



Ceratocystiopsis spp. associated with pine- and spruce-infesting bark beetles in Norway

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Received: 29 November 2021 / Revised: 27 April 2022 / Accepted: 30 April 2022 / Published online: 4 June 2022
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Abstract

The genus *Ceratocystiopsis* (*Ophiostomatales*, *Ascomycota*) includes 21 species, which can be found mainly in association with bark beetles in the Northern Hemisphere. A survey of *Ceratocystiopsis* species associated with bark beetles infesting *Picea abies* and *Pinus sylvestris* in Norway yielded 126 isolates, representing *Ceratocystiopsis neglecta* and *Ceratocystiopsis rollhanseni*, and four species described herein as *Ceratocystiopsis chalcographii*, *Ceratocystiopsis debeeria*, *Ceratocystiopsis norroenii* and *Ceratocystiopsis troendelagii*. The new taxa were morphologically characterised and phylogenetically analysed on the basis of sequence data of multiple loci (ITS, LSU, beta-tubulin (*TUB2*), calmodulin (*CAL*) and translation elongation factor 1-alpha (*TEF1*) genes). *Ceratocystiopsis norroenii* and *C. rollhanseni* were the most frequently isolated species, and the latter species had the wider vector range.

Keywords Beetle–fungus relationships · Ophiostomatoid fungi · *Picea abies* · *Pinus sylvestris* · Four new taxa

Introduction

Ceratocystiopsis (Upadhyay and Kendrick (1975) (*Ascomycota*, *Ophiostomatales*) is based on *Ophiostoma minutum* Siemaszko (1939), which was described based on perithecia found on the wood of *Picea abies* infested with *Ips typographus* in the Białowieża Forest, Poland (Siemaszko 1939). Conidial states were not described.

Ceratocystiopsis taxonomy has a turbulent history, and species of ceratocystiopsis-like fungi have been accommodated in various genera including *Ceratocystiopsis* (Upadhyay 1981; Marmolejo and Butin 1990; Hsiao and Harrington 1997; Zipfel et al. 2006; Strzałka et al. 2020; Nel et al. 2021), *Ceratocystis* (Griffin 1968; Olchowecki

and Reid 1974), *Cornuvesica* (Viljoen et al. 2000) and *Ophiostoma* (Siemaszko 1939; Hausner et al. 1993, 2003; Kirschner and Oberwinkler 1999). Important historical developments in the taxonomy of *Ceratocystiopsis* included erecting *Ceratocystiopsis* for species that have short-necked perithecia and falcate-shaped ascospores with gelatinous sheaths and a *Hyalorhinocladiella* anamorph (Upadhyay and Kendrick 1975); and reducing the genus to synonymy with *Ophiostoma*, based on partial SSU and LSU rDNA sequences by Hausner et al. in 1993. *Ceratocystiopsis* was later on re-instated based on morphological characters and the analysis of combined partial nuclear LSU and β -tubulin gene sequences (Zipfel et al. 2006). Currently, *Ceratocystiopsis* is one of nine more or less clearly defined genera in the *Ophiostomataceae* (De Beer and Wingfield 2013; De Beer et al. 2013a, b, 2016; Hyde et al. 2020). It is also worth mentioning that *Ceratocystiopsis* may also produce cucullate ascospores (De Beer and Wingfield 2013) and occasionally sporothrix-like asexual morphs (Bridges and Perry 1987; Marmolejo and Butin 1990).

As currently circumscribed, *Ceratocystiopsis* accommodates 21 species (De Beer et al. 2013b; Strzałka et al. 2020; Nel et al. 2021; Chang et al. 2021; Wang et al. 2021), that have a wide geographic distribution including records from Africa (Nel et al. 2021), Asia (e.g. Yamaoka et al. 1997; Wang et al.

Section editor: Hans-Josef Schroers

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2020; Chang et al. 2021; Wang et al. 2021), Europe (e.g. Hausner et al. 1993; Kirisits 2004, Jankowiak 2005; Linnakoski et al. 2012) and North America (Olchowecki and Reid 1974; Bridges and Perry 1987; Marmolejo and Butin 1990; Hsiau and Harrington 1997; Hausner et al. 2003). However, they are mostly known and abundant in the Northern Hemisphere conifer ecosystems of Europe and North America.

Ceratocystiopsis minuta is the most frequently reported and widespread species. However, Plattner et al. (2009), based on molecular phylogenetic analyses, showed that European and Asian strains of *C. minuta* clustered in at least three well defined, closely related lineages (C, D, and E), whereas ‘*C. minuta*-like’ strains from North America were unrelated. Hence, *C. minuta* (clades C, D and E *sensu* Plattner et al. 2009) is a species complex that probably includes several cryptic yet undescribed species. No type material of *C. minuta* was originally designated (Siemaszko 1939). Reid and Hausner (2010) proposed an epitypification based on UAMH 11218 (= a dried culture of UM1532 = WIN(M)1532), originally isolated from *P. abies* infested by *I. typographus* in the Biebrzański National Park (Poland). This strain belongs to clade D of Plattner et al. (2009) that included also five other strains from Poland and one from Japan. The Polish strains were collected from *P. abies* infested by *I. typographus* while the Japanese strain was obtained from an adult beetle of *I. typographus japonicus* on *Picea jezoensis*. The two Polish strains of *C. minuta* in clade E had the same origin as Polish strains in clade D. In turn, clade C contained eight strains that originated from Austria, Scotland and Japan and these were found in association with different *Ips* species on *Larix* spp. and *Picea* spp. (Plattner et al. 2009).

Ceratocystiopsis species occupy a relatively narrow range of habitats. Most species have been recorded on conifers in association with bark and wood boring beetles (e.g. Olchowecki and Reid 1974; Hsiau and Harrington 1997; Yamaoka et al. 1997; Kirisits 2004; Linnakoski et al. 2012; Nel et al. 2021; Wang et al. 2021). Other species have been described from hardwood trees (Olchowecki and Reid 1974; Li et al. 2018, Strzałka et al. 2020).

Very little is known about the distribution of *Ceratocystiopsis* species in Norway. To date, only *C. minuta* and *C. rollhanseniana* have been reported from Norway either, from *P. abies* infested by *I. typographus* (Solheim 1986, 1992, 1993) or galleries created by unknown bark beetle species on *Pinus sylvestris* (Hausner et al. 2003).

The aim of this study was to characterize the morphology, phylogenetic affinities and taxonomy of *Ceratocystiopsis* isolates found in Norway during a survey of bark beetles and their galleries on conifers from October 2014 to September 2015.

Materials and methods

Fungal isolates

Twenty-two species of bark beetles were collected from several locations in Norway between October 2014 and September 2015 (Table 1, Fig. 1). The species list of beetles collected from all sampling sites is shown in Table 1. The bark beetles were collected by hand from *Picea abies* and *Pinus sylvestris*. Bark beetle adults (alive) were excised from the bark and galleries with sterilised tweezers and stored individually in sterile 1.5 ml microcentrifuge tubes for subsequent fungal isolations. Each beetle was removed from its storage microtube with sterilised tweezers and was morphologically identified with taxonomical keys (Lekander et al. 1977; Ehnström and Axelsson 2002). Later, each bark beetle was divided into three parts, elytra, head and the rest, before placing the parts in three separate Petri dishes on malt extract agar [MEA; 6.25 g malt Bacto™ agar powder (Beckton, Dickinson, Sparks, USA), 10 g agar (Bacto™ agar powder from VWR International, Leuven, Belgium), 0.5 L deionized water] without antibiotics. Emerging cultures were purified by transferring small pieces of mycelium or spore masses from individual colonies to fresh MEA. Fungi were incubated at 20 °C, then subcultured onto 2% MEA (20 g Bacto™ malt extract, 20 g agar Bacto™ agar powder (Becton Dickinson and Company, Franklin Lakes, USA) in 1 L deionized water) and stored at 4 °C. After 2 weeks of incubation at 20 °C, the purified fungal cultures were grouped according to their morphological characteristics. Depending on the number of isolates belonging to the same morphotype, 2–19 isolates per morphotype were chosen for molecular identification (Table 2). The frequency of occurrence was calculated according to the following formula: $F = (NS/NTs) \times 100$, where F is the frequency of occurrence (%) of the fungus, NS is the number of beetles from which a particular fungus was isolated and NTs is the total number of beetles.

The cultures are maintained in the culture collection of the Norwegian Institute of Bioeconomy (NIBIO), Norway. Ex-type isolates of new species described in this study were deposited in the collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Type specimens were deposited in the Mycological Herbarium (O), Natural History Museum, University of Oslo, Norway. The ex-type strains of *C. neglecta* and *C. rollhanseniana* were sourced from the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), and from the culture collection of the University of Manitoba (WIN) in Canada (Table 2). Taxonomic descriptions and nomenclatural data were registered in MycoBank (www.Mycobank.org) (Robert et al. 2013).

Table 1 Bark beetle sampling sites with (in bold) and without *Ceratocystiopsis* species

Sampling site	Latitude	Longitude	Municipality	County	Bark beetle species	Host
1	69.818212	25.157902	Porsanger	Finnmark	<i>Pityogenes quadridens</i> , <i>Orthotomicus suturalis</i>	<i>Ps</i>
2	69.551479	25.831215	Karasjok 4	Finnmark	<i>Ips acuminatus</i> , <i>Pityogenes bidentatus</i>	<i>Ps</i>
3	69.447422	25.589316	Karasjok 5	Finnmark	<i>Tomicus piniperda</i>	<i>Pa</i>
4	69.465227	25.568562	Karasjok 6	Finnmark	<i>P. quadridens</i> , <i>P. bidentatus</i>	<i>Ps</i>
5	69.886036	23.245540	Alta 1	Finnmark	<i>T. piniperda</i>	<i>Ps</i>
6	69.870668	23.262178	Alta 2	Finnmark	<i>T. piniperda</i>	<i>Ps</i>
7	69.830809	23.467727	Alta 3	Finnmark	<i>Hylurgops palliatus</i>	<i>Pa</i>
8	69.568020	20.217645	Lyngen	Troms	<i>H. palliatus</i> , <i>Trypodendron lineatum</i>	<i>Ps</i>
9	69.069233	18.487633	Målselv 2	Troms	<i>H. palliatus</i> <i>T. piniperda</i>	<i>Ps</i>
10	69.060888	18.995351	Målselv 5	Troms	<i>I. acuminatus</i>	<i>Ps</i>
11	69.001709	18.995351	Målselv 6	Troms	<i>I. acuminatus</i>	<i>Ps</i>
12	68.701934	16.300628	Kvæfjord	Troms	<i>Dryocoetes hectographus</i> , <i>T. piniperda</i> , <i>T. lineatum</i>	<i>Ps</i> , <i>Pa</i>
13	64.454948	14.018562	Lierne 1	Trøndelag	<i>Cryphalus saltuarius</i> , <i>Pityophthorus micrographus</i>	<i>Pa</i>
14	64.403248	13.548321	Lierne 2	Trøndelag	<i>Pityogenes saalasi</i> , <i>Polygraphus subopacus</i> , <i>C. saltuarius</i>	<i>Pa</i>
15	61.910603	11.979645	Engerdal	Hedmark	<i>P. bidentatus</i> , <i>T. piniperda</i>	<i>Ps</i>
16	61.728590	11.344890	Rendalen	Hedmark	<i>Pityogenes chalcographus</i> , <i>T. piniperda</i>	<i>Ps</i>
17	61.362270	12.227550	Trysil	Hedmark	<i>P. chalcographus</i> , <i>T. piniperda</i>	<i>Ps</i>
18	60.374962	12.533580	Grue 1	Hedmark	<i>Hylastes brunneus</i>	<i>Ps</i>
19	60.373333	12.461444	Grue 2	Hedmark	<i>P. quadridens</i> , <i>P. bidentatus</i> ,	<i>Ps</i>
20	60.352141	12.536538	Grue 3	Hedmark	<i>Orthotomicus laricis</i> , <i>T. piniperda</i>	<i>Ps</i>
21	60.237895	10.986056	Nannestad	Akershus	<i>P. chalcographus</i> , <i>Polygraphus poligraphus</i>	<i>Pa</i>
22	59.668680	10.718710	Ås	Akershus	<i>H. palliatus</i> , <i>Dryocoetes autographus</i> , <i>Hylastes cunicularius</i> <i>P. poligraphus</i>	<i>Pa</i>
23	59.639736	10.654259	Frogn 1	Akershus	<i>Crypturgus cinereus</i> , <i>C. pusillus</i> , <i>Orthotomicus proximus</i>	<i>Ps</i>
24	59.634348	10.668942	Frogn 2	Akershus	<i>Ips sexdentatus</i> , <i>O. proximus</i> , <i>P. quadridens</i>	<i>Ps</i>
25	60.581978	9.9339731	Ringerike	Buskerud	<i>P. micrographus</i>	<i>Ps</i>
26	59.743666	9.564444	Flesberg	Buskerud	<i>P. chalcographus</i>	<i>Ps</i>

Microscopy and growth studies

Isolates selected for molecular analyses and as herbarium specimens were morphologically characterised. Isolates were grown on 2% MEA. In attempts to induce the formation of ascomata, autoclaved twigs of host trees including bark were placed at the centres of agar plates containing 2% MEA. Fungal cultures were derived from single conidia. To promote the production of ascomata, all isolates of each taxon were crossed among themselves in all possible combinations, following the technique described by Grobbelaar et al. (2010). These cultures were incubated at 25 °C and monitored regularly for the appearance of fruiting structures.

