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ORIGINAL ARTICLE

Microstrobilinia castrans, a new genus and species of the *Sclerotiniaceae* parasitizing pollen cones of *Picea* spp.

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Abstract
The fungal pathogens of spruce are well known in Europe and elsewhere. Therefore, it was surprising to discover a new fungal species and genus in Central Europe that attacks the pollen cones of three spruce species. The new ascomycete forms apothecia on stromatized pollen cones of Norway spruce (*Picea abies*) and Serbian spruce (*Picea omorika*) in mountain areas and on West Himalayan spruce (*Picea smithiana*) planted in urban lowland regions of Switzerland, Germany, and Italy. It was also detected in France, based on metabarcode sequences deposited in the GlobalFungi database. Its sudden appearance and the different origins of the host trees in Europe and Asia leave the origin of the fungus unclear. The new fungus might be a neomycete for Europe. A phylogenetic analysis using SSU, LSU, ITS, *RPB2*, and *TEF1* sequences classified the fungus as a member of *Sclerotiniaceae* (*Helotiales*, *Leotiomycetes*). However, it differs morphologically from the other genera of this family in having an ascus without apical apparatus containing four mainly citriform spores with 16 nuclei each. Furthermore, it is the only known cup fungus that parasitizes pollen cones of conifers by stromatizing their tissue and infecting pollen grains. The fungus does not seem to cause major damage to the spruce populations, as only a few pollen cones per tree are affected. All this leads us to describe the newly discovered fungus as the new species and new genus *Microstrobilinia castrans*, the fungus that castrates pollen cones of spruce.

Keywords GlobalFungi database · *Helotiales* · Multinucleate ascospores · Neomycete · New genus · New species · Phenology · Pollen parasite

Introduction

The conifer genus *Picea* (*Pinaceae*) includes about 35 species that occur in the northern temperate and boreal regions of the world. However, in mountainous areas, some species reach far into the south, such as *Picea chihuahuana* Martinez in Mexico (Schmidt 2004; Schmidt-Vogt 1987). In Europe, *Picea abies* (L.) H. Karst., the native Norway spruce, is one of the most important timber species with a long history of economic use and forestry cultivation. This has led to its widespread distribution and high frequency in plantations and sylvicultural settings outside its natural distribution range. In addition, several non-native spruce species, e.g., the North American *Picea pungens* Engelm. and *Picea sitchensis* (Bong.) Carrière, are planted in forests or as ornamental trees in parks and gardens in Europe. Due to the economic importance of spruce trees, their diseases and parasites are very well investigated (e.g., Kujala 1950; Schmidt-Vogt 1989; Butin 2011; Farr and Rossman 2022).

All spruce species are monoecious conifers with large woody female seed cones and small male pollen cones. Several fungi are known to parasitize on the seed cones, such as the rust fungi *Thekopsora areolata* (Fr.) Magnus and *Chrysomyxa pyrolata* (Körn.) G. Winter (Butin 2011; Phillips and Burdekin 1992). Others use the woody cones as substrate without causing obvious damages. Examples are *Phragmotrichum chailletii* Kunze occurring on the scales of still hanging cones and *Piceomphale bulgarioides* (P. Karst.) Svrček or *Strobilurus esculentus* (Wulfen) Singer on fallen cones. The seeds can also become infected and killed by *Caloscypha fulgens* (Pers.) Boud. (Paden et al.

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1978; Kolotelo et al. 2001). However, no fungus has been known to grow exclusively on the pollen cones of spruce species until now (Farr and Rossman 2022).

In early spring 2018, the apothecia of an unknown cup fungus were first discovered on the pollen cones of two *Picea smithiana* (Wall.) Boiss (West Himalayan spruce) planted in the arboretum of the Swiss Federal Institute for Forest, Snow and Landscape Research WSL in Birmensdorf, Switzerland. This spruce species is native from Afghanistan to Nepal where it grows at altitudes of 2000–4000 m above sea level (asl.) in mixed forests (Schmidt-Vogt 1987). The ascomycete was thereafter also found on *P. smithiana* in other parks in Switzerland. A more detailed investigation revealed that this new fungus also occurs on the native *P. abies* in the Swiss Alps and Jura mountains and in mountainous regions of southern Germany and northern Italy. Additionally, it was found on the non-native *Picea omorika* (Panč.) Purk. (Serbian spruce) planted in parks and gardens.

Despite intensive literature research and consultation of the specialist for inoperculate Ascomycetes Hans-Otto Baral (Tübingen, Germany, personal communication) and his expert network, no fungal species or genus could be found that matched the characteristics of the newly discovered fungus. The aim of this study was therefore to clarify the taxonomy of the newly discovered pathogen, what resulted in the description of a new monotypic genus.

