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Ophiostoma dentifundum sp. nov. from oak in Europe, characterized using molecular phylogenetic data and morphology

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Previous phylogenetic studies based on ITS sequence data have shown that *Ophiostoma* species with *Sporothrix* anamorphs include several species complexes. Isolates from oak in Poland and Hungary, which have previously been referred to as *O. stenoceras*, as well as isolates morphologically similar to *S. inflata* formed the basis of this study. Identification was based on sequences for the ITS region of rDNA operon and partial β-tubulin gene. Analyses showed that isolates from Poland and Hungary reside in a well resolved clade, separate from those in the *O. stenoceras*-complex. The morphology of these isolates was compared with those of strains in the *O. stenoceras* complex and *S. inflata*. Morphological differences in teleomorph and anamorph structures were found between the isolates from Poland and Hungary and those in the *O. stenoceras*-complex. Growth characteristics and the presence of the teleomorph in culture could be used to separate this fungus from isolates in the *S. inflata*-complex. The fungus from Poland and Hungary is described here as *O. dentifundum* sp. nov. It is phylogenetically most closely related to isolates of *S. inflata*, which represent four well defined groups based on morphology and DNA sequence phylogeny.

INTRODUCTION

Ophiostoma species with *Sporothrix* anamorphs are common inhabitants of the phloem and wood of both hardwood and coniferous trees and are frequently isolated from stained wood, worldwide. One common species belonging to this group is *O. stenoceras*, which consists of a complex of several species. At least seven species morphologically similar to *O. stenoceras* have been described. These include *O. abietinum*, *O. albidum*, *O. fusiforme*, *O. lunatum*, *O. narcissi*, *O. nigrocarpum* and *O. ponderosae*. Recent phylogenetic analyses based on DNA sequence data have shown that these *Ophiostoma* species group in different complexes. Despite slight morphological differences between these taxa (Mathiesen-Käärik 1953, 1960, Aoshima 1965, Griffin 1968), SSU and ITS/5.8S sequences of the rDNA operon have shown that *O. albidum* and *O. ponderosae* are synonyms of *O. stenoceras* (De Beer *et al.* 2003). *O. narcissi*, *O. fusiforme* and *O. lunatum* are relatively closely related to *O. stenoceras*, while *O. abietinum* and *O. nigrocarpum* are very distantly related to this species (Pipe, Brasier & Buck 2000, de Beer *et al.* 2003, Aghayeva *et al.* 2004). All of the above mentioned species produce only *Sporothrix* anamorphs, which makes them more similar to each other than to species having other anamorphs or synanamorphs such as *Leptographium* and *Pesotum*.

The genus *Sporothrix* represents a heterogeneous and polyphyletic assemblage of anamorphs of endomycetes, true ascomycetes and phragmobasidiomycetes (de Hoog 1993). *Sporothrix* species, with the type species *S. schenckii*, form simple structures with conidiogenous cells proliferating sympodially and giving rise to a single conidium at each successive conidiogenous locus (de Hoog 1974, 1993). Various studies, including those based on DNA sequence data, have confirmed the relationship between *Ophiostoma* and *Sporothrix* species and that *Sporothrix s. str.* resides in the ascomycetes (Weijman & de Hoog 1975, Berbee & Taylor 1992, Middelhoven, Guého & de Hoog 2000, de Beer *et al.* 2003). The recent description of two new *Ophiostoma* species with *Sporothrix* anamorphs has

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confirmed that fungus known as *O. stenoceras*, represents several closely related species (Aghayeva *et al*. 2004).

In this study we consider a collection of two *Ophiostoma* isolates from oak in Poland and Hungary with a distinct *Sporothrix* anamorph. These isolates are compared with other similar fungi based on morphological differences and sequence data for the ITS regions including the 5.8S gene of the ribosomal DNA operon and the partial β-tubulin gene.

MATERIALS AND METHODS

Isolates examined

Strains used in this study originated from Asia, Europe, North and South America, and South Africa (Table 1). Eleven isolates from different geographical locations and different substrates were compared with *Ophiostoma fusiforme*, *O. lunatum*, *O. stenoceras*, *Sporothrix schenckii* and *Sporothrix* sp. Two strains (CMW 13016, CMW 13017) resembling and treated as *O. stenoceras* in previous studies (Kowalski & Butin 1989, Kowalski, pers. comm., Pipe *et al*. 2000) were shared with us by Clive M. Brasier (Forestry Commission Research Agency, Alice Holt Lodge, Farnham, Surrey, UK) and Claude Delatour (Forest Pathology Laboratory, INRA Champenoux, Champenoux, France) as well as Tadeusz. Kowalski (Department of Forest Pathology, Hugo Kołłątaj University of Agriculture, Kraków, Poland). *S. inflata* isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht). Dried reference specimens linked to key cultures have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa. All isolates are stored in the fungal genetic resources collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative strains have also been deposited at the CBS.

DNA sequence comparisons

DNA was isolated from single-ascospore or single-conidial isolates as previously described by Aghayeva *et al*. (2004). The internal transcribed spacer regions (ITS1 and ITS2), including the 5.8S gene, were amplified using primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al*. 1990). Amplicons were also obtained for the β-tubulin gene using primers Bt2A and Bt2B (Glass & Donaldson 1995). The reaction mixture was the same for both amplified regions and the PCR reactions and conditions for ITS1 and 2 regions, including the 5.8S gene were as described in Aghayeva *et al*. (2004). The partial β-tubulin gene was amplified using an initial denaturation step of 1 min at 94 °, followed by 30 cycles of denaturation for 1 min at 94 °, primer annealing for 1 min at 50–55 ° (primers annealed at different temperatures depending on isolate), a chain elongation step of 1 min at 72 °, and a final elongation step of 5 min at 72 °, followed the 30 amplification cycles. PCR products were electrophoresed on a 1% (w/v) agarose gel containing ethidium bromide and amplicons were visualized under UV light. PCR fragments were purified by gel filtration through Sephadex G50 columns (Sigma-Aldrich, Chemie, Steinheim, Germany) to remove unincorporated dye-labelled nucleotides.