Morphological features were examined by mounting fungal tissue in 80% lactic acid on glass slides and observed using a Nikon Eclipse 50i microscope (Nikon® Corporation, Tokyo, Japan) with an Invenio 5S digital camera (DeltaPix®, Maalov, Denmark) to capture photographic images. Colour designations were based on the colour charts of Kornerup and Wanscher (1978).

For each taxonomically relevant structure, fifty measurements were made, when possible, using the Coolview 1.6.0 software (Precoptic®, Warsaw, Poland). Averages, ranges and standard deviations were calculated for the measurements, and these are presented in the format '(min–)(mean–SD)–(mean + SD)(–max)'.

Growth characteristics for the novel species were determined by analysing the radial growth for two isolates (two for each species). Agar disks (5 mm diam.) were cut from the actively growing margins of fungal colonies and these disks were placed at the centres of plates containing 2% MEA. Four replicate plates for each of the six putative new species were incubated at temperatures between 5 and 35 °C at 5 °C intervals. The radial growth (two measurements perpendicular to each other per plate) was determined 14 days after inoculation, and growth rates were calculated as mm/day.

PCR, sequencing and phylogenetic analyses

Morphotypes were initially identified by sequencing the internal transcribed spacer 1 and 2 (ITS), 28S ribosomal large

Table 2 Isolates used in the present study

Fungal species	Isolate no. ¹		Tree host	Insect vector	Site ²	GenBank accessions ³				
	CBS	CMW				NIBO or WIN	ITS	LSU	TUB2	CAL
<i>Ceratocystiopsis chalcographii</i> sp. nov.	147954 ^T	57280	<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Nannestad	OL309930	OL309930	OL309955	OL310002	OL310034
Taxon 1	147953	57281	<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Nannestad	OL309931	OL309931	OL309956	OL310003	OL310035
	147955 ^A	57279	<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	OL309932	OL309932	OL309957	OL310004	OL310036
		57282	<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	OL309933	OL309933	OL309958	OL310005	OL310037
			<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Nannestad	–	–	OL309959	OL310006	OL310038
			<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Nannestad	–	–	OL309960	–	–
			<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	–	–	OL309961	–	–
			<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	–	–	OL309962	–	–
<i>Ceratocystiopsis norveicii</i> sp. nov.	147956 ^T	57285	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Målselv 5	OL309934	OL309934	OL309963	OL310007	OL310039
Taxon 2	147957 ^A	57287	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Målselv 6	OL309935	OL309935	OL309964	OL310008	OL310040
		57283	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Karasjok 6	OL309936	OL309936	OL309965	OL310009	OL310041
		57286	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Karasjok 6	OL309937	OL309937	OL309966	OL310010	OL310042
		57284	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Målselv 6	OL309938	OL309938	OL309967	OL310011	OL310043
			<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Frogn 2	–	–	OL309968	–	–
			<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Målselv 5	–	–	OL309969	–	–
			<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Målselv 6	–	–	OL309970	–	–
			<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Porsanger	–	–	OL309971	–	–
<i>Ceratocystiopsis debeeria</i> sp. nov.	147959 ^A	57290	<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	OL309939	OL309939	OL309972	Not obtained	OL310044
Taxon 3	57289	57289	<i>Pinus sylvestris</i>	<i>Pityogenes chalcographus</i>	Rendalen	OL309940	OL309940	OL309973	OL310012	OL310045
		57291	<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Flesberg	OL309941	OL309941	OL309974	OL310013	OL310046
		57288	<i>Picea abies</i>	<i>Pityogenes chalcographus</i>	Flesberg	OL309942	OL309942	OL309975	OL310014	OL310047
<i>Ceratocystiopsis troendelagii</i> sp. nov.	147962 ^T	57294	<i>Picea abies</i>	<i>Polygraphus subopacus</i>	Lierne	OL309943	OL309943	OL309976	Not obtained	OL310048
Taxon 4	147963 ^A	57295	<i>Picea abies</i>	<i>Pityogenes saatali</i>	Lierne	OL309944	OL309944	OL309977	OL310015	OL310049
<i>Ceratocystiopsis neglecta</i>	147960	57292	<i>Picea abies</i>	<i>Hylurgops palliatus</i>	Ås	OL309945	OL309945	OL309978	OL310016	OL310050
Taxon 5	147961	57293	<i>Picea abies</i>	<i>Polygraphus subopacus</i>	Lierne	OL309946	OL309946	OL309979	OL310017	OL310051
			<i>Picea abies</i>	<i>Hylurgops palliatus</i>	Ås	–	–	OL309980	OL310018	OL310052
			<i>Picea abies</i>	<i>Hylurgops palliatus</i>	Ås	–	OL309947	OL309981	OL310019	OL310053
	100596 ^T		<i>Picea abies</i>	<i>Hylurgops palliatus</i>	Baden-Württemberg	MH874319	–	OL309982	OL310020	OL310054
<i>Ceratocystiopsis rolhanseni</i>	147950	57276	<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Porsanger	–	OL309948	OL309983	OL310021	OL310055
Taxon 6	147952	57278	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	OL309949	OL309984	OL310022	OL310056
	147951	57277	<i>Pinus sylvestris</i>	<i>Orthotomicus proximus</i>	Frogn 1	–	OL309950	OL309985	OL310023	OL310057

Table 2 (continued)

Fungal species	Isolate no. ¹		Tree host	Insect vector	Site ²	GenBank accessions ³				
	CBS	CMW NIBO or WIN				ITS	LSU	TUB2	CAL	TEF1
		N2015-0946/3/1	<i>Pinus sylvestris</i>	<i>Orthotomicus proximus</i>	Frogn 1	–	OL309951	OL309986	OL310024	OL310058
		N2015-1162/2/1	<i>Pinus sylvestris</i>	<i>Crypturgus pusillus</i>	Frogn 1	–	OL309952	OL309987	OL310025	OL310059
		N2015-1169/1/2/1	<i>Pinus sylvestris</i>	<i>Crypturgus pusillus</i>	Frogn 1	–	OL309953	OL309988	OL310026	OL310060
		N2015-0003/2/3	<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Grue	–	–	OL309989	OL310027	OL310061
		N2015-0859/3/2	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	–	OL309990	OL310028	OL310062
		N2016-0836/3/4	<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Porsanger	–	–	OL309991	OL310029	OL310063
		N2015-0847/3/2	<i>Pinus sylvestris</i>	<i>Pityogenes quadridentis</i>	Karasjok 6	–	–	OL309992	OL310030	OL310064
		N2015-0493/3/2	<i>Pinus sylvestris</i>	<i>Ips acuminatus</i>	Mällselv 5	–	–	OL309993	–	–
		N2015-0846/2/4	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 6	–	–	OL309994	–	–
		N2015-0849/1/1	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 6	–	–	OL309995	–	–
		N2015-0853/1/1	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	–	OL309996	–	–
		N2015-0855/3/2	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	–	OL309997	–	–
		N2015-0857/2/1	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	–	OL309998	–	–
		N2015-0857/3/1	<i>Pinus sylvestris</i>	<i>Pityogenes bidentatus</i>	Karasjok 4	–	–	OL309999	–	–
		N2015-0938/2/3	<i>Pinus sylvestris</i>	<i>Orthotomicus proximus</i>	Frogn 1	–	–	OL310000	–	–
		N2015-0947/1/2	<i>Pinus sylvestris</i>	<i>Orthotomicus proximus</i>	Frogn 1	–	OL309954	OL310001	–	–
		WIN110,UAMH9797	<i>Pinus sylvestris</i>	Bark beetle galleries	Kongsvinger	EU913719	EU913679	EU913758	OL310031	OL310065
		WIN113,UAMH9774 ^T	<i>Pinus sylvestris</i>	Bark beetle galleries	Tangen	EU913718	EU913678	EU913757	OL310032	OL310066
		WIN117	<i>Pinus sylvestris</i>	Bark beetle galleries	Kongsvinger	–	–	–	OL310033	OL310067

¹ CBS Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMW Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; NIBO (N) Culture collection of Norwegian Institute of Bioeconomy (NIBIO), Norway; UAMH: The UAMH centre for global microfungi biodiversity, University of Toronto, Toronto, Canada; WIN Culture Collection of the University of Manitoba (Winnipeg), Canada. ^T ex-holotype isolates, ^A additional specimen examined

² All isolates were collected in Norway except *C. neglecta* CBS 100596 which was collected in Germany

³ ITS = internal transcribed spacer regions 1 and 2 of the nuclear ribosomal DNA operon, including the 5.8S region; LSU = internal transcribed spacer region 2 and the 28S large subunit of the rDNA gene; TUB2 = Beta-tubulin; CAL = Calmodulin; TEF1 = Translation elongation factor 1-alpha; Bold type = GenBank accession numbers of sequences obtained in the present study

