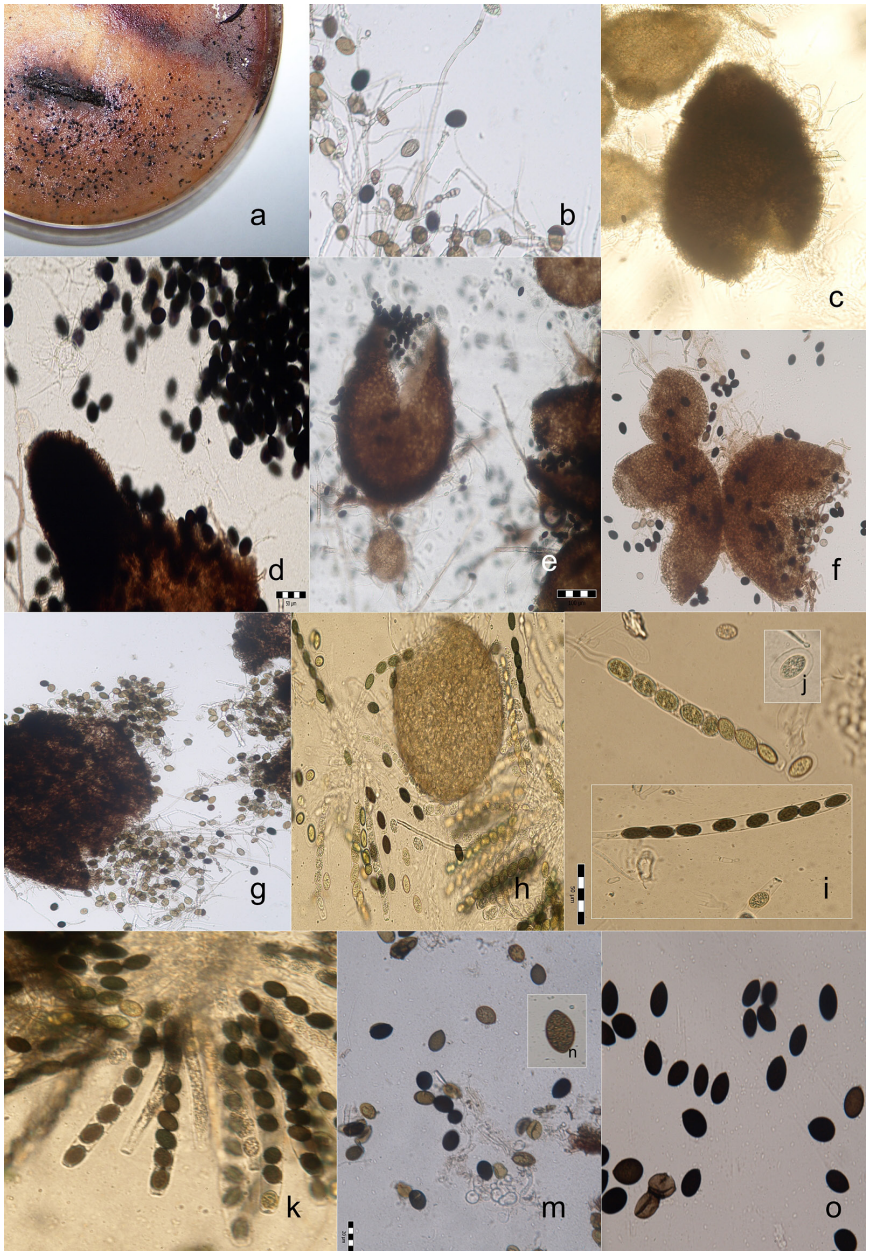

Figure 1. *Sordaria macrospora* on *Pinus nigra*: (**a–b**) anamorph: (**a**) colony on PDA after 2 weeks; (**b**) vegetative hyphae; (c–o) teleomorph: (**c**) ascomata with setae; (**d**) perithecial neck; (**e–g**) opening of perithecium; (**h–k**) rosettes of asci; (**i**) 8-spored ascus; (**j**) ascospore sheath; (**m**) immature ascospores; (**n**) granular content of ascospores; (**o**) mature ascospores. Scale bars: m = 20 µm, d, i = 50 µm, e = 100 µm