PCR products were sequenced using the above-mentioned primers and the ABI Prism® BigDye™ Terminator v3.0 Ready Reaction Cycle sequencing kit (Applied Biosystems, Foster City, CA). DNA sequencing reactions were run on the ABI PRISM®3100 Genetic Analyser or the ABI PRISM® 377 DNA sequencer. Data were edited in Sequence Navigator. All phylogenetic analyses were performed using PAUP v. 4.0 (Swofford 2002). A partition homogeneity test was conducted to determine, if sequence data of the two gene regions could be combined. A heuristic search using TBR branch swapping (MULPAR on) was applied to determine the most parsimonious trees. Trees were rooted using *S. schenckii* as an outgroup taxon. Confidence levels of the branching points were determined using 1000 bootstrap replicates.

Morphology

Strains were grown on 2% malt extract agar (MEA; 20 g l^{-1} malt extract Biolab, Merck, Midrand, South Africa; 20 g agar Biolab, Merck; 1000 ml dH_2O). Cultures were incubated at room temperature for 10 days. Teleomorph and anamorph structures were mounted on glass slides in lactophenol and examined using phase contrast microscopy. 50 measurements were made from each taxonomically informative structure from the isolate that was chosen to define the new species. For *Sporothrix inflata*, 25 measurements were made for each isolate. Three-day-old slide cultures (Riddell 1950), mounted in lactophenol, were prepared to study the anamorph structures. Colony colours were determined using the charts of Rayner (1970).

Growth in culture was studied on 2% MEA (Table 2). Discs (5 mm diam) were taken from the margins of one-week-old cultures and transferred to the centres of each of three 90 mm Petri dishes containing 20 ml 2% MEA. Growth comparisons were conducted at temperatures ranging from 5–35 ° at 5 ° intervals for 10 d in the dark. Measurements of colony diameter were made for each isolate by taking two readings from each of three replicate plates each day, commencing on the second day. Growth of strains was compared based on average colony diameters computed from the three replicate plates per isolate. Isolates that did not grow at low or high temperatures were subsequently maintained at 25 ° to test their viability.

Ten single ascospore and ten single conidial cultures were prepared for the two *Ophiostoma* sp. isolates from Poland and Hungary (CMW 13016, CMW 13017) to

Table 1. Isolates of *Ophiostoma* and *Sporothrix* used in this study.

Species	Isolate no.[a]	Other no.[b]	Substrate	Origin	Collector or supplier	Growth[f]	GenBank accession no.	
							ITS	β-tubulin
O. fusiforme	CMW 9968[c]	CBS 112912	*Populus nigra*	Azerbaijan	*D. N. Aghayeva*	T	AY280481	AY280461
	CMW 8281	CBS 112909	*Castanea sativa*	Azerbaijan	*D. N. Aghayeva*	NT	AY280482	AY280462
	CMW 8285	CBS 112910	*Castanea sativa*	Azerbaijan	*D. N. Aghayeva*	NT	AY280483	AY280463
	CMW 7131	CBS 112925, HA 206	*Quercus petraea*	Austria	*E. Halmschlager*	T	AY280497	AY280464
	CMW 10565	CBS 112926, CTK 102	*Larix deciduas*	Austria	*T. Kirisits*	T	AY280484	AY280465
O. lunatum	CMW 10563[c]	CBS 112927, CTK 101	*Carpinus betulus*	Austria	*T. Kirisits*	T	AY280485	AY280466
	CMW 10564	CBS 112928, IC/P/CH/1	*Larix deciduas*	Austria	*T. Kirisits*	T	AY280486	AY280467
Sporothrix sp.	CMW 1468	C 1211	*Dendroctonus ponderosae*	Canada	*Y. Hiratsuka, Y. Yamaoka*	T	AF484457	AY280468
	CMW 109	C 232	*Pinus echinata*	USA	*F. Hinds*	T	AY280487	AY280469
	CMW 110	C 235	*Pinus echinata*	USA	*F. Hinds*	T	AY280488	AY280470
O. stenoceras	CMW 3202[c]	C 1188, CBS 237.32	Pine pulp	Norway	*H. Robak*	T	AF484462	AY280471
	CMW 2344	–	*Eucalyptus smithii*	South Africa	*G. H. Kemp*	T	AY280491	AY280472
	CMW 2524	–	*Acacia mearnsii*	South Africa	*Z. W. de Beer*	NT	AF484459	AY280473
	CMW 11192	C 965	Sap	New Zealand	*R. Farrell*	NT	AY280492	AY280474
	CMW 11193	C 966	Wood	New Zealand	*R. Farrell*	NT	AY280493	AY280475
Ophiostoma sp.	**CMW 13016[d]**	CBS 115790, H 2133	*Quercus* wood	Hungary	*C. Delatour*	T	AY495434	AY495445
	CMW 13017[d]	CBS 115865, 40 C2, 13976	*Quercus* robur	Poland	*T. Kowalski*	T	AY495435	AY495446
S. inflata	**CMW 12526**	CBS 156.72	Greenhouse soil	The Netherlands	*H. Kaastra-Höweler*	T	AY495425	AY495436
	CMW 12527[c]	CBS 239.68	Wheat-field soil	Germany	*W. Gams*	T	AY495426	AY495437
	CMW 12528	CBS 427.74	*Lilium* sp.	The Netherlands	*G. J. Bollen*	T	AY495427	AY495438
	CMW 12529[e]	CBS 553.74	Soil	Canada	*R. A. A. Morall*	T	AY495428	AY495439
	CMW 12531	CBS 792.73	Soil	Chile	*J. Grinbergs*	NT	AY495429	AY495440
	CMW 12532	CBS 793.73	Meadow soil	Germany	*A. von Klopotek*	T	AY495430	AY495441
	CMW 12535	CBS 841.73	Soil	Chile	*J. Grinbergs*	NT	AY495431	AY495442
	CMW 12536[d]	CBS 110895	*Quercus petraea*	Austria	*E. Halmschlager, T. Kowalski*	T	AY495432	AY495443
	CMW 12537[d]	CBS 110896	*Quercus robur*	Austria	*E. Halmschlager, T. Kowalski*	T	AY495433	AY495444
S. schenckii	CMW 7612	MRC 6862	Human sporothrichosis	South Africa	*H. F. Vismer*	NT	AY280494	AY280476
	CMW 7614	MRC 6867	Human sporothrichosis	South Africa	*H. F. Vismer*	T	AY280495	AY280477
	CMW 7615	MRC 6956	Human sporothrichosis	South Africa	*H. F. Vismer*	T	AY280496	AY280478