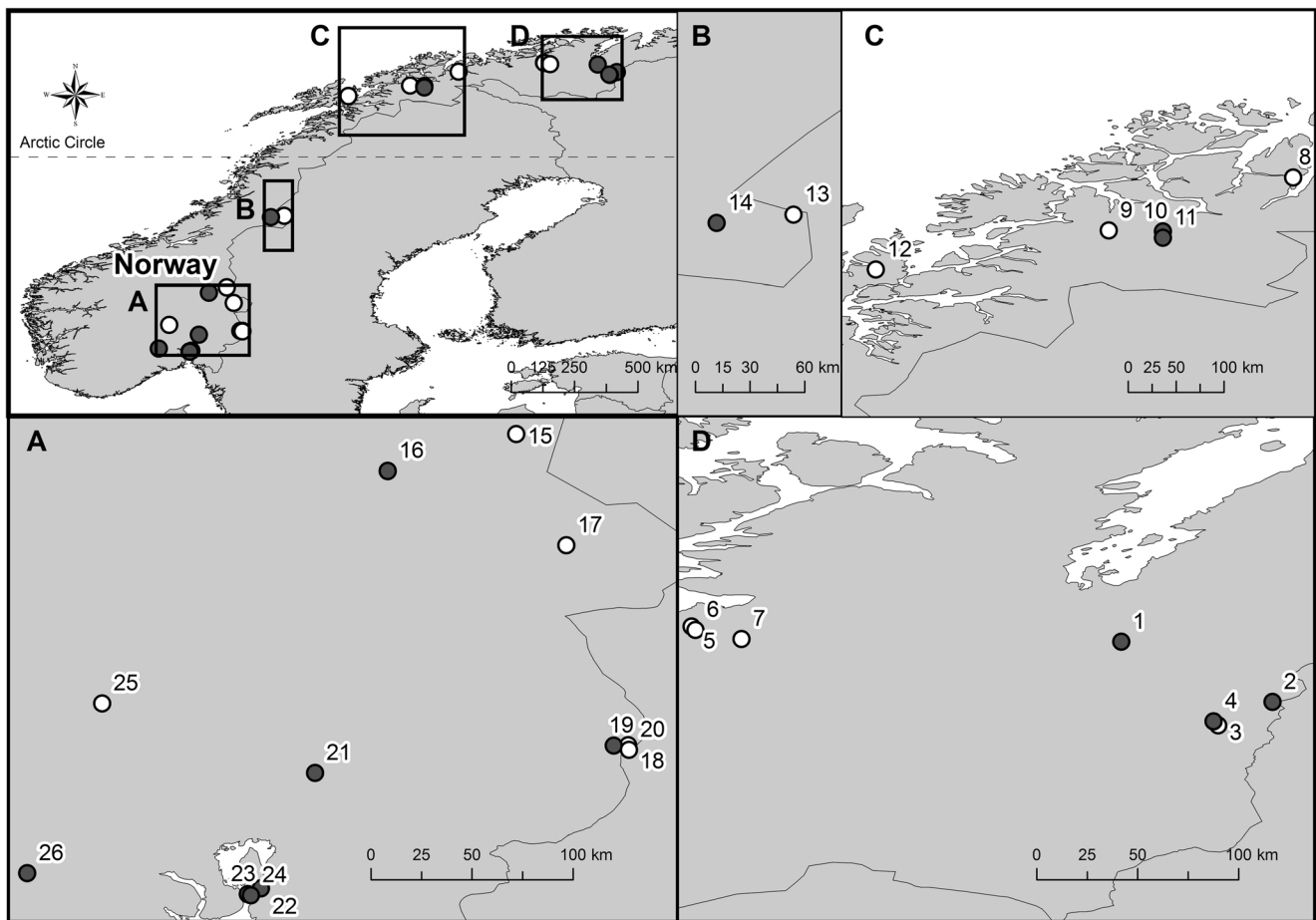


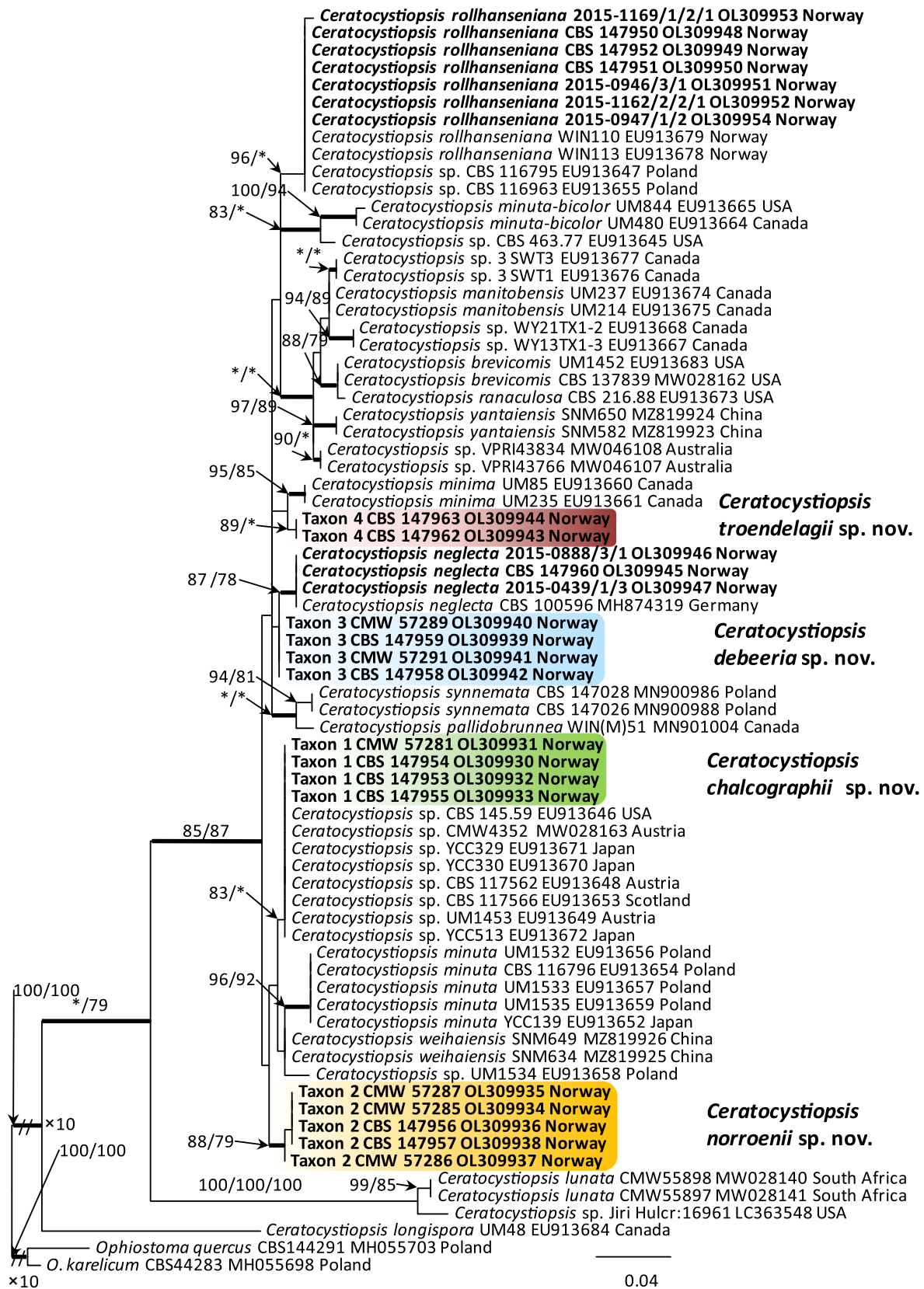
Fig. 1 The sample sites in Norway where isolations from bark beetles yielded *Ceratocystopsis* species (black) or not (white)

subunit (LSU) or partial β -tubulin (*TUB2*) gene region of representative isolates (Table 2). DNA extractions were performed as described by Aas et al. (2018). For more detailed analysis, two additional loci were also amplified and sequenced and subjected to phylogenetic analyses: partial calmodulin (*CAL*) and partial translation elongation factor 1-alpha (*TEF1*) sequences. The primers used for PCR and sequencing of the various loci were as follows: ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for the ITS region; LR0R and LR5 (Vilgalys R; Vilgalys and Hester 1990) for the LSU region; T10 (O'Donnell and Cigelnik 1997) or Bt2a together with Bt2b (Glass and Donaldson 1995) for *TUB2*; F-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998) were used for *TEF1*; CL1 and CL2a (O'Donnell et al. 2000) or CL3F and CL3R (De Beer et al. 2016) were used for *CAL*. Protocols for PCR amplification and DNA sequencing were as described previously (Aas et al. 2018).

All phylogenetic analyses were performed independently for each locus (Figs. 2, 3, 4, 5 and 6). Resulting trees were visually compared for topological incongruence. Loci showing no topological incongruences (i.e. ITS+LSU+*TUB2*) were combined and analysed as a concatenated construct (Fig. 7).

For phylogenetic analyses, sequence alignments were performed using the online version of MAFFT v7 (Katoh and Standley 2013). The ITS, LSU, *TUB2*, *CAL* and *TEF1* datasets were aligned using the E-INS-i strategy with a 200PAM/ $\kappa=2$ scoring matrix, a gap opening penalty of 1.53 and an offset value of 0.00. The alignments were checked manually with BioEdit v.2.7.5 (Hall 1999). The resulting alignments and trees were deposited into TreeBASE (<http://purl.org/phylo/treebase/phylovs/study/TB2:S28950>).

Phylogenetic trees were inferred for each of the datasets using three different methods: maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). For ML and BI analyses, the best-fit substitution models for each aligned dataset were established using the corrected Akaike information criterion (AICc) in jModelTest 2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012). ML analyses were carried out with PhyML 3.0 (Guindon et al. 2010), utilizing the Montpellier online server (<http://www.atgc-montpellier.fr/phyml/>). The ML analysis was combined with bootstrapping (1000 bootstrap pseudoreplicates) to assess node support values and the overall reliability of the tree topology. The best evolutionary substitution models were as follows: GTR+I+G for ITS (-lnL = 4817.94), LSU (-lnL = 1452.82)



◀ **Fig. 3** Phylogram obtained from maximum likelihood (ML) analyses of the LSU data for the *Ceratocystiopsis* spp. Sequences obtained during this study are presented in bold type. Bootstrap values $\geq 75\%$ for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values $< 75\%$. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Ophiostoma karelicum* and *O. quercus* represents the outgroup in analyses of LSU data

and *CAL* (-lnL = 3350.13); GTR+G for *TUB2* (-lnL = 4368.71) and ITS+LSU+*TUB2* (-lnL = 9793.62); and HKY+I+G for *TEF1* (-lnL = 5374.80).

MP analyses were performed using PAUP* 4.0b10 (Swofford 2003). Gaps were treated as a fifth state. Bootstrap analysis (1000 bootstrap replicates) was conducted to determine the levels of confidence for the nodes within the inferred tree topologies. Tree bisection and reconnection (TBR) was selected as the branch swapping option. The tree length (TL), Consistency Index (CI), Retention Index (RI), Homoplasy Index (HI) and Rescaled Consistency Index (RC) were recorded for each analysed dataset after the trees were generated.

BI analyses using Markov Chain Monte Carlo (MCMC) methods were carried out with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). Four MCMC chains were run in parallel for 10 million generations applying the best-fit model for each dataset. Trees were sampled every 100th generations, resulting in 100,000 trees. Tracer v1.4.1 (Rambaut and Drummond 2007) was utilized to determine the burn-in value for each dataset. The remaining trees were utilized to generate a 50% majority rule consensus tree, which allowed calculating posterior probability values for nodes.

Results

Collections of bark beetles and fungi

Altogether, 459 beetles, representing 22 species, were collected from Norway during this study (Table 1). Among the bark beetles carrying *Ceratocystiopsis* species, only *Pityogenes chalcographus* occurred on both pine and spruce. Five bark beetle species with *Ceratocystiopsis* species were found on pine, four on spruce. The isolations from bark beetles yielded a total of 126 isolates tentatively identified as *Ceratocystiopsis* spp. (Table 3). Fifty isolates were collected from *Ips acuminatus*, 21 isolates from *Orthotomicus proximus*, 15 isolates from *P. chalcographus* and 12 isolates from *Crypturgus cinereus*. The additional twenty-eight isolates were collected from *C. pusillus*, *Hylurgops palliatus*, *Pityogenes bidentatus*, *P. saalasi*, *P. quadridens* and *Polygraphus subopacus* (Table 3). No *Ceratocystiopsis* species were detected in samples from *Cryphalus saltuarius*, *Dryocoetes autographus*, *D.*

hectographus, *Hylastes brunneus*, *H. cunicularis*, *Ips sexdentatus*, *Orthotomicus laricis*, *O. suturalis*, *Pityophthorus micrographus*, *Polygraphus poligraphus*, *Tomicus piniperda* and *Trypodendrum lineatum* (Table 1).