tivated on PDA medium: the fungus formed sparse aerial mycelium of pale white colour, after 2 weeks of inoculation dark pycnidia were formed (Figure 1a). Vegetative hyphae were hyaline, 4–5 µm wide, septate, branched, lacking chlamydospores. Conidia did not appear (Figure 1b). After 2 weeks, black perithecia, pyriform, setose, solitary (Figure 1c) with a central ostiole were formed (Figure 1e), size 370–400 (500) × 250–300 µm (Figures 1f, g), perithecial neck was positively phototropic, 35 (60)–150 µm (Figure 1d). Setae occurred relatively scarcely, they were brown or hyaline, smooth-walled, strait with globose or subglobose apices, 2 × 55 (68) µm (Figure 1c). Asci with truncate apex and small apical rings were unitunicate, aseptate, cylindrical, with a non-amyloid apical thickening (Figures 1h, i, k), 8 spored (Figure 1j), 160–175 × 20 µm in size and formed rosettes (Figures 1h, k). Ascus was elongated to release the ascospores, which were uniseriate, linearly arranged (Figure 1i), first green to pale brown coloured (Figure 1h), broadly ellipsoidal, later brown, smooth-walled with granular contents (Figures 1m, n) without guttules, surrounded by a gelatinous sheath (Figure 1j). As the asci mature, they swell and fill the upper part of the perithecium. One of the asci stretched and pushed through the ostiolar opening, while its base remained attached to the perithecial wall, 50 µm in size (Figure 1e). Mature ascospores were dark-brown with

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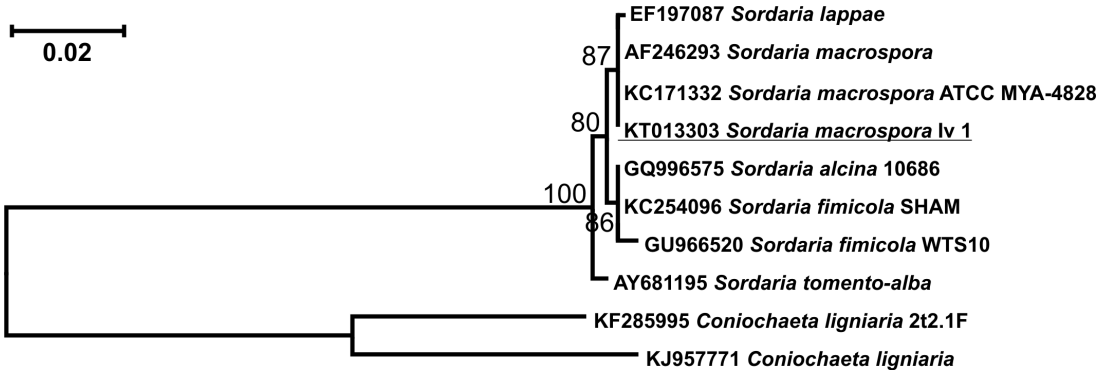

Figure 2. Phylogenetic relationship of *Sordaria macrospora* and related species inferred from internal transcribed spacer nucleotide sequences. The tree was constructed using Neighbor Joining algorithm. The numbers at nodes are bootstrap values after 1000 repetitions. The sequence obtained in this study is underlined

granular contents, 20 (22)–28 × 13–14 (20) µm in size, with a colourless basal germ slit (Figures 1m, o). Paraphyses were absent. These results differ in the size of ascospores, which are circular or elliptical (mill-stone shaped) in *C. malacotricha* and saucer-shaped in *C. ligniaria*.

To confirm the phylogenetic classification of *S. macrospora* Iv 1 isolate, phylogenetic analyses were performed using partial LSU and ITS sequences. *S. macrospora* Iv 1 isolate partial LSU sequence was identical to the LSU sequences of several *Sordaria* species (e.g. *S. macrospora*, *S. fimicola*, and *S. humana*) but showed only 91% similarity to the *Coniochaeta* spp. LSU sequences (data not shown). The ITS sequence of the *S. macrospora* Iv 1 isolate was 100% identical to *S. macrospora* ITS sequences (e.g. isolate ATCC MYA-4828, GenBank accession No. KC171332) and it differed from *S. fimicola* by two positions in ITS1 region. Sequence similarity between ITS sequences of *S. macrospora* Iv 1 and *Coniochaeta* spp. was less than 80%. The phylogenetic analysis placed the ITS sequence of the *S. macrospora* Iv 1 isolate clearly to the *S. macrospora* branch with high bootstrap support (Figure 2) confirming identification based on morphological traits.