[a] CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

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[c] Ex-type culture.

[d] Isolate used in previous studies (Kowalski & Butin 1989, Kowalski, pers. comm., Pipe *et al.* 2000, Halmschlager & Kowalski 2003).

[e] This ex-type culture of *Humicola dimorphospora*.

[f] Growth: T, tested and NT, not tested.

Taxa presented in **bold** represent those for which sequences were generated in this study.

Table 2. Characters distinguishing the species of *Ophiostoma* with *Sporothrix* anamorphs compared in this study.

	O. fusiforme Aghayeva *et al.* (2004)	*O. lunatum* Aghayeva *et al.* (2004)	*O. dentifundum* this study	*Ophiostoma stenoceras* Robak (1932)
Perithecia	Starting to form in about 30 d, maturing quickly, sometimes with 2 necks	Starting to form in about 40 d, maturing quickly, rarely with 2 necks	Starting to form in about 25–30 d, maturing quickly	Starting to form in about 1 wk, maturing after week. Abnormal specimens have up to five necks in agar
Base diam	121.5–274 μm	59.5–178(–205) μm	(122–)153–216(–261) μm	130–250 μm
Neck				
Length	301.8–985(–1168) μm	162–554(–700) μm	(439–)567–1345(–1571) μm	420–1500 μm
Width at base	21.8–33.7(–45) μm	15–33(–40) μm	(20–)24–36(–41) μm	20 μm
Width at apex	9.1–13.5(–18) μm	7–11(–14) μm	(7–)10–17(–20) μm	8–12 μm
Ornamental hyphae at the base				
Length	16.6–94.5(–143) μm	11.9–106.8 μm	(24–)41–64(–90) μm	–
Width	1.7–.2(–2.5) μm	1–2.5(–4) μm	1–2 μm	
Ostiolar hyphae				
Length	23.4–68.4(–96) μm	14–60(–62) μm	(18–)20–52(–55) μm	20–48(–60) μm
Width	1–2(–3) μm	1–1.5(–2.5) μm	1–2.5 μm	2.5–3 μm
Ascospores				
Length	3.4–4.3(–5.5) μm	3–4 (4.5) μm	(2–)2.5–3.5(–4) μm	2–3 μm
Width	1–1.5(–2) μm	1–1.5 μm	1–1.5 μm	1–1.5 μm
Colour of culture	White, becoming dull white or blackish after formation of perithecia	White, becoming dull white or blackish after formation of perithecia	At first hyaline, later white or white with black dots of perithecia	Greyish or white, first slightly domed, later quite flat
Colony diam*	33.5 mm*	31.5 mm*	31.3 mm*	29.9 mm*
Texture of culture	Smooth, finely floccose, aerial hyphae present	Smooth, finely floccose, aerial hyphae present	Smooth, finely floccose, aerial hyphae present	Quite flat, aerial hyphae rarely occurring, surface covered by a thick layer of conidia, giving the cultures a yeast-like or porridge-like appearance
Conidiogenous cells	14.5–54(–72) × (1–)1.5–2 μm	11.5–35(–59.5) × 1–1.5 μm	(20–)23–85(–90) × 0.5–1.5 μm	–
Denticles	Sharp 0.5–1 μm	Two types: (1) inconspicuous 0.5 μm; (2) cylindrical, up to 4.5 μm	Distinct 0.5–1 μm	–
Conidia	Guttuliform to fusiform	Curved, crescent shape	Fusiform, sometimes slightly curved with pointed (toothed) base	Broadly ellipsoidal, basally pointed
Length	3.5–6(–8) μm	2–5(−6) μm	(4–)4.5–7.5(–10) μm	3.5–7 μm
Width	1–2 μm	1–1.5 μm	1–1.5 μm	2–3.5 μm

* Measurements of colony diam after 10 d growth on MEA at 25 ° in the dark.

reveal their modes of sexual reproduction. Isolates producing perithecia were considered as self fertile.