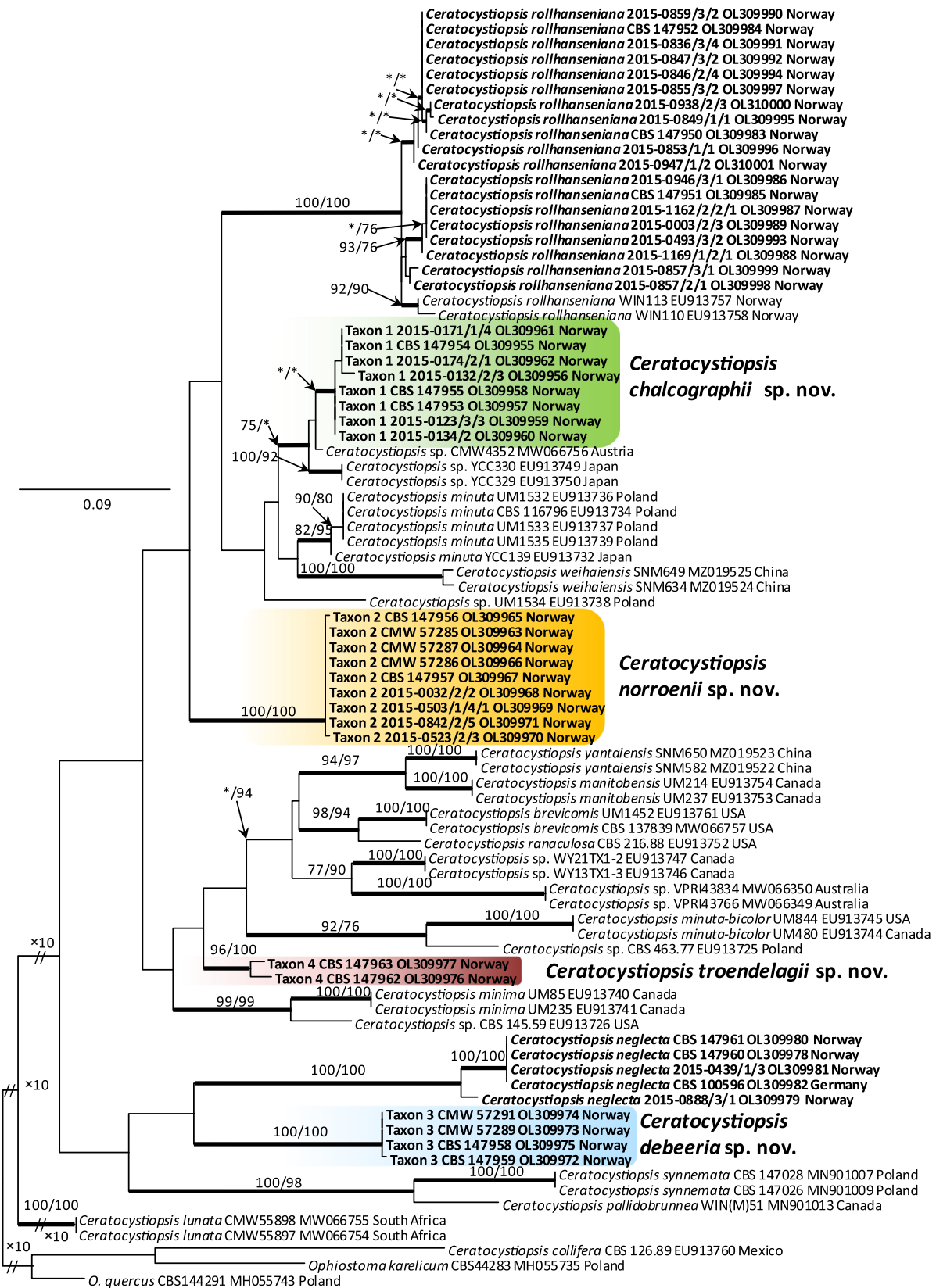
Based on preliminary morphological observations, the isolates could be arranged into six taxa. The frequencies of occurrence of *Ceratocystiopsis* spp. varied between ten bark beetle species (Table 3). Taxon 6, represented by 55 isolates, was isolated from six different bark beetle species with frequencies of occurrence ranging from 11 to 72% from the respective beetle species. It was the dominant species associated with *P. bidentatus* (72% of specimens carried the fungus) and *O. proximus* (70%), and taxon 6 also occurred at a relatively high frequency on *C. cinereus* (40%). Fifty isolates belonged to taxon 2. This taxon was found only in high frequency on *I. acuminatus* (54%). The remaining 16 isolates represented four *Ceratocystiopsis* taxa and were collected from four beetle species at a frequency $\leq 11\%$ (Table 3).

Phylogenetic analyses

Alignments for the ITS data set contained 738 characters; for the LSU data 496 characters; for the *CAL* data 613 characters; for the *TEF1* data 785 characters; for the *TUB2* data 526 characters for the ITS+LSU+*TUB2* data 1839 characters (including gaps). The exon/intron arrangement of the *CAL* data included intron 3, exon 4, intron 4, exon 5, intron 5, exon 6 and intron 6. The exon/intron arrangement of the *TEF1* data included intron 3, exon 4, intron 4 and exon 5. The aligned *TUB2* gene region consisted of exons 3 to 6, interrupted by introns 3, 4 and 5.

DNA sequence data were obtained for 45 isolates representing all six identified morphological groups (Table 2). BLAST analyses of the ribosomal DNA sequences confirmed their affinities with *Ceratocystiopsis*. Sequences from our 45 representative isolates were combined with those of other members of *Ceratocystiopsis*. They formed six independent, well-supported terminal clades representing six phylogenetic species (Taxa 1–6) in the ITS, LSU, *TUB2*, *CAL* and *TEF1* phylogenetic inferences (Figs. 2, 3, 4, 5 and 6). Based on the phylogenetic analyses of the ITS and LSU data sets (Figs. 2 and 3), the isolates emerged as two known and four undescribed taxa.

Four isolates recovered from *P. abies* and *P. sylvestris* infested by *P. chalcographus* (i.e. taxon 1) in the ITS and LSU trees grouped in the '*C. minuta*' clade C (Plattner et al. 2009). This clade C includes European and Japanese strains of *Ceratocystiopsis minuta* obtained from conifer-infesting bark beetles (Plattner et al. 2009). In the *TUB2* tree, eight isolates of taxon 1 clustered into a distinct clade next to a *Ceratocystiopsis* strain (CMW4352) from Austria and Japanese isolates of *Ceratocystiopsis* spp. (YCC330 and



YCC329) that belonged to the ‘*C. minuta*’ clade C according to Plattner et al. (2009) (Fig. 4). Although only poorly

supported, this clade appears to share a common node with strains assigned to *C. minuta* s. str. (the ‘*C. minuta*’ clade D

Fig. 4 Phylogram obtained from Maximum Likelihood (ML) analyses of *TUB2* data for the *Ceratocystiopsis* spp. Sequences obtained during this study are presented in bold type. Bootstrap values $\geq 75\%$ for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values $< 75\%$. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Ophiostoma karelicum* and *O. quercus* represents the outgroup in analyses of the *TUB2* data

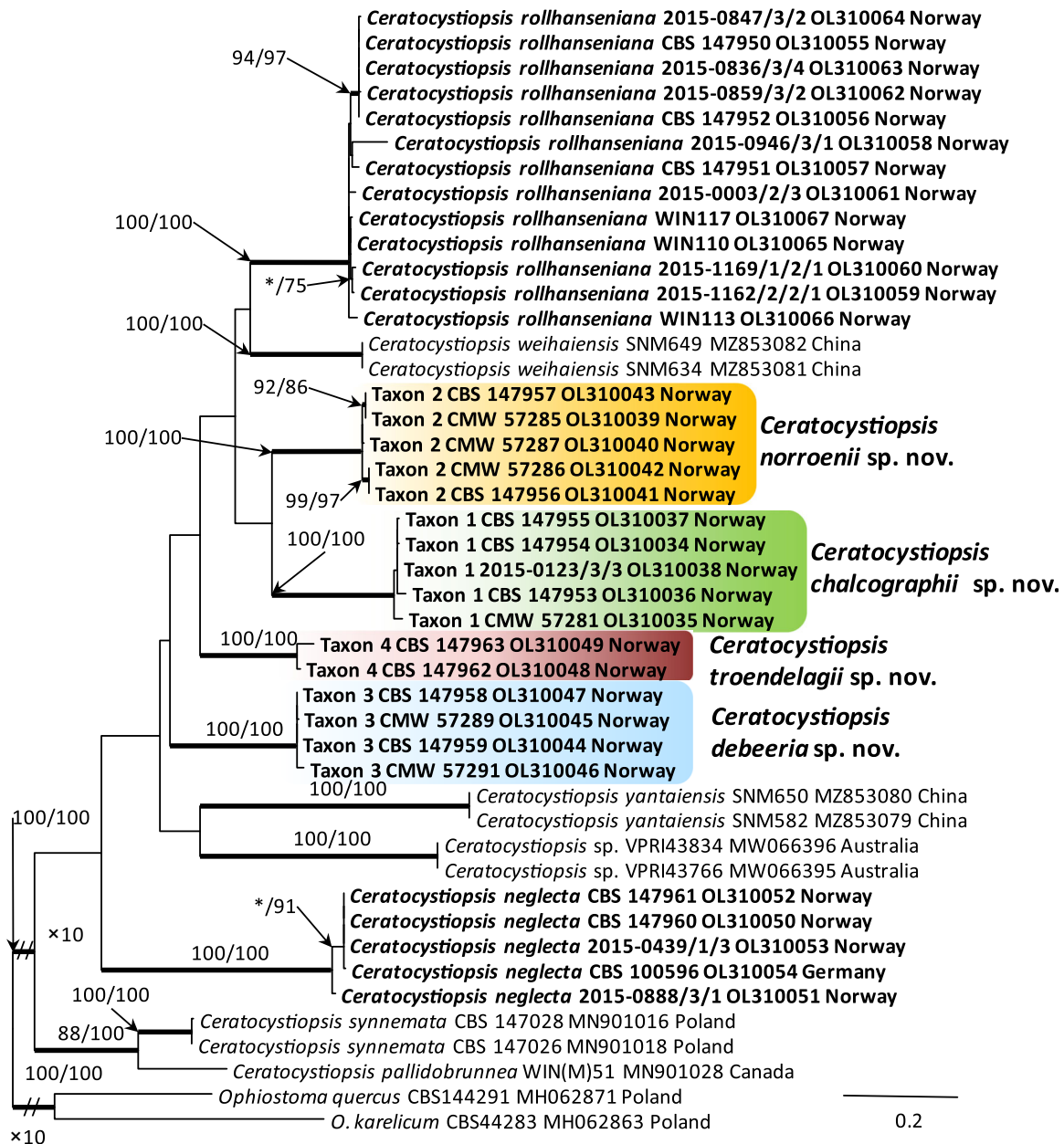


Fig. 5 Phylogram obtained from Maximum Likelihood (ML) analyses of *TEF1* data for the *Ceratocystiopsis* spp. Sequences obtained during this study are presented in bold type. Bootstrap values $\geq 75\%$ for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95

including UM1532) and *Ceratocystiopsis weihaiensis* (Fig. 4).

Nine isolates obtained from *P. sylvestris* infested by *I. acuminatus* (i.e. taxon 2) form a well-supported lineage, distinct from all the other known species of *Ceratocystiopsis* in the ITS, LSU, *TUB2*, *CAL* and *TEF1* analyses (Figs. 2, 3, 4, 5 and 6). In the ITS tree, five isolates of taxon 2 clustered into a distinct clade next to *Ceratocystiopsis* strains CBS 116963 and UM 1534 from Poland that belonged to the '*C. minuta*' clade C and E, respectively (Fig. 2). Six isolates representing

obtained from Bayesian Inference (BI) analyses. *Bootstrap values $< 75\%$. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Ophiostoma karelicum* and *O. quercus* represents the outgroup in analyses of the *TEF1* data