DISCUSSION

Wood and needles of the *Pinus* species showing necrosis symptoms are often colonised by different species of fungi. According to the study of fungal species on pine sapwood samples, the most common fungi were *Coniochaeta ligniaria* (Grev.) Cooke (Råberg *et al.* 2007) or *Coniochaeta malacotricha* (Niessl) Trav. [syn. *Rosellinia malacotricha* Niessl, *Sordaria malacotricha* Awd., *Helminthosphaeria malacotricha* Kirschst.] (Arx & Müller 1954; Munk 1957; Kobayashi & Katsu 1970; Checa *et al.* 1988; Kirchner 1998; Fakirova 2004). The first fungus isolated from fruit body on the wood of dead stems of *Picea abies* L. Karst. is characterised by pale to intense salmon to orange colours of cultures, abundant aerial mycelium, and irregularly shaped ellipsoidal conidia. These features distinguish this fungus from *C. malacotricha* with pale colours (nearly white to pale salmon) and sparse to absent aerial mycelium and smaller, 3–6 × 2–4 µm, often ovoidal and apiculate conidia (Weber 2002; Asgari & Zare 2006).

Results achieved by Crous *et al.* (2009) with the fungus *Sordaria macrospora* Auersw. (Sordariales, Sordariomycetes, Ascomycota), which formed a fast-growing culture at first white with abundant aerial mycelium with hyaline, thin-walled, septate, irregularly branched hyphae, absence of conidia, are in accordance to our observations. *S. macrospora* does not produce any conidiospores and is homothallic (self-fertile), what means that no mating partner is needed to develop mature fruiting bodies (Nowrousian *et al.* 2004).

These features distinguish this fungus from *C. malacotricha* the perithecia of which are egg-shaped or conical and gregarious (Chlebicki 1991) or immature without sac and spores (Weber 2002).

According to Crous *et al.* (2009), the fungus *Sordaria macrospora* has fruit bodies ascocarps with narrow opening: perithecia superficial, pear-shaped, dark brown to black, 400–700 µm. Ascocarps wall is composed of several layers of brown, isodiametric cells, often covered with soft hairs on the surface.

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Table 1. Comparison of the morphological characteristics of two *Coniochaeta* species (*C. ligniaria*, *C. malacotricha*) identified on different hosts with signs (symptoms) of a new pathogen *Sordaria macrospora* on *Pinus nigra*