RESULTS

DNA sequence comparisons

The aligned sequences for the ITS region resulted in a data set of 563 characters, of which 426 characters were constant, 132 were parsimony-informative and 5 variable characters were parsimony-uninformative. Approximately 546 bp were amplified for most strains. The sequence data set for the two *Ophiostoma* isolates (CMW 13016 and CMW 13017) was of 536 bp in size. The sequenced fragments for *Sporothrix inflata* isolates were of different size. Sequences for the β-tubulin data set were more variable and sequenced fragments were approximately 288–542 bp in size, depending on the isolate. Four most parsimonious trees, all with the same general topology, were retained for both data sets. Results of the partition homogeneity test showed that the sequenced regions of ITS1/2, including 5.8S and partial β-tubulin genes could be combined ($P = 0.580$). Thus, the combined data set contained a total of 870 characters, of which 561 were constant, 305 were parsimony-informative, and four were parsimony-uninformative. Two most parsimonious trees were retained and the tree length was 534 steps (consistency index/CI = 0.835, retention index/RI = 0.961, homoplasy index/HI = 0.165). The general topology of the trees was the same and one of these is presented in Fig. 1.

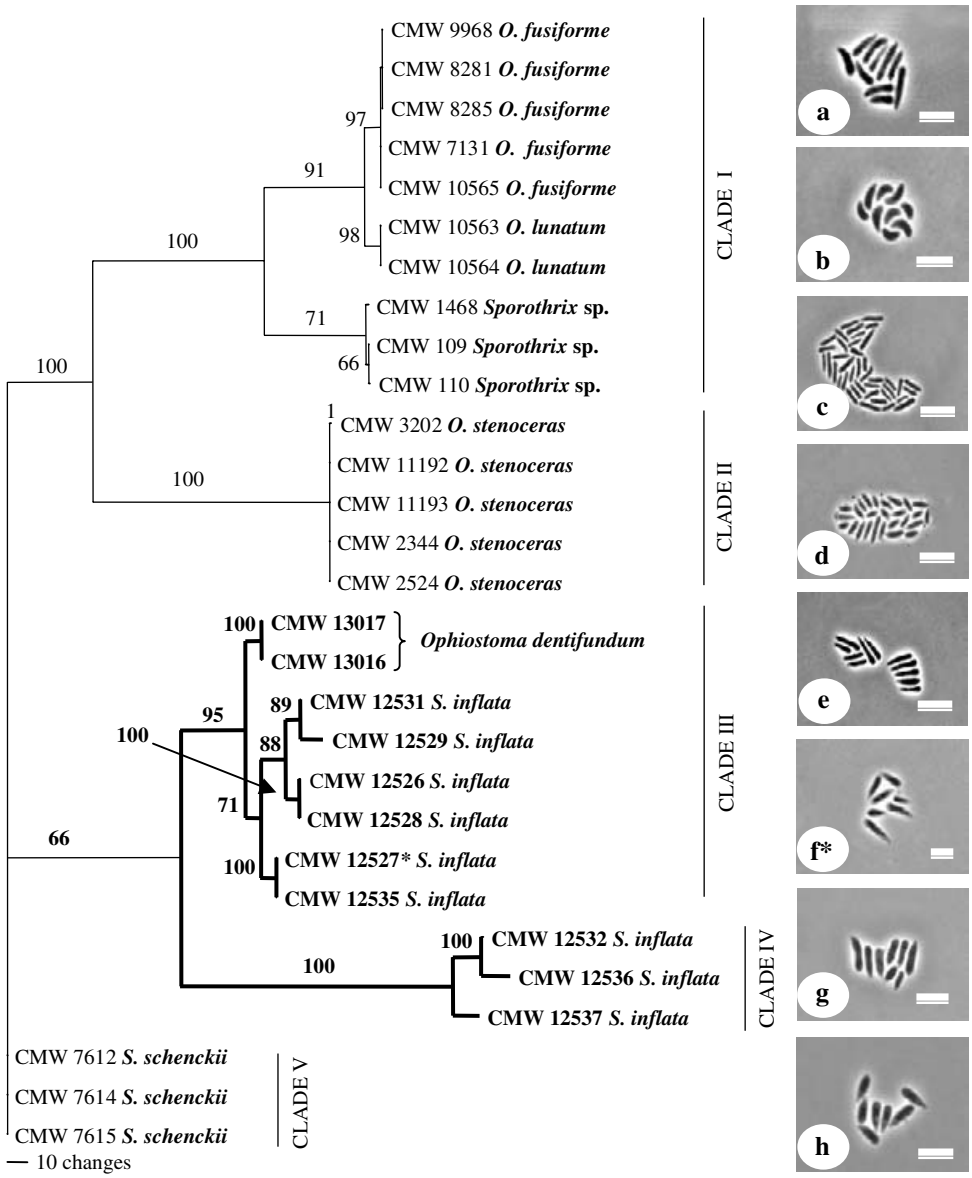

Fig. 1. One of the two most parsimonious trees obtained by a heuristic search of the combined data set of the 5.8S gene, including ITS1/2 rRNA operon regions, and the partial β-tubulin gene (534 steps, CI = 0.835, RI = 0.961, HI = 0.165). Bootstrap values, determined after 10^3 replications are indicated at the branching points. Bold lines indicate isolates primarily used in this study. *The ex-type isolate of *Sporothrix inflata*. *S. schenckii* was used as outgroup. Conidia of the taxa are shown right of the respective clades: a, CMW9968; b, CMW10564; c, CMW1468; d, CMW3202; e, CMW13017; f, CMW12527 *; g, CMW12536 and h, CMW7614. Bars = 5 μm.