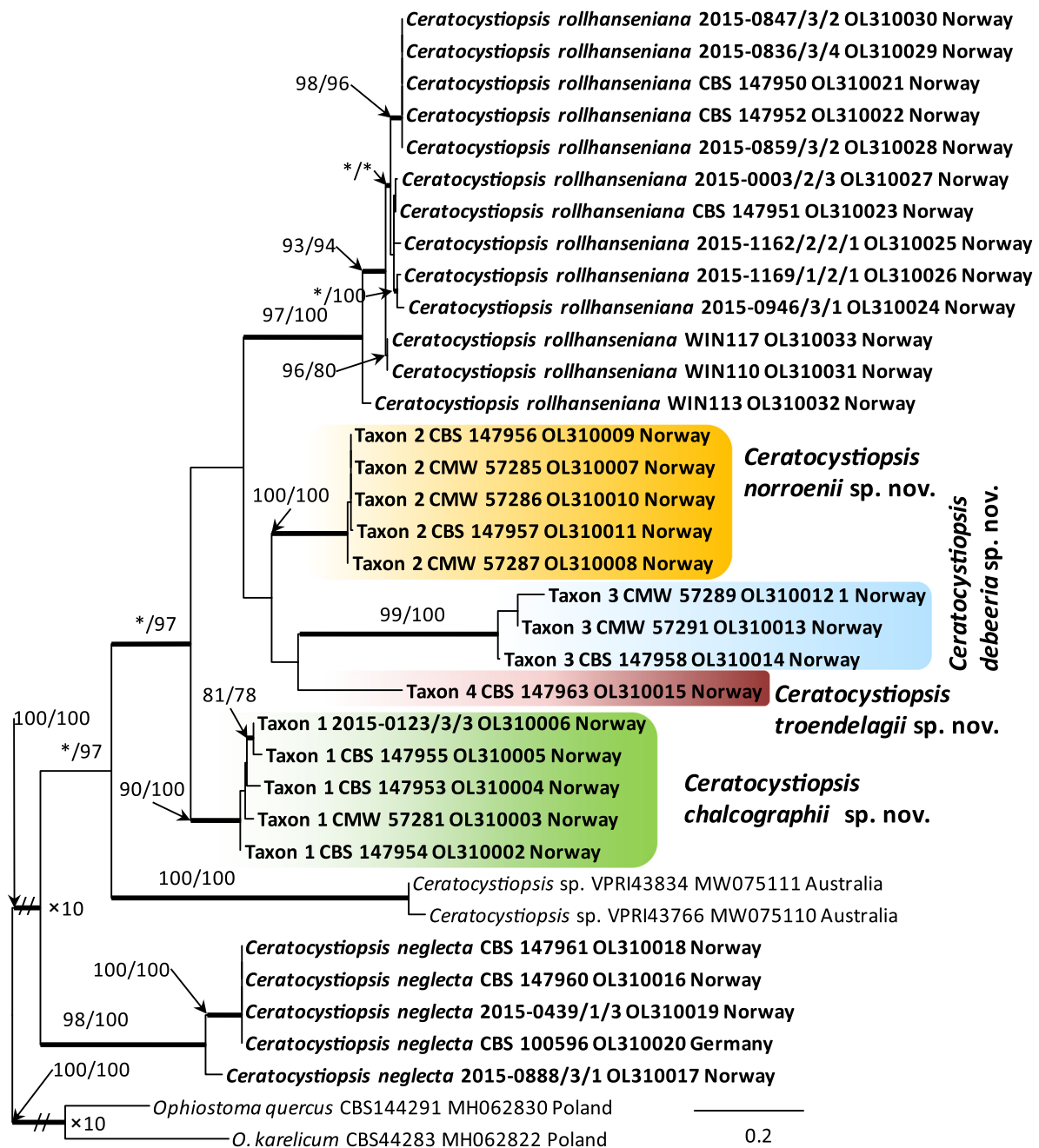


Fig. 6 Phylogram obtained from Maximum Likelihood (ML) analyses of CAL data for the *Ceratocystiopsis* spp. Sequences obtained during this study are presented in bold type. Bootstrap values $\geq 75\%$ for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95

obtained from Bayesian Inference (BI) analyses. *Bootstrap values $< 75\%$. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Ophiostoma karelicum* and *O. quercus* represents the outgroup in analyses of the CAL data

taxon 3 and taxon 4 also form well-supported lineages distinct from other known species of *Ceratocystiopsis* (Figs. 2, 3, 4, 5 and 6). Taxon 3 appears to represent a phylogenetically distinct lineage, potentially sharing ancestry with *C. neglecta* (Figs. 2, 3 and 4). Members of taxon 4 form a lineage adjacent to *C. minima* within the LSU based phylogenetic tree (Fig. 3). However, the other datasets do not support monophyly for these two species (Figs. 2, 4 and 7).

Fig. 7 Phylogram obtained from Maximum Likelihood (ML) analyses of the combined datasets of ITS+LSU+TUB2 data for the *Ceratocystiopsis* spp. Sequences obtained during this study are presented in bold type. Bootstrap values $\geq 75\%$ for ML and Maximum Parsimony (MP) analyses are presented at nodes as follows: ML/MP. Bold branches indicate posterior probabilities values ≥ 0.95 obtained from Bayesian Inference (BI) analyses. *Bootstrap values $< 75\%$. The tree is drawn to scale (see bar) with branch length measured in the number of substitutions per site. *Ophiostoma karelicum* and *O. quercus* represents the outgroup in analyses of the combined datasets of ITS+LSU+TUB2

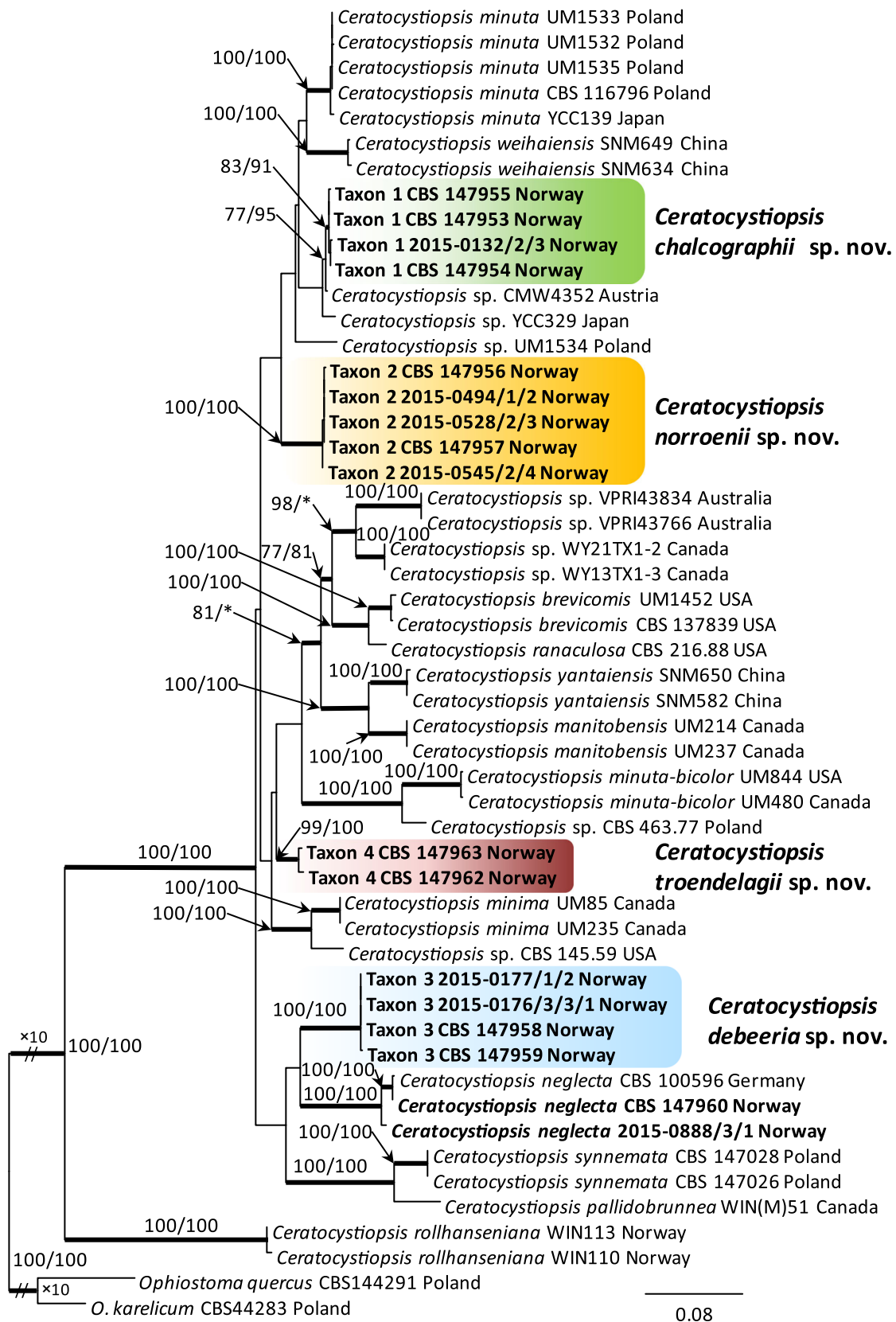


Table 3 Absolute number and frequencies of occurrence (%) of *Ceratocystiopsis* isolates obtained from 10 bark beetle species during the course of this study

Beetle species	Fungus species	<i>Picea abies</i> (Pa)				<i>Pinus sylvestris</i> (Ps)					Total	
		1	2	3	4	5	6	7	8	9		10
	<i>C. chalcographii</i> taxon 1					8(11%)						8
	<i>C. norroenii</i> taxon 2						49(54%)				1(11%)	50
	<i>C. deberia</i> taxon 3					7(9%)						7
	<i>C. trendelagii</i> taxon 4			1(3%)	1(3%)							2
	<i>C. neglecta</i> taxon 5		3(3%)		1(3%)							4
	<i>C. rollhanseniana</i> taxon 6	12(40%)					6(29%)	1(11%)	21(70%)	13(72%)	2(22%)	55

Bark beetle species: 1 = *Crypturgus cinereus*; 2 = *Hylurgops palliatus*; 3 = *Pityogenes saalasi*; 4 = *Polygraphus subopacus*; 5 = *Pityogenes chalcographus*; 6 = *Crypturgus pusillus*; 7 = *Ips acuminatus*; 8 = *Orthotomicus proximus*; 9 = *Pityogenes bidentatus*; 10 = *Pityogenes quadridens*

The analyses of the combined ITS+LSU+TUB2 datasets clearly separated taxa 1 to 4 from the other known species of *Ceratocystiopsis* and also from each other (Fig. 7). Nineteen isolates of taxon 5 grouped with the ex-type isolate of *C. rollhanseniana* (UM113) and other isolates of this species based on LSU (Fig. 3). The TUB2, CAL and TEF1 data also confirmed that those isolates represented *C. rollhanseniana* (Figs. 4, 5 and 6). The ITS, LSU, TUB2, CAL and TEF1 datasets confirmed that four isolates of taxon 6 grouped with ex-type isolate of *C. neglecta* (Figs. 2, 3, 4, 5 and 6).

Taxonomy

The morphological characterization and phylogenetic comparisons based on five genetic loci, showed that four herein described morphotypes (taxon 1, 2, 3 and 4) are distinct from each other and from other known *Ceratocystiopsis* taxa. Therefore, they are described here as new species:

Ceratocystiopsis chalcographii Jankowiak & H. Solheim, sp. nov., Fig. 8

Mycobank: 841844

Etymology: The epithet *chalcographii* (Latin) refers to the species name of the bark beetle vector of this fungus, *Pityogenes chalcographus*.

Diagnosis: This species is closely related to *C. minuta* and *C. norroenii*. *Ceratocystiopsis chalcographii* has longer perithecial necks (36–124 µm vs. 20–45 µm), ostiolar hyphae (7.7–16.4 vs. to 12 µm) and ascospores (9.9–15 vs. 10–13 µm) compared to *C. minuta*. *Ceratocystiopsis chalcographii* has obovoid conidia, whereas *C. minuta* has oblong conidia (Reid and Hausner 2010). *Ceratocystiopsis chalcographii* has longer conidia (2.9–6 µm) compared to *C. minuta* (2–4 µm) (Reid and Hausner 2010). In addition, conidiophores of *C. chalcographii* are simple or slightly branched while *C. minuta* produces simple or verticillate to irregular branched conidiophores (Reid and Hausner 2010). *Ceratocystiopsis norroenii* also produces branched conidiophores; however, this taxon could be distinguished from *C. minuta* by oblong and larger

conidia (2.7–5 × 1.1–2.3 vs. 2–4 × 1.2 µm). In addition, *C. norroenii* does not produce a sexual morph.

Type: Norway, Nannestad, from *Pityogenes chalcographus* infesting *Picea abies*, 15 Oct. 2014, H. Solheim and M.E. Waalberg (O-F-259424 – holotype; CBS 147954 = CMW 57280 – ex-type culture).

Description: *Perithecia* abundantly produced on media and sterilised spruce twigs, formed by single isolates, variable in shape, with a globose or sub-prolate black base, (46–)55–101(–125) µm diam, ending in black, straight or slightly curved necks, (36–)55–88(–124) µm long, (20.3–)23.9–32.3(39.7) µm diam at the base and (5.9–)8.2–13(–17) µm diam at the apex, ending in a tuft of 5–8 *ostiolar hyphae*, hyaline, aseptate, straight, tapering toward the apex, (7.7–)9.2–13(–16.4) µm long. Asci not observed. *Ascospores* one-celled, hyaline, including the sheath falcate in side view (9.9–)11.6–14(–15) × (1–)1.2–1.6(–1.8) µm, accumulating in white-coloured mucoid masses at tip of neck.