	Host plant				
	Pinus nigra	–	*Pinus sylvestris*	*Pinus sylvestris*	*Picea abies*
Causal agent	*Sordaria macrospora*	*Sordaria macrospora*	*Coniochaeta malacotricha*	*Coniochaeta malacotricha*	*Coniochaeta ligniaria*
Ascomata	*Perithecium pyriform* 370–400(500) × 250–300 µm, neck 35–60(150) µm	Perithecium superficial, pear-shaped, 400–700 µm, ostiolum 30 × 12 µm	Perithecium egg-shaped or conical, gregarious, 230–300 µm diameter, 320–400 µm high	immature perithecia (without sac and spores)	black setose perithecium remaining often immature (without asci and spores)
Setae	brown or hyaline, straight, smooth-walled 55(68) × 2 µm	soft setae	black, shining hairs 50–60 × 3 µm	dark brown, pointed 30–60 × 3.5–4 µm, wall 0.8–1 µm thick	25–65(–70) × 2–4 µm, middle to dark brown, with 0.5–1 µm thick wall, smooth, obtuse or tapering
Paraphyses	absent	absent	paraphyses indistinct	absent	absent
Asci	8 spored, cylindrical, unitunicate, 160–175 × 20 µm	unitunicate, 8-spored cylindrical, uniseriate with a non-amyloid apical thickening	cylindrical, 8-spored, 80–92 × 11–12 µm	absent	absent
Ascospores	uniseriate, brown, 1-celled, granular contents, germ slit, 20 (22)28 × 13(14)–20 µm, surrounded by a gelatinous sheath	brown, ellipsoidal, smooth, surrounded by a gelatinous sheath 25–35 × 17–22 µm, a colourless basal germ pore	circular or elliptical (mill-stone shaped) flat, brown with dark brown large refractive oil drop, 12–13.5 × 9.6–10 × 5.6 µm	absent	almond or lemon shaped, brown, 11–17 × 6-8 × 5–6 µm Germ slit 14 µm
Guttules/oil drops	–	–	large oil drop	2 or some tiny guttules	–
Colonies on PDA	pale white, abundant aerial mycelium	first white, abundant aerial mycelium	–	colonies with pale colours, nearly white to pale salmon-coloured, sparse, no aerial mycelium	aerial whitish, sometimes sparse or lacking, white to yellowish or orange, salmond-coloured, pink or isabelline
Hyphae	4–5 µm, hyaline	hyaline, thin-walled, branched	–	1.5–3.5 (–4) µm	2-4 µm hyaline, multiguttulate, septate, smooth-walled
Conidia	–	–	–	smaller, ovoid and apiculate (2.5–)3–4(–5) × 1–2 µm	ellipsoidal to cylindrical, hyaline, smooth-walled, mostly biguttulate, 1-celled (3–)3.5–6 (–8) × (1)1.5–2.5 µm
Chlamydospora	lacking	–	–	absent	absent
Authors	our experiment	Crous *et al.* (2009)	Chlebicki (1991)	Weber (2002)	Weber (2002)

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Ostiole is erect, up to 150 µm wide. Paraphyses are absent, periphyses lining the ostiole. Unitunicate asci are cylindrical with a non-amyloid apical thickening containing 8 ascospores. These were in one row – uniseriate, brown, ellipsoidal, smooth, surrounded by a gelatinous sheath, with a colourless basal germ pore, 25–35 × 17–22 µm in size. This hyaline, gelatinous sheath, ranging from narrow, irregular and indistinct to prominent, is present in some species of *Coniochaeta*, it is absent in others and not noted in most (Mahoney & La Favre 1981). According to other authors (Petrini & Fischer 1988; Engh *et al*. 2007), fungus *Sordaria macrospora* isolated from *Pinus sylvestris* forms black perithecia embedded in fuzzy white hyphae. Ascospores are mostly dark, ascus bears a distinctive apical pore. No conidia are present. Crous *et al*. (2009) observed ascomata formed after 8–10 days at room temperature under daylight. A gelatinous layer around the ascospores was visible only in water.

Ascospore size and shape are important taxonomic characteristics valuable for distinguishing species, although there is a considerable variation within species. Ascospores of *Sordaria fimicola* are about 12 × 20 µm, those of *S. macrospora* are about 17 × 31 µm, and of *S. brevicollis* are about 10 × 18 µm (Fields 1970).

C. ligniaria has spores broadly spindle or lemon-shaped with tapering ends (Weber 2002), or ellipsoidal to ovoid, giving larger sizes (Mahoney & La Favre 1981; Checa *et al*. 1988). On the wood of *Picea abies* dead stems Weber (2002) detected perithecia of the fungus *C. ligniaria* with ascospores sizing 11–17 × 6–8 × 5–6 µm, tapering or obtuse dark brown setae, 15–40 × 2–5 µm wide in the widest part, wall 0.8–1 µm thick. The key in Asgari *et al.* (2007) shows that *C. ligniaria* ascomata are covered with rigid, pointed setae, ascospores broadly ellipsoidal to subglobose, 9–20 × 8–15 × 4–8 µm. Cannon *et al.* (1985) they occur on wood of coniferous fungus *C. ligniaria* with scattered or aggregated globose ascomata 200–340 µm, covered with black thick-walled setae, usually less than 20 µm long. Asci 154–180 × 10–11 µm were cylindrical, 8-spored, fairly long-stalked, with rounded to truncate apex and conspicuous apical ring. Ellipsoidal, brown, thick-walled ascospores were arranged uniseriately, size (12) 13.5–16.5 × (6.5) 7–9 µm × 6–8 µm, with a prominent germ slit extending over the whole length of one face of the spore.