The phylogram generated based on the combined data revealed five major clades. Clade I had 100% bootstrap support and contained three subclades (Fig. 1a–c). This clade clearly represented three distinct but closely related species, including *O. fusiforme* (CMW 9968, CMW 8281, CMW 8285, CMW 7131, CMW 10565), *O. lunatum* (CMW 10563, CMW 10564) and a *Sporothrix* species (CMW 1468, CMW 109, CMW 110). Clade II (Fig. 1d) included isolates of *O. stenoceras* (CMW 3202, CMW 11192, CMW 11193, CMW 2344, CMW 2524). This fungus is relatively closely related to the *Ophiostoma* species in Clade I, based on sequence data and morphological characteristics previously presented (Aghayeva *et al.* 2004) and supported in the present study (Fig. 1a–d). Clade III and Clade IV (bootstrap support 95% and 100% respectively) are distant from the first two clades in the phylogenetic tree. Clade III incorporated four well-supported subclades (Fig. 1e–f). The first subclade included the two *Ophiostoma* isolates (CMW 13016, CMW 13017, Fig. 1e) from oak in Poland and Hungary and is strongly supported (bootstrap, 100%). The other three subclades contained isolates of *S. inflata*, one from Canada (CMW 12529), two from Chile (CMW 12531, CMW 12535), one from Germany (CMW 12527, Fig. 1f) and two from The Netherlands (CMW 12526, CMW 12528). One of two Chilean isolates (CMW 12531) grouped with an isolate from Canada (bootstrap value 89%) in the second subclade. Both isolates from The Netherlands grouped together

CLADES	EXON	INTRON C	EXON	INTRON D	EXON	INTRON E	EXON
I	28	-	42	-	54	57	130
II	28	-	42	-	54	61	130
III	28	94–96	42	-	54	66–67	130
IV	28	115, 123, 116	42	57–58	54	66	130
V	28	86	42	-	54	79	130

Fig. 2. Map of partial β-tubulin gene. Black boxes indicate the introns, and striped boxes indicate the exons. Labelled arrows, Bt2a & Bt2b above the diagram indicate the primers used for PCR amplification and sequencing. The lengths of the exons and introns are indicated in base pairs below the diagram. −, missing introns.

with 100 % bootstrap value in the third subclade. The ex-type isolate of *S. inflata* from Germany (CMW 12527, Fig. 1f) and the other isolate from Chile (CMW 12535) resided in the fourth subclade (bootstrap, 100 %). Clade IV included an isolate of *S. inflata* from Germany (CMW 12532) and two from Austria (CMW 12536, CMW 12537, Fig. 1g) and this clade also had strong statistical support (bootstrap, 100 %). Clade V included three isolates of *S. schenckii* (CMW 7612, CMW 7614, CMW 7615, Fig. 1h), which were used as outgroup in this and a previous study (Aghayeva *et al.* 2004).

The Bt2 primer set amplified fragments that varied in size. These polymorphisms result from size variations in the DNA sequence of this region. The number, position and size of introns varied between the different isolates (Fig. 2). Positions of the introns were determined by comparing putative amino acid codons derived from the DNA sequence data, with the published amino acid sequence for the β-tubulin protein data set of *Neurospora crassa* and other eukaryotes (Glass & Donaldson 1995).

Sequences of the isolates in Clade I included only one intron (Intron E), which was 57 bp in size. Clade II, represented by *O. stenoceras*, included isolates with a single intron (Intron E), 61 bp in size. The sequence data obtained for isolates in Clade III differed from those of the two previous Clades in that another intron (Intron C) was present in addition to Intron E. Intron C was approximately 95 bp in *Ophiostoma* sp. and 94–96 bp long in *S. inflata*, Intron E was 67 bp in *Ophiostoma* sp. and ranged from 66–67 bp in size in isolates of *S. inflata*. The sequences of other *S. inflata* isolates residing in Clade IV contained a third intron (Intron D) of 57–58 bp (depending on the isolate) residing within the second exon. The sequences of outgroup isolates in Clade V were similar to those in Clade III, having two introns (Intron C and E). The additional intron regions are probably the result of single deletion or insertion events and were, therefore, excluded from the final DNA data set. Thus only the exon sequences and the sequence of intron E were used for DNA sequence analysis.

Morphological comparisons

Ophiostoma isolates from Poland and Hungary residing in the first subclade of Clade III, were previously referred to as *O. stenoceras* (Kowalski & Butin 1989, Kowalski, pers. comm., Pipe *et al.* 2000), but they were found to represent a distinct taxon (Fig. 3). This fungus has a teleomorph that is similar to that of *O. stenoceras* and an anamorph that is similar to *O. fusiforme*. Isolates were self-fertile and formed perithecia in single conidial and single ascospore cultures. Perithecial necks were similar to those of *O. stenoceras* but substantially longer than those in *O. fusiforme* and *O. lunatum* (Table 2). The widths of necks at the base were wider in the isolates from Poland and Hungary. These isolates also differed slightly from other species in ascospore size: the ascospores were larger than those of *O. stenoceras*, but smaller than those of *O. fusiforme* and *O. lunatum*. Anamorph characteristics, such as the shape and size of conidiogenous cells and conidia, resembled those found in *O. fusiforme*. In these isolates, protoperithecia began to form later than those in *O. stenoceras* isolates, but they also matured rapidly on MEA.

Colony morphology for all *S. inflata* isolates used in this study was very similar in 10 d old cultures. Typical colonies were white, floccose and smooth. However, *S. inflata* isolates differed from each other in colony texture, colour as well as in shape and size of conidia in 20 d old cultures. At this age, CMW 12531 from Chile residing in the second subclade of Clade III was olive-gray (23′′′′) to olivaceous black (21′′′m) with a floccose mycelium. Isolate CMW 12529 from Canada was creamy (19′f), to brown-olivaceous, with smooth mycelium and tufts at the centre.