Asexual morph hyalorhinocla-diella-like. *Conidiophores* mononematous, micronematous or semi-macronematous, abundantly formed, arising from vegetative hyphae, simple or seldom slightly branched, upright, straight or undulate, producing conidiogenous cells at their apices. *Conidiogenous cells* integrated, hyaline, proliferating percurrently, not denticulate, (1.7–)6.9–23.4(–40.4) × (0.7–)1–1.8(–2.2) µm. *Conidia* hyaline, aseptate, obovoid, the upper part swollen, apex rounded, tapering toward base, sometimes pyriform, (2.9–)3.6–5(–6) × (0.9–)0.9–1.2(–1.9) µm, without sheath.

Colonies with optimal growth at 30 °C on 2% MEA, with radial growth rate of 1.11 (± 0.05) mm/day. **Colonies** white, margin smooth, flocculose, due to abundant aerial mycelium with white lumps of slimy conidia; reverse pale orange (5A3). **Hyphae** hyaline to pale grey in colour, smooth, submerged in medium, not constricted at septa, 1–2.6 (mean 1.7 ± 0.4) µm diam.

Ecology: Isolated from *Pityogenes chalcographus* infesting *Picea abies* and *Pinus sylvestris*.

Habitat: *Picea abies* and *Pinus sylvestris* forests.

Distribution: Currently only known from Norway.

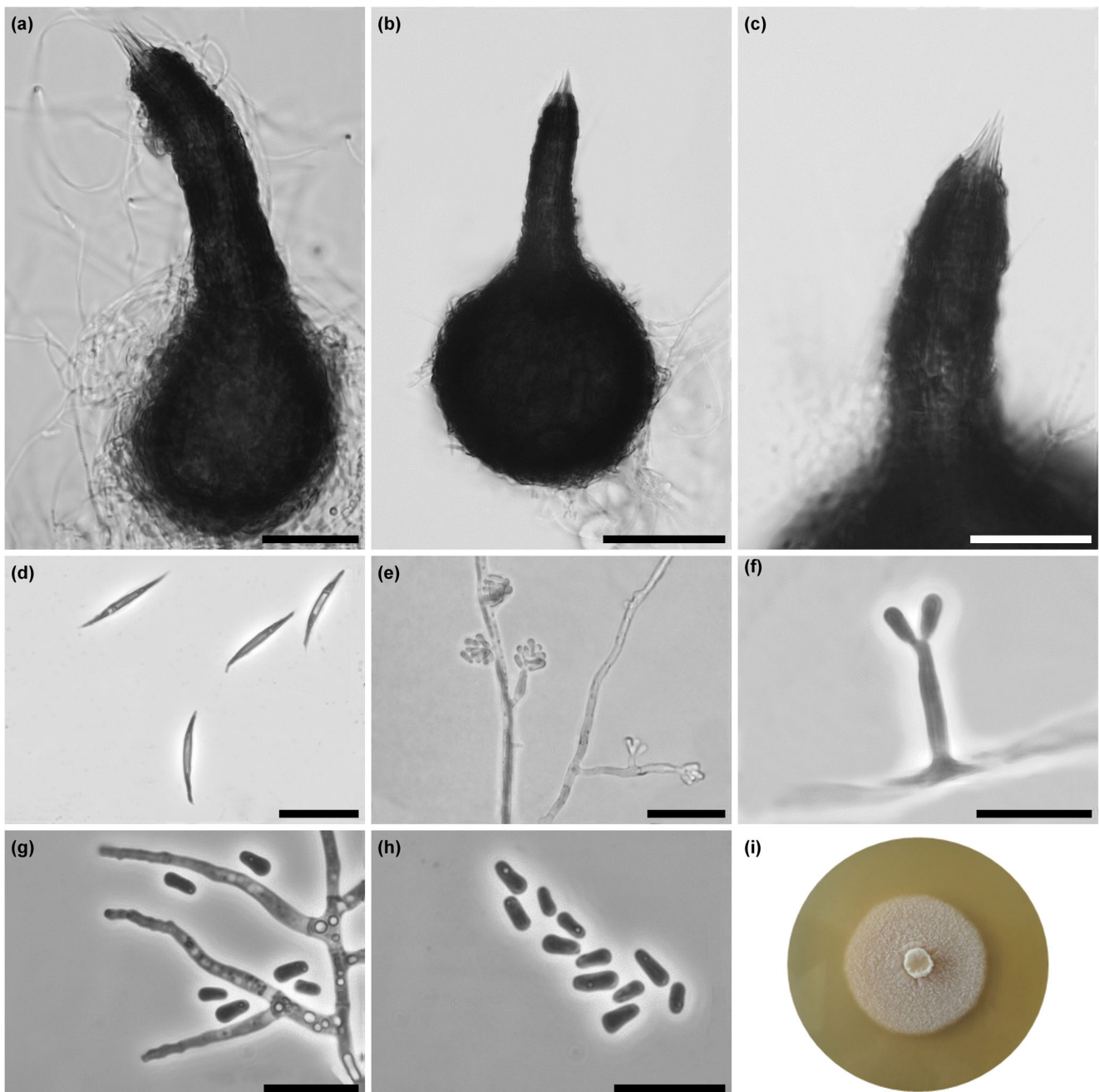


Fig. 8 Morphological characteristics of *Ceratocystiopsis chalcographii* (CBS 147954, Taxon 1). **a–b** Perithecium; **c** Ostiolar hyphae; **d** Ascospores; **e–f** Micronematous conidiophores; **g** slightly branched

micronematous conidiophores; **h** Conidia; **i** 14-day-old culture on 2% MEA. Scale bars: **a** = 25 μ m; **b** = 50 μ m; **c** = 25 μ m; **d** = 10 μ m; **e** = 25 μ m; **f–g** = 10 μ m

Notes: *Ceratocystiopsis chalcographii* and *C. norroenii* formed two distinct, well-supported clades within the European and Japanese putative strains of *C. minuta* (clades C and E according to Plattner et al. 2009) (Figs. 2, 3 and 4). They can be both differentiated from *C. minuta* by the morphology of their sexual and asexual morphs. *Ceratocystiopsis norroenii* is distinct from *C. minuta* by producing oblong and larger conidia and *C.*

chalcographii produces avoid and longer conidia on simple conidiophores. *Ceratocystiopsis minuta* produces oblong smaller conidia on highly branched conidiophores (Reid and Hausner 2010) and the ascocarps in *C. chalcographii* have longer necks, longer ostiolar hyphae and the ascospores are larger compared to those produced by *C. minuta* (see diagnosis and description above, Reid and Hausner 2010).

Ceratocystiopsis chalcographii was found only in association with *P. chalcographus* on Scots pine in Rendalen and on Norway spruce in Nannestad and was isolated from bark beetles infesting *P. abies* and *P. sylvestris* with a frequency of 11% (Table 3). This species is one of two *Ceratocystiopsis* species isolated from *P. chalcographus* (i.e. *Ceratocystiopsis chalcographii* and *Ceratocystiopsis debeeria*).

Additional specimen examined: Norway: Rendalen, from *Pityogenes chalcographus* infesting *Pinus sylvestris*, 29 Oct. 2014, H. Solheim, (O-F-259425, culture CBS 147955 = CMW 57282).

Ceratocystiopsis norroenii Jankowiak & H. Solheim, sp. nov., Fig. 9

Mycobank: 841845

Etymology: The epithet *norroenii* (Latin) refers to the word Norrøn, used for the North in Norse mythology.

Diagnosis: See comparisons between *C. norroenii* and *C. minuta* under *C. chalcographii*.

Type: Norway, Karasjok, from *Ips acuminatus* infesting *Pinus sylvestris*, 21 Jun. 2015, H. Solheim (O-F-259426 – holotype; CBS 147956 = CMW 57283–ex-type culture).

Description: Sexual morph not observed.

Asexual morph hyalorhinoclaidiella-like. *Conidiophores* micronematous or macronematous, abundantly formed. Micronematous conidiophores arising from vegetative hyphae, simple or branched (1 branches), hyaline, upright, straight or undulate, producing apical conidiogenous cells; forming often a single conidium only, formed either laterally on vegetative hyphae or on short lateral branches; *conidiogenous cells* integrated, hyaline, proliferating percurrently, sometimes denticulate (1.4–)12.1–28.1(–33.3) × (0.9–)1.2–1.7(–2.1) μm; macronematous conidiophores arising from vegetative hyphae, hyaline, (22.2–)29.6–48.2(–64.9) long, and (8.5–)11.2–19.4(–15.8) μm wide at the apex, arising from a basal cell, (4.3–)6.2–11.7(–16) × (1.4–)1.7–2.6 (–3.1) μm, irregularly branched or penicillate, with 1 or 2 branches producing 3–4 apical conidiogenous cells per branch; *conidiogenous cells* integrated,

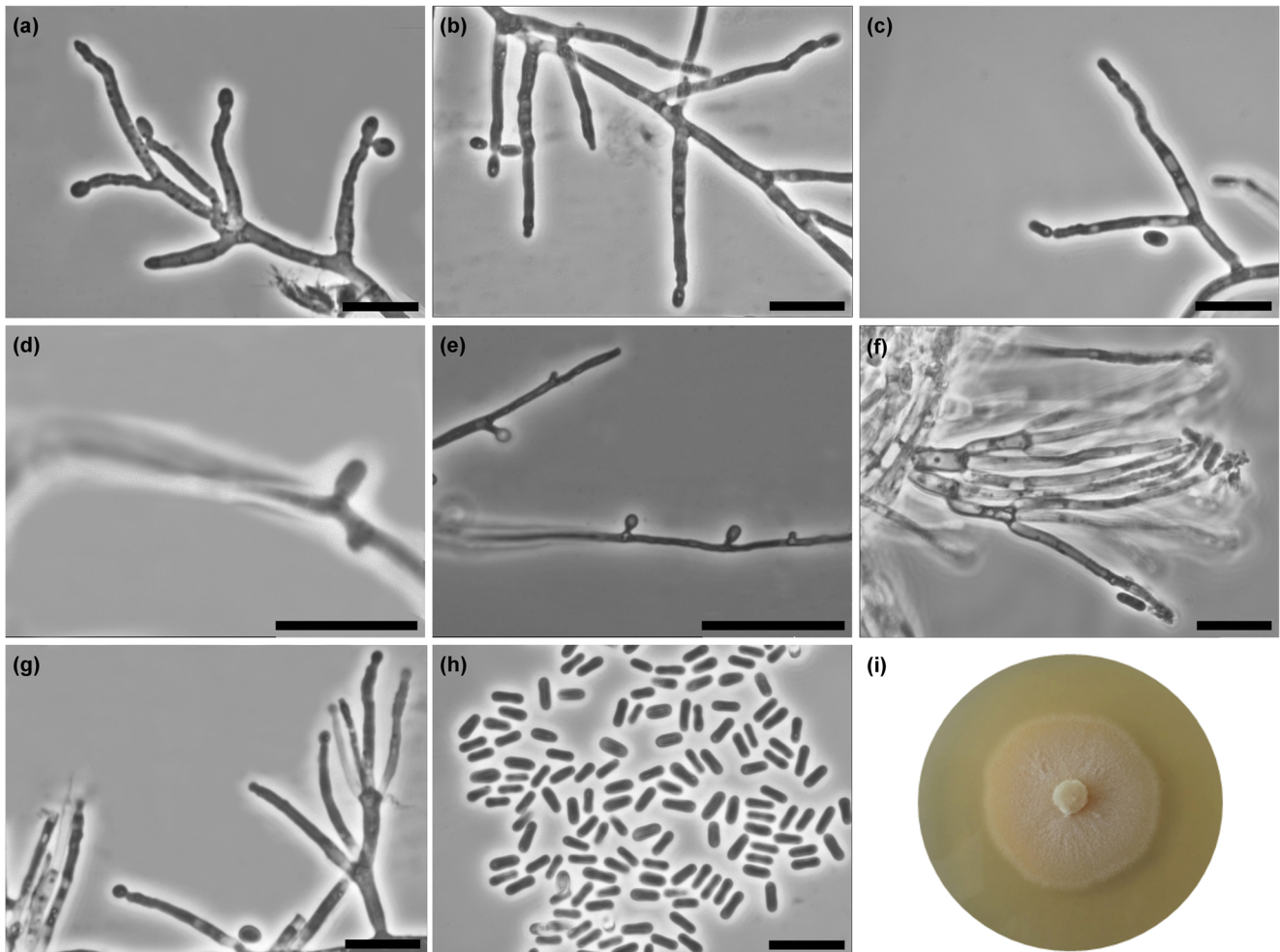


Fig. 9 Morphological characteristics of *Ceratocystiopsis norroenii* (CBS 147956, Taxon 2). **a–c** Simple (**a**, **b**) and slightly (**c**) branched micronematous conidiophores; **d–e** Conidia arising directly from

hyphae; **f–g** branching macronematous conidiophores; **h** conidia; **i** 14-day-old culture on 2% MEA. Scale bars: **a–d** = 10 μm, **e** = 25 μm, **f–h** = 10 μm

hyaline, proliferating percurrently, sometimes denticulate $(13.1\text{--}15.9\text{--}27.2\text{--}(35.8) \times (1\text{--}1.2\text{--}1.5\text{--}(1.8) \mu\text{m}$; *conidia* hyaline, smooth, unicellular, oblong, sometimes slightly obovoid, $(2.7\text{--}3.2\text{--}4.1\text{--}(5.1) \times (1.1\text{--}1.2\text{--}1.6\text{--}(2.3) \mu\text{m}$, without mucilaginous sheath.