The results achieved by other authors lead to *C. malacotricha*, except that the ascospores of that species have guttules. Arx and Müller (1954) presented brown ascospores sizing 9–12 × 6–7 µm with dark brown, large defractive oil drop. According to Checa *et al.* (1988), fungus *C. malacotricha* formed pyriform to subglobose ascocarps (270–290 × 200–235 µm) densely covered with aculeate setae. Ascospores were broadly ellipsoidal to subcircular, measuring 11–12 × 9–10 µm. According to other authors (Munk 1957; Kobayashi & Katzu 1970; Rogers & Grand 1971), *C. malacotricha,* occurring on the wood of *Pinus sylvestris,* has millstone-shaped ascospores that are broadly elliptical in face view and narrowly elliptical in side view. Asgari *et al.* (2007) determined ascospores of a similar size of 10–14 × 9–13 × 6–8 µm and ascomata densely covered with setae with a broad base and pointed apex. On bark of coniferous trees Cannon *et al*. (1985) found out scattered, superficial, globose to pyriform ascomata of the fungus *C. malacotricha*, 300–430 µm in size, covered in irregular thick-walled spines to 60 µm long. Asci were not seen. Ascospores were dark brown, strongly flattened, 10.5–13 × 9–10.5 × 6–7.5 µm in size and had the face view oblong to oblong-elliptical and the side view elliptical, with a prominent germ slit completely encircling the spore.

Table 2. Morphometric measurements of ascomata, setae, asci, and ascospores of the fungus *Sordaria macrospora*

	Nitra-Zobor)	Nitra-Chrenová	Nitra- Sihoť	Nitra -Kynek	Boundary value for all localities (µm)
	value/*n* (µm)				
Ascomata	400 × 280	500 × 300	370 × 250	380 × 280	370–400(500) × 250–300
Setae	65 × 2	68 × 2	55 × 2	60 × 2	55(68) × 2
Asci	165 × 20	175 × 20	160 × 20	170 × 20	160–175 × 20
Ascospores	27 × 19	28 × 20	20 × 13	22 × 14	20(22) 28 × 13(14)–20
Hyphae	4.4	5	4	4.7	4–5
Colonies (%)	10	10	10	10	10

n = 30 repetitions of measurement

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The fungus *Sordaria macrospora* formed 10% of the cultivated isolates, followed up by the fungi of the genus *Pinus nigra* (Juhásová *et al.* 2004; Ivanová & Bernadovičová 2010; Pastirčáková *et al.* 2014). An important finding is that this fungus was identified for the first time as a new pathogen in association with the damaged needles of *Pinus nigra* in Slovakia. Comparison of the morphological characteristics of two *Coniochaeta* species (*C. ligniaria*, *C. malacotricha*) identified on different coniferous host trees with signs (symptoms) of the new pathogen *Sordaria macrospora* isolated on *Pinus nigra* is in Table 1. Comparison of morphometric measurements of ascomata, setae, asci, and ascospores of the newly obtained fungus *S. macrospora* is in Table 2.

Both LSU and ITS regions represent information-rich sequences for fungal identification. The LSU region is generally considered less variable than the ITS region, which can limit taxonomic resolution at the species levels (Begerow *et al.* 2010). In our experiment LSU sequences could clearly differentiate between Coniochaeta and Sordaria genera, but not among the *Sordaria* species. ITS sequences, however, clearly differentiated among the *Sordaria* species, and phylogenetic analysis confirmed the classification of *S. macrospora* Iv 1 isolate.

The ITS sequence comparisons of *S. macrospora* Iv 1 isolate against GenBank database indicated that homologous sequences were recovered from multiple plants worldwide, e.g. from *Trifolium subterraneum* in Spain (GenBank accession No. KP698327), from kiwi fruit and *Camellia sinensis* in China (GenBank accession No. KC807208 and No. JQ809681, respectively), or from *Simmondsia chinensis* in the USA (GenBank accession No. KP335242).

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