The shape of the conidiogenous cells and conidia differed slightly between isolates CMW 12531 and CMW 12529. In CMW 12531, conidiogenous cells were

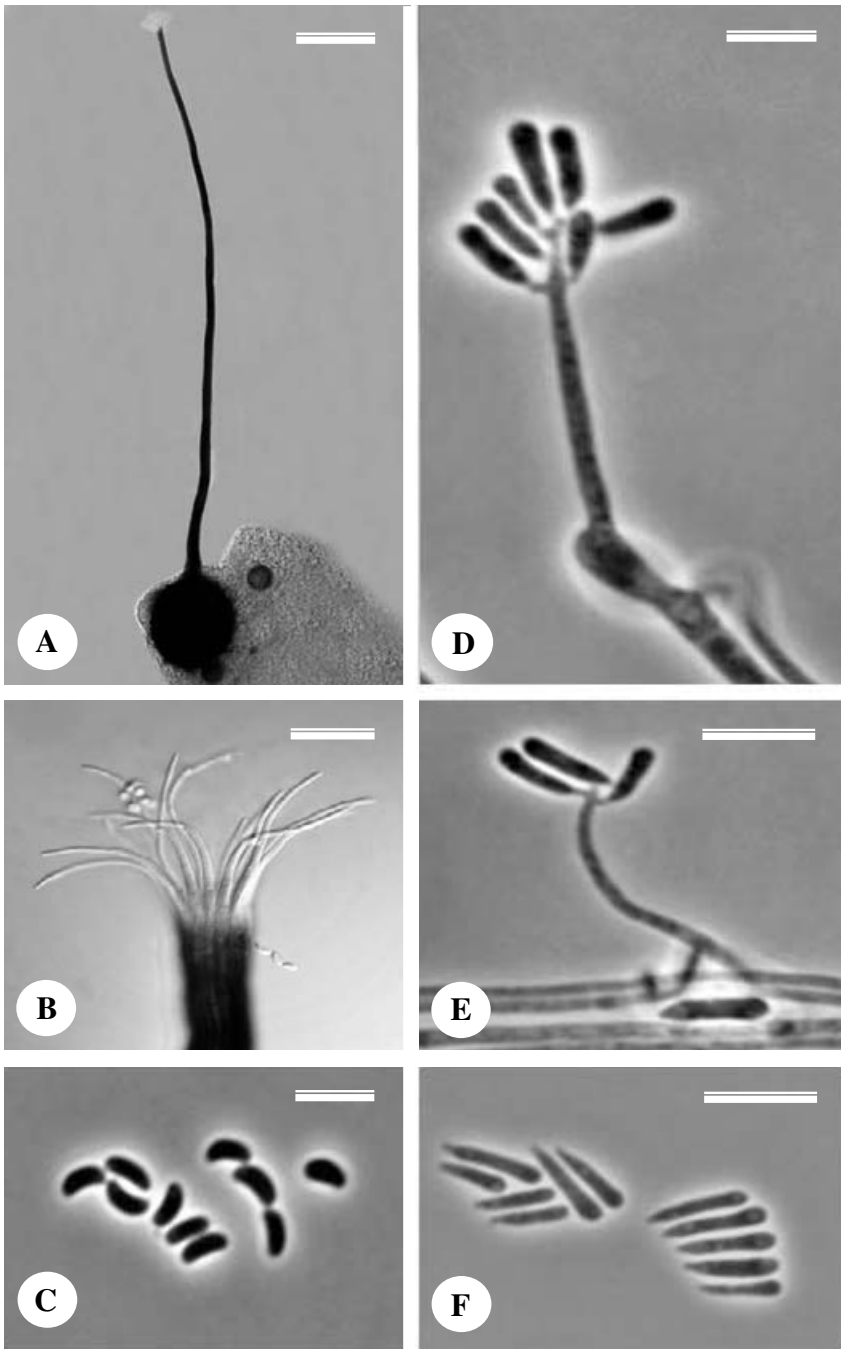

Fig. 3. Morphological characteristics of *Ophiostoma dentifundum* (PREM 57850, CMW 13016) (A) perithecium with globose base, and ornamental hyphae at the base and long neck; (B) ostiolar hyphae; (C) allantoid ascospores in side view; (D–E) conidiogenous cell with conidia; (F) conidia. Bars: A = 100 μm, B, D, E, F = 10 μm; and C = 5 μm.

scattered, arising laterally or terminally from hyphae and they were often monoblastic. Denticles were very rare and hardly visible. Conidia were oblong, somewhat clavate, and formed by sympodial growth of the apex of the conidiogenous cells. CMW 12529 produced conidiogenous cells that were straight or occasionally slightly undulating, with the apical part consisting of a cluster of blunt denticles. Conidia were hyaline, primarily globose to obovoid, later obovoid to oblong with short, blunt basal scars.

Isolates from The Netherlands (CMW 12526 and CMW 12528) residing in the third subclade of Clade III had colonies that were creamy, yellowish (19d), floccose, and somewhat lanose. Conidiogenous cells and conidia were similar in shape and size in these two isolates. Conidiogenous cells were long, straight, and undulating; denticles were small and rare. Conidia were fusiform with pointed bases. The ex-type strain of *S. inflata* (CMW 12527) and the isolate from Chile (CMW 12535) residing in the fourth subclade had colonies that were white, olivaceous grey (23′′′′f). Conidiogenous cells and conidia in these isolates were similar in shape and size, and their morphology was consistent with the description of *S. inflata* by de Hoog (1974).