Colonies with optimal growth at 25 °C on 2% MEA, with radial growth rate of 1.11 mm (± 0.15 mm) per day. *Colonies* orange white (5A2), reverse pale orange (5A3), margin smooth, with suede-like surface, but occasionally funiculose; *hyphae* hyaline, smooth, submerged in medium and aerial mycelium rarely present, 0.5–1.8 (mean 1 ± 0.3) μm diam.

Ecology: Isolated from *Ips acuminatus* and *Pityogenes quadridens* infesting *Pinus sylvestris*.

Habitat: *Pinus sylvestris* forests.

Distribution: Currently only known from Norway.

Notes: See comparisons between *C. norroenii* and *C. minuta* under *C. chalcographii*. *Ceratocystiopsis norroenii* was frequently isolated from *Ips acuminatus* and *Pityogenes quadridens* infesting Scots pine growing in the north part of Norway.

Additional specimen examined: **Norway**: Målselv, from *Ips acuminatus* infesting *Pinus sylvestris*, 21 Jun. 2015, H. Solheim (O-F-259427, culture CBS 147957 = CMW 57284).

Ceratocystiopsis debeeria Jankowiak & H. Solheim, sp. nov., Fig. 10

Mycobank: 841846

Etymology: Named for Prof. Wilhelm de Beer who has contributed greatly to the taxonomy of ophiostomatoid fungi.

Diagnosis: *Ceratocystiopsis debeeria* is the closest phylogenetic relative of *C. neglecta*, from which it is differentiated by the absence of perithecia in *in vitro* culture, simple or slightly irregularly branched conidiophores, and by conidial size, which is $2.7\text{--}5.2 \times 0.9\text{--}1.7 \mu\text{m}$ in the former and $3\text{--}4.5 \times 1\text{--}1.5 \mu\text{m}$ in the latter.

Type: **Norway**, Flesberg, from *Pityogenes chalcographus* infesting *Picea abies*, 31. Oct. 2014, M.E. Waalberg and R.A. Lindseth (O-F-259428 – holotype; CBS 147958 = CMW 57288–ex-type culture).

Description: *Sexual morph* not observed.

Asexual morph hyalorhinocla-diella-like. *Conidiophores* mononematous, abundantly formed, micronematous, arising from vegetative hyphae, simple or seldom slightly and irregularly branched, upright, straight or undulate, producing apical conidiogenous cells; *conidiogenous cells* integrated, hyaline, proliferating percurrently, rarely denticulate $(7.8\text{--}10.6\text{--}25.8\text{--}(37.9) \times (0.8\text{--}1\text{--}1.4\text{--}(1.8) \mu\text{m}$; *conidia* hyaline, aseptate, obovoid with the upper part swollen, apex round, tapering toward base, sometimes oblong, without mucilaginous sheath, $(2.7\text{--}3.1\text{--}4.2\text{--}(5.2) \times (0.9\text{--}1.1\text{--}1.4\text{--}(1.7) \mu\text{m}$.

Colonies with optimal growth at 25 °C on 2% MEA, with radial growth rate of 0.82 (± 0.18) mm/day; no growth at 35 °C. *Colonies* yellowish white (2A2), reverse pale orange (5A3), margin smooth, with suede-like surface; *hyphae* hyaline to pale grey (B1) in colour, smooth, submerged in the medium and aerial mycelium rarely observed, 0.8–2.9 (mean 1.6 ± 0.4) μm diam.

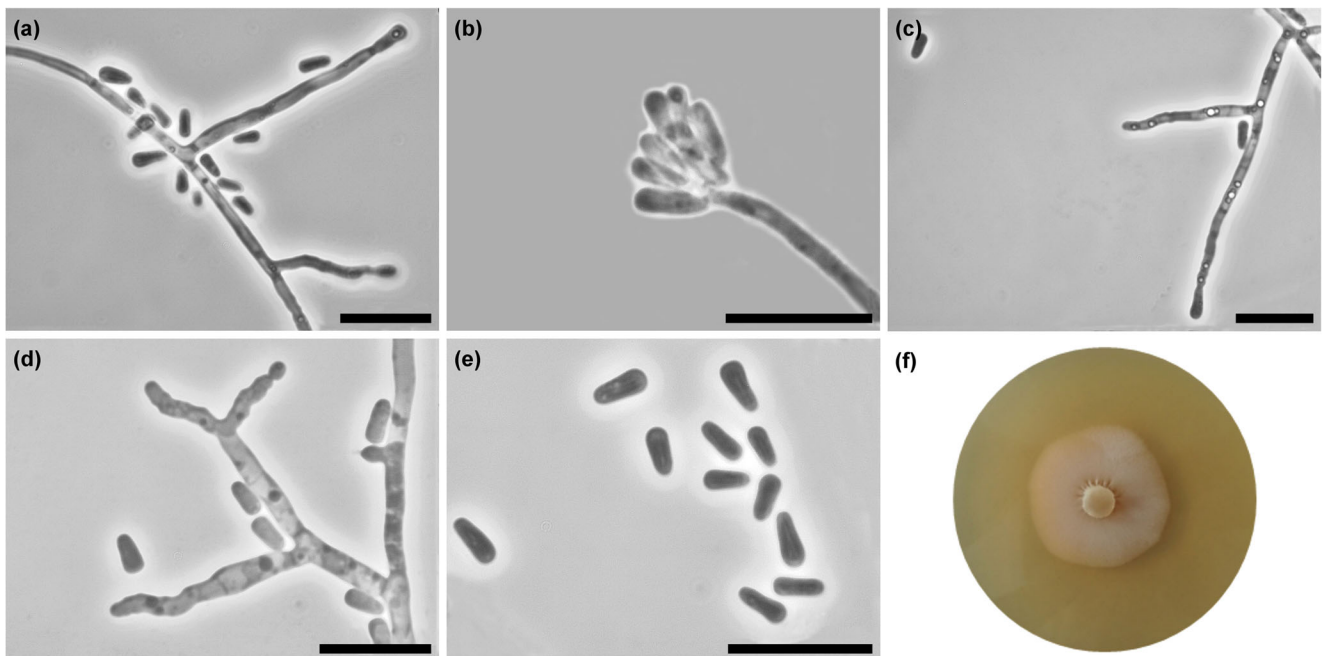


Fig. 10 Morphological characteristics of *Ceratocystiopsis debeeria* (CBS 147958, Taxon 3). **a–d** Simple (**a**, **b**) and slightly branched (**c**, **d**) micronematous conidiophores; **e** Conidia; **f** 14-day-old culture on 2% MEA. Scale bars: **a–e** = 10 μm

Ecology: Isolated from *Pityogenes chalcographus* infesting *Picea abies* and *Pinus sylvestris*.

Habitat: *Picea abies* and *Pinus sylvestris* forests.

Distribution: Currently only known from Norway.

Notes: In a phylogenetic perspective, *C. debeeria* is closely related to *C. neglecta* (Figs. 2, 3 and 4). *Ceratocystiopsis debeeria* is characterised by the absence of perithecia in *in vitro* culture, which are present in *C. neglecta* (Kirschner

and Oberwinkler 1999). In addition, *C. debeeria* and *C. neglecta* can be distinguished by their conidial size.

Ceratocystiopsis debeeria appears to be rare and it was only isolated on a few occasions from *Pityogenes chalcographus* infesting *Picea abies* in Rendalen and *Pinus sylvestris* in Flesberg.

Additional specimen examined: Norway, Rendalen, from *Pityogenes chalcographus* infesting *Pinus sylvestris*, 29

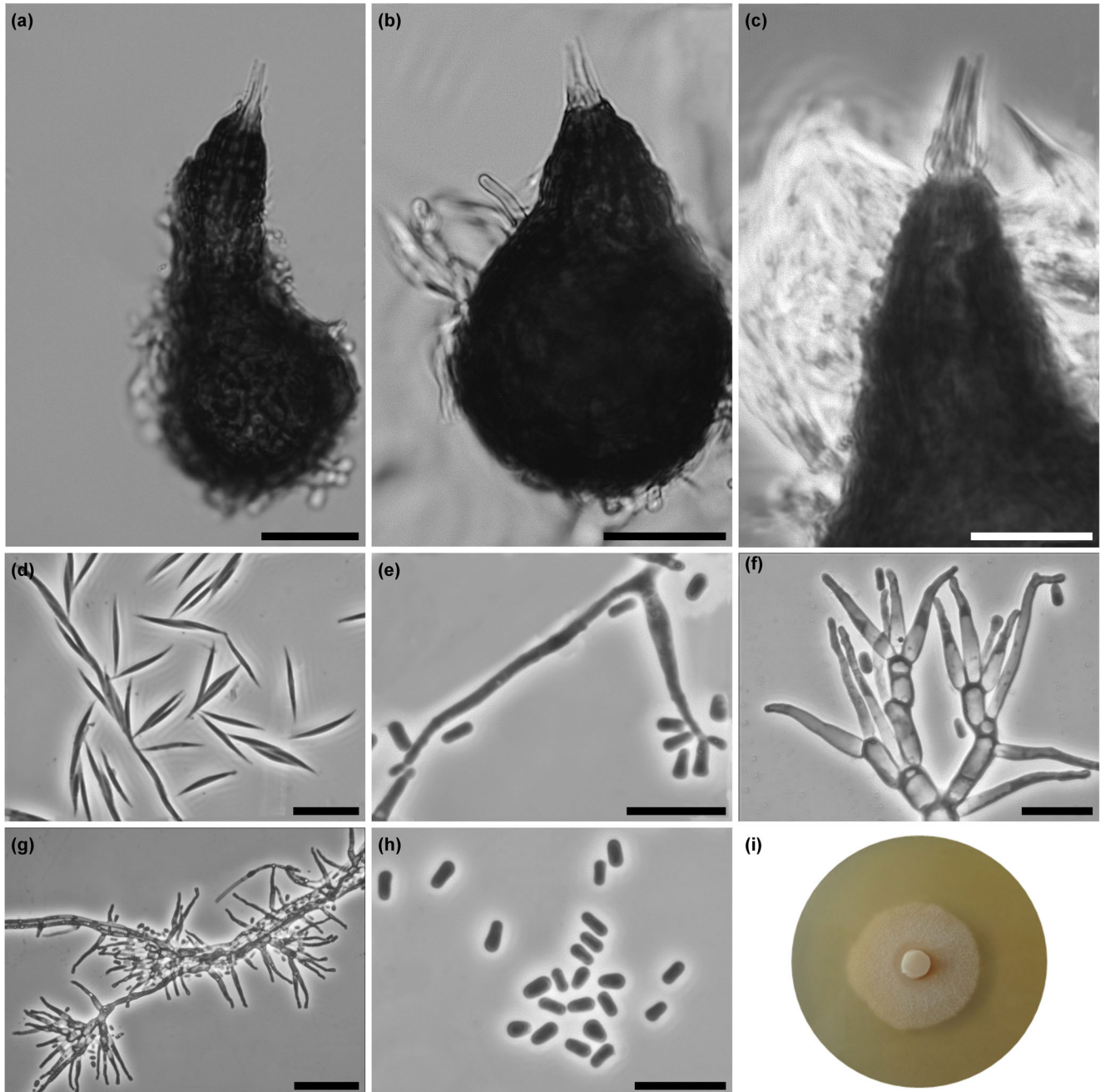


Fig. 11 Morphological characteristics of *Ceratocystiopsis troendelagii* (CBS 147962, Taxon 4). **a, b.** Perithecium; **c** Ostiolar hyphae; **d** Ascospores; **e** Microneumatous conidiophores; **f–g** branch

macroneumatous conidiophores; **h** Conidia; **i** Fourteen-day-old culture on MEA. Scale bars: **a–b** = 25 μ m; **c–f** = 10 μ m; **g** = 25 μ m; **h** = 10 μ m

Oct. 2014, *H. Solheim* (O-F-259429, culture CBS 147959 = CMW57290).