The *S. inflata* isolates (CMW 12532, CMW 12536, CMW 12537) in Clade IV were different in culture colour and texture, but the shape and size of the conidiogenous cells and conidia was similar. The colony of CMW 12532 was creamy brownish 19″i, later greyish sepia 17i or greyish olive 21‴, floccose with few concentric rings at the edges (rarely observed at 20 °). CMW12536 had a colony that was grey or creamy grey, with concentric rings of different colours: izabella 19″I, hazel 11′K, or olivaceous 23‴″b, lanose at the centre, and smooth towards the edges. The colony of CMW12537 was greyish olive 21‴, hazel 11′K, to olivaceous, later with small white patches of farinaceous mycelium. Dark, thick-walled conidia referred to as the '*Humicola*-type' by Halmschlager & Kowalski (2003) were also observed rarely in CMW 12532 and CMW 12536.

Growth in culture of isolates of *O. fusiforme*, *O. lunatum*, *O. stenoceras*, as well as the two isolates (CMW 13016, CMW 13017) of the unknown *Ophiostoma* sp. from Hungary and Poland, was very similar at 25 °. Isolates treated in this study as representing *S. inflata* differed in growth characteristics. *S. inflata* isolate CMW 12529 residing in the second subclade of Clade III grew best at 25 °, isolates (CMW 12526, CMW 12527, CMW 12528) residing second and third subclade grew best at 20 °, while those in Clade IV (CMW 12532, CMW 12536, CMW 12537) grew most rapidly at 25 °. The growth of *S. inflata* isolates CMW 12526 and CMW 12528 was slower than that of other isolates and reached only 21 mm diam in 10 d at 20 °.

TAXONOMY

Isolates from Hungary and Poland treated in this study clearly represent a distinct taxon, which we describe here as new:

Ophiostoma dentifundum Aghayeva & M. J. Wingf., **sp. nov.** (Fig. 3)

Etym.: *denti-* (Latin, tooth), and *-fundum* (base), referring to the toothed bases of the conidia.

Coloniae *in vitro* in MEA ad 25 ° in 10 diebus in tenebris diametrum medium 31.4 mm attingunt. Non crescunt infra 10 ° et supra 30 °. Mycelium aerium primo plerumque laeve, mox subtiliter floccosescens. Coloniae promi hyalinae, deinde albae vel cum punctis nigris peritheciorum, pagina inferiori sine colore, sine odore. Perithecia post 25–30 dies evoluta, superficialia vel partim in substrato (agaro) inclusa, sparsa. Bases globosae, nigrae, (122–)153–216(–261) μm diametro, pilis hyphalibus brunneis vel nigris, (24–)41–64(−90) × 1–2 μm. Collum basin versus niger, (439–)567–1345(–1571) μm longa, basi (20–)24 × 36(–41) μm, apice (7–)10 × 17(–20) μm. Hyphae ostiolares multae, hyalinae, divergentes, (18–)20–52(–55) × 1–2.5 μm. Ascosporae hyalinae, unicellulares, lateraliter visae allantoideae vel reniformes, (2–)2.5–3.5(–4) × 1–1.5 μm. Cellulae conidiogenae hyalinae, septatae, sparsae (20–)23–85(90) × 0.5–1.5 μm. Conidia recta in denticulis, holoblastica, hyalina. unicellularia, fusiformia (4–)4.5–8(10) × 1–1.5 μm.

Typus: **Hungary**: wood of *Quercus* sp., 1990, *C. Delatour* (PREM 57850 – holotypus; *cultura viva* CMW 13016, H 2133, CBS 115790).

Anamorph: *Sporothrix* sp.

Colonies in vitro on MEA attaining an average diameter of 31 mm in 10 d at 25 ° in the dark. No growth below 10 ° or above 30 °. Aerial mycelium at first usually smooth, soon becoming finely floccose. Colonies at first hyaline, later white or white with black groups of perithecia, reverse without distinct colour, aroma absent. *Perithecia* (Fig. 3a) developing after 25–30 d, superficial or partly embedded in substrate/agar, numerous, scattered. Bases globose, black, (122–)153–216(–261) μm diam; ornamented with brown to black, septate, thin- to thick-walled, unbranched hyphal hairs of variable length, (24–)41–64(–90) × 1–2 μm. Necks black at base, brown at apex, hyaline just below the ostiolar hyphae, straight or slightly curved (439–)567–1345(–1571) μm long, (20–)24–36(–41) μm wide at base, (7–)10–17(–20) μm at tip. *Ostiolar hyphae* (Fig. 3b) numerous, hyaline, divergent, (18–)20–52(–55) × 1–2.5. *Ascospores* hyaline (Fig. 3c), 1-celled, allantoid to reniform in side view, (2–)2.5–3.5(–4) × 1–1.5 μm.

Conidiogenous cells (Fig. 3d, e) micronematous, mononematous, hyaline, septate, scattered, arising ortho- or slightly plagiotropically from hyphae in terminal or side branch positions, (20–)23–85(–90) × 0.5–1.5 μm, apical part forming conidia by sympodial growth, denticles 0.5–1 μm long, occasionally proliferating below the apex and giving rise to a secondary conidiogenous cell. *Conidia* (Fig. 3f) produced directly on denticles, arising sympodially, holoblastic, hyaline, 1-celled, fusiform, apices rounded, conidia slightly swollen at points of attachment, tooth-like, with pointed bases, sometimes slightly curved at the base (4–)4.5–7.5(–10) × 1–1.5 μm, formed singly.