Ceratocystiopsis troendelagii Jankowiak & H. Solheim, sp. nov., Fig. 11

Mycobank: 841847

Etymology: The epithet *troendelagii* (Latin) refers to the area in Mid-Norway, Trøndelag, from which this fungus was collected.

Type: **Norway**, Lierne, from *Polygraphus subopacus* infesting *Picea abies*, 8 Aug. 2015, *T. Kvamme* and *Å. Lindelöv* (O-F-259430 – holotype; CBS 147962 = CMW57294 ex-type culture).

Diagnosis: The species is characterised by having a sexual and an asexual, hyalorhinocliadiella-like morph, differentiated from the closely related species *C. minima* by smaller perithecial base diameter 25–54 µm vs. 40–85 µm, mainly obovoid vs clavate or oblong conidia, which are also shorter (2.7–5.2 µm vs. 2.5–8.0) µm.

Description: *Perithecia* abundantly produced on media and sterilised spruce twigs, formed by single isolates, with a globose or sub-globose black base, (25–)33–45(–54) µm diam, ending in black, straight or slightly curved necks, (9–)11–29(–72) µm long, (12.5–)15–20(24.6) µm at the base and (4–)4.7–7.7(–10.7) µm diam at the apex, with 3–6 *ostiole* hyphae hyaline, aseptate, straight, (3.9–)7.2–10.5(–11.8) µm long, tapering extensively toward the apex. Asci not observed. *Ascospores* one-celled, hyaline, including the sheath falcate in side view (7–)8.8–11.8(–13.5) × (0.7–)0.8–1.1(–1.3) µm, accumulating in a white-coloured drop at tip of necks.

Asexual morph hyalorhinocliadiella-like. *Conidiophores* micronematous or macronematous, abundantly formed. Micronematous conidiophores arising from vegetative hyphae, simple, hyaline, upright, straight or undulate, producing apical conidiogenous cells; *conidiogenous cells* integrated, hyaline, proliferating percurrently, sometimes denticulate, (5.9–)8.7–20.4(–30.4) × (0.8–)1.3–2.2(–2.7) µm; macronematous conidiophores arising from vegetative hyphae, hyaline, (17.8–)21.9–34.8(–41.2) long, and (4.4–)11.7–28.8(–37.7) µm wide at the apex, arising from a basal cell, (3.5–)5.1–10.9(–16.5) × (1.7–)2.2–3.3(–4.1) µm, irregularly branched with 2–3 branches, producing 2–4 apical conidiogenous cells per branch; *conidiogenous cells* integrated, hyaline, proliferating percurrently, sometimes denticulate, (10.5–)12.1–17.9(–23.6) × (1.4–)1.6–2.2(–2.7) µm; *conidia* hyaline, smooth, unicellular, oblong or obovate (2–)2.6–3.5(–4.1) × (0.8–)1.1–1.8(–2.2) µm, without mucilaginous sheath.

Colonies with optimal growth at 20 °C on 2% MEA with radial growth rate 0.72 (± 0.05) mm/day; no growth at 35 °C. **Colonies** yellowish white (2A2), reverse pale orange(5A3), margin smooth, with suede-like surface, occasionally funiculose; *hyphae* hyaline, smooth, submerged in medium

and aerial mycelium rarely observed, 0.7–2.2 (mean 1.2 ± 0.4) µm diam.

Ecology: Isolated from *Pityogenes saalasi* and *Polygraphus subopacus* infesting *Picea abies* and *Pinus sylvestris*

Habitat: *Picea abies* and *Pinus sylvestris* forests

Distribution: Currently only known from Norway.

Notes: *Ceratocystiopsis troendelagii*, based on LSU data is phylogenetically related to *C. minima* (Fig. 3) but formed a distinct clade in both the ITS and the *TUB2* based tree (Figs. 2 and 4). It can be differentiated from *C. minima* by dimension of the perithecial base and conidial morphology.

Ceratocystiopsis troendelagii was the rarest *Ceratocystiopsis* species isolated in this study. It was isolated twice in the same plot, once from *Pityogenes saalasi* and once from *Polygraphus subopacus*. Both bark beetles have a northern distribution in Fennoscandia.

Additional specimen examined: **Norway**, Lierne, from *Pityogenes saalasi* infesting *Picea abies*, 8 Aug. 2015, *T. Kvamme* and *Å. Lindelöv* (O-F-259431, culture: CBS 147963 = CMW57295).

Discussion

We collected 22 species of bark beetles from *Picea abies* and *Pinus sylvestris* located in Norway. From these beetles, we recovered 126 isolates that based on morphology can be assigned to *Ceratocystiopsis*, representing six species. Both molecular and morphological data revealed four undescribed species, herein described as *C. chalcographii*, *C. debeeria*, *C. norroenii* and *C. troendelagii*, and two known species, *C. neglecta* and *C. rollhanseni*.

Description of these new species brings the total number of recognized species in this genus to 25, of which seven occur in Norway. These include the six species recovered in this study as well as *C. minuta*, which was previously found in association with *I. typographus* (Solheim 1986, 1992, 1993). *Ceratocystiopsis rollhanseni* was described by Hausner et al. (2003) based on the Norwegian isolates collected by James Reid from *P. sylvestris*.

Ceratocystiopsis norroenii and *C. rollhanseni* were the most frequently isolated *Ceratocystiopsis* species. However, these species appear to have different distribution and autecology including insect associations. *Ceratocystiopsis norroenii* appears to be a consistent associate of *I. acuminatus* on *P. sylvestris* while *C. rollhanseni* has broader host/vector associations including different bark beetle species found on *P. abies* and *P. sylvestris*. Our phylogenetic analysis of the LSU sequences (Fig. 3) showed that some Polish isolates (CBS 116963, CBS 116796) previously identified as *C. minuta* and collected from *P. abies* infested by *I. typographus* actually represented *C. rollhanseni*, showing a wider distribution in Europe.

Ceratocystiopsis norroenii belongs to the *C. minuta sensu lato* lineage that includes several sublineages in Europe and Japan (Plattner et al. 2009), representing a different, morphologically well-defined phylogenetic species. To stabilize the concept of *C. minuta*, an epitype was designated by Reid and Hausner (2010), RJ705 = UAMH 11218 = WIN(M) 1532. The *C. norroenii* lineage was not represented in Plattner et al. (2009), suggesting that the *C. minuta* species complex could accommodate numerous morphologically similar species. For example, *C. weihaiensis*, a species from China, was also shown to be phylogenetically close to *C. minuta* (Chang et al. 2021).

Ceratocystiopsis chalcographii belongs also to the *C. minuta* species complex. These isolates were recovered from *P. chalcographus* on *P. abies*. Several isolates from Scotland, Austria and Japan, previously identified as *C. minuta* and placed in clade D (Plattner et al. 2009), were shown to have ITS, LSU and *TUB2* sequences identical or very similar to those of *C. chalcographii* (Figs. 2, 3 and 4). Therefore, *C. chalcographii* may have a wider geographical distribution in Europe and possibly Asia. This species also shares an identical LSU sequence with an isolate from the USA (CBS 145.59). More studies are needed on the taxonomic/genetic diversity of the *C. minuta* species complex to resolve the various cryptic species that might be part of this group. In this study, *P. chalcographus* is also the only vector of *C. debeeria*, suggesting in this case species-specific ectosymbiont relationships between them.

Ceratocystiopsis troendelagii and *C. neglecta* were found only on a few occasions in our study. *Ceratocystiopsis troendelagii* seems to be preferably associated with *P. saalasi* and *P. subopacus*, while *C. neglecta* was found associated with *H. palliatus* and *P. subopacus*. This is the first report of *C. neglecta* in association with *P. subopacus*. Previously, *C. neglecta* was only known from different bark- and ambrosia beetles including *C. cinereus*, *C. pusillus*, *D. autographus*, *H. palliatus*, *I. typographus*, *P. chalcographus* and *T. lineatum* infesting *P. abies* and *P. sylvestris* in Germany (Kirschner 2001; Kirschner and Oberwinkler 1999).

Most of the formally described species of *Ceratocystiopsis* are known only from conifers, although *C. lunata* and *C. synnemata* were recently collected from hardwood-infesting beetles (Li et al. 2018; Strzałka et al. 2020; Nel et al. 2021). The results of the present study confirm that *Ceratocystiopsis* species have a close affinity to conifers.

Based on morphological characters and DNA sequence data, four new species of ophiostomatalean fungi were described and one is reported from Norway for the first time. The relationships between fungi and bark beetles are continuously studied in Europe and worldwide (Kirisits 2004; Linnakoski et al. 2012; Jankowiak et al. 2020; Marincowitz et al. 2020; Chang et al. 2021; Nel et al. 2021; Trollip et al. 2021). Our survey led to the discovery and description of four

new species, suggesting that there is still a lot of unknown taxonomic and ecological diversity to be uncovered for the ophiostomatalean fungi in Europe.

Acknowledgements We would like to thank and acknowledge the following for their assistance across various aspects of this study. Terje B. Dahl, Torstein Kvamme, Geir Kvammen, Åke Lindelöv Ruben A. Lindseth, Tor H. Sundt and Max E. Waalberg, for their assistance in field sampling. Anne E. Nielsen, Max E. Waalberg and Gro Wollebæk participated in laboratory work. T.K. helped with the identification of bark beetles.

Abbreviations BI: Bayesian Inference; *TUB2*: beta-tubulin; *CAL*: Calmodulin; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; ITS: The internal transcribed spacer; LSU: The large ribosomal subunit (28S); MEA: Malt extract agar; ML: Maximum likelihood; MP: Maximum Parsimony; NCBI: National Center for Biotechnology Information; NIBIO: Culture collection of Norwegian Institute of Bioeconomy, Norway; O: the Mycological Herbarium, Natural History Museum, University of Oslo, Norway; *s.l.*: *sensu lato*; *s.str.*: *sensu stricto*; *TEF1*: Translation elongation factor 1- α ; UAMH: The UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Canada; WIN(M or UM): Culture Collection of the University of Manitoba (Winnipeg); Canada.

Author contribution HS conceived the idea of this study, performed isolations and DNA sequencing and edited the final version of the manuscript. RJ performed DNA sequencing, performed microscopy for the species descriptions and edited the description text and wrote the manuscript. PB performed phylogenetic analysis. JM performed DNA sequencing, GH performed DNA sequencing and edited the final version of the manuscript.

Funding Open access funding provided by Norwegian Institute of Bioeconomy Research. This work was funded by the Norwegian biodiversity information centre on the project ‘Ophiostomatoid fungi in Norway’ (No. 51-14) and Norwegian Institute of Bioeconomy Research, (No. 132001). This work was supported by the Ministry of Science and Higher Education of the Republic of Poland (SUB/040013/D019).

Data availability All data generated or analysed during this study are included in this published article and/or is available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate Not applicable.

Adherence to national and international regulations Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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