Additional specimen examined: **Poland**: Oberschlesien, forest district Wierklaniec, on *Quercus robur*, 24 June 1990 [reisolation from a living *Q. robur* tree, which had previously been inoculated with the original strain, isolate no. 13976 (fungal genetic resources collection, Department of Forest Pathology, Hugo Kołłątaj University of Agriculture, Kraków, Poland) obtained from an ascospore mass from perithecia occurring on the wood of a dead *Q. robur* tree, near Tscenstocchau, forest district Herby, 23 Aug. 1986, *T. Kowalski*], (PREM 57849; living cultures CMW 13017, 40 C2, CBS 15865).

DISCUSSION

This study has shown that isolates previously treated as *Ophiostoma stenoceras* represent several related but distinct species. This is consistent with reports showing that *O. stenoceras* represents a complex of species (Pipe *et al.* 2000, Aghayeva *et al.* 2004). Morphological characteristics as well as DNA sequence comparisons

for two gene regions enabled us to show that isolates from oak in Poland and Hungary represent an undescribed species of *Ophiostoma* with a *Sporothrix* anamorph. This species, *O. dentifundum*, is phylogenetically most closely related to *S. inflata* and both of these species are phylogenetically distant from taxa residing in the *O. stenoceras*-complex. We have also shown, based on phylogenetic comparisons and morphological characteristics that isolates previously treated as *S. inflata* from different parts of the world and different substrates, represent several distinct lineages.

O. dentifundum was originally isolated from *Quercus* sp. and treated as *O. stenoceras*, which has been considered a common sapstain fungus on oak in Europe (Kowalski & Butin 1989, Pipe *et al.* 2000). The recognition of species morphologically similar to *O. stenoceras*, but distinct from it in this and a previous study (Aghayeva *et al.* 2004), raise questions regarding reports of *O. stenoceras* from oak in Europe (Kowalski & Butin 1989, Kirschner 1998, Pipe *et al.* 2000). It is unclear whether such reports refer to *O. dentifundum*, *O. fusiforme*, or possibly *O. lunatum*. Further surveys are required to resolve this question and to fully document the incidence and relative frequency of *Ophiostoma* species with *Sporothrix* anamorphs on oak in Europe. Thus far, *O. dentifundum* is only known from the two isolates examined in this study, and the ecology of this fungus, in particular its hosts and vectors, if any, require attention in future investigations. Based on the recent recognition of three new species superficially resembling *O. stenoceras* in this and a previous study (Aghayeva *et al.* 2004), it is likely that there are more cryptic species in the *O. stenoceras* complex in different ecological niches in various parts of the world.

The anamorph of *O. dentifundum* has conidiogenous cells and conidia that are very similar to those of *O. fusiforme*, and they somewhat resemble *O. stenoceras*. The conidia of *O. dentifundum* are fusiform to broadly ellipsoidal with pointed bases that are tooth-like. The apices of the conidia are slightly swollen, unlike those of *O. fusiforme* and *O. stenoceras*.

The teleomorph of *O. dentifundum* most closely resembles *O. stenoceras* in shape and size of the ascomatal bases and necks. The ascospores are slightly smaller than those of *O. fusiforme*, but longer than those of *O. stenoceras*. Phylogenetic comparisons showed that *O. dentifundum* is different to other *Ophiostoma* spp. with *Sporothrix* anamorphs considered in this study. The new species did not group with any of these *Ophiostoma* spp., but was closely related to *S. inflata*, which resides in a separate clade.

This study has shown that isolates previously treated as *S. inflata* reside in four different groups, supported by high bootstrap values. One of the suclades in *S. inflata s. lat.* included two isolates, the ex-type strain of *S. inflata* from Germany and an isolate from Chile; we consider these two isolates to represent *S. inflata s. str.* Other isolates from Chile, Canada, and The Netherlands residing in two neighbouring subclades had a culture morphology and growth rate different to that of *S. inflata s. str.* The *S. inflata* isolates in Clade IV from Germany and Austria were similar in growth rate but differed from the other *S. inflata* isolates in optimum temperatures for growth. Dark, thick walled conidia were also occasionally observed in these isolates; such conidia previously led to the description of the fungus as *Humicola dimorphospora* (Roxon & Jong 1974). '*Humicola*-type' conidia were also mentioned by Halmschlager & Kowalski (2003) in their cultures of *S. inflata*. *H. dimorphospora* is the anamorph of species in the *Chaetomiaceae*, and we thus prefer reference to the second conidial type as blastoconidial rather than *Humicola*-type. No teleomorph structures were found associated with isolates of *S. inflata*. Pfennig & Oberwinkler (1993) suggested that *O. bragantinum* could be a teleomorph of *S. inflata s. lat.*, but no teleomorph has been observed, either in the original or any single spore culture of *S. inflata* used in this study.

Phylogenetic comparisons of ITS1/2 including 5.8S gene region and partial β-tubulin gene provided sufficient characters to differentiate between *S. inflata* isolates. In particular, there was significant variation in the β-tubulin gene sequences, resulting from a variable number of introns in this genic region. Isolates in clade III had two introns (C and E), while those in clade IV had three introns (C, D and E). This suggests that the fungus referred to as *S. inflata*, probably represents at least four independent evolutionary lineages.

This study has provided added evidence to show that fungi treated as *O. stenoceras* and *S. inflata* represent heterogeneous complexes. We provided a name for *O. dentifundum* as the fungus appears to be well characterized, especially due to the presence of a teleomorph and its consideration in previous studies. Further studies and especially additional collections are required to fully define the *Sprothrix* spp. for which we have chosen not to provide names here.

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