Materials and methods

Sampling

Pollen cones stromatized by the new fungus and bearing primordial to mature apothecia were regularly collected from two *P. smithiana* trees in the arboretum of the Swiss Federal Institute WSL. To determine host specificity, distribution, and abundance of the encountered fungus, various *Picea* species also of other regions in Switzerland, several sites in southern Germany, and one site in Italy were explored (Table S1). These included individual *Picea abies* trees in pastures and at forest edges in lowland and mountainous forests. Planted exotic *Picea omorika* and *P. smithiana* trees were examined in urban areas. For the latter, the official tree inventories of 30 Swiss cities and municipalities were evaluated to find locations of *P. smithiana*. This tree species was only registered in the city of Zurich (Stadt Zürich 2021), in the canton of Geneva (Wyler 2021) and in Lausanne (canton Vaud) (Table S1). Also, *P. smithiana* trees were examined in the Swiss National Arboretum of the Vallon de l'Aubonne (www.arboretum.ch). At all sites, other spruce species growing in the vicinity of *P. smithiana*, especially *P. abies* and *P. omorika*, were closely inspected. The collection points (Table S1) were visualized in QGIS 3.16 using (i) free vector maps from www.naturalearthdata.com; (ii) the distribution map of *Picea abies* from Caudullo et al. (2017, 2018) for the map of Europe; and (iii) free geodata from the Swiss Federal Office of Topography (swisstopo) for the map of Switzerland. A selection of vouchers was deposited in the fungal herbarium of ETH Zurich (ZT Myc). The remaining samples are stored at WSL (Table S1). Five collections (including the type collection) representing the three host tree species and main regions of Switzerland were chosen for cultivation and molecular investigations (Table 1).

Fungal isolations from different sources

Single ascospore isolations were obtained from ten stromatized pollen cones with mature apothecia from each of the 5 representative samples (Table 1). To induce sporulation, cones were incubated in moist chambers for 1 day at 20 °C. One apothecium was separated from each cone using tweezers and fixed with double-sided adhesive tape upside down on the underside of the lid of a 9 cm Petri dish containing WA + S (Table 2). After half a day at 20 °C, 10 individual germinating spores per apothecium were picked under a binocular microscope (100 × magnification) with a fine needle and each was transferred to a fresh DMA + S (Table 2) plate (in total 500 isolates). For the isolation of the fungus from the stromatized pollen cones, one cone per representative sample was cut open and 4 small pieces (about 1 mm^3) of blackened tissue were removed from the inside with a sterile scalpel and placed on a DMA + S plate (in total 20 isolates). All cultures were then incubated at 20 °C for 2 weeks. For each of the 5 representative samples, one single spore culture and one culture from the cones were chosen for comparison of ITS sequences (in total 10). Multi-locus phylogenetic analyses were based on a total of 5 isolates (1 × from pollen cone, 4 × from single spores) (Table 1). A single spore culture of *Elliottinia kerneri* (Wettst.) L.M. Kohn was produced and analyzed in the same way. The isolates were deposited in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands (Table 1).

To investigate which parts of the pollen cones are infected by the fungus at what time, ten freshly developed, still immature pollen cones showing early symptoms of an infection (deformations, brown discolorations, and white mycelium between the scales) were collected from one *P. smithiana* tree at the WSL site on May 2, 2019. Ten healthy looking freshly developed pollen cones were taken as control. These 20 cones were surface disinfected by shortly wiping off the surface with 70% (v/v) ethanol before they were longitudinally cut in half with a scalpel. At the tip and the central part of each cone, small pieces of tissue were removed from (i) outer scales, (ii) pollen sacs and (iii) the central axis (Fig. S1), resulting in six isolation tests per healthy and six per pollen cone with early symptoms (2 × 60 tissue pieces). All tissue pieces were

Table 1 Specimens used in the phylogenetic study, including GenBank accession numbers of ITS, LSU, SSU, *RPB2*, and *TEF1* sequences

Species	Country	Canton	Host	Vaucher	Culture no	Isolation source	GenBank accession no				
							ITS	**LSU**	**SSU**	***RPB2***	***TEF1***
Botrytis cinerea (*Botryotinia fuckeliana*)	–		–	OSC 100012			DQ491491	AY544651	AY544695	DQ247786	DQ471045
Cenangiopsis quercicola	Denmark		*Quercus robur*	TAAM 178677			LT158425	KX090811	KX090862	KX090713	KX090663
Cenangium acuum	Germany		*Pinus sylvestris*	TAAM 198515			LT158439	KX090822	KX090873	KX090720	KX090674
Cenangium ferruginosum	Montenegro		*Pinus nigra*	TAAM 198451			LT158471	KX090840	KX090892	KX090739	–
Ciboria caucus	Norway		*Salix caprea*	isolate 1572.1			Z73766	Z73740	Z73712	–	–
Ciboria viridifusca	Estonia		*Alnus* sp.	TAAM 165962			LT158429	KX090812	KX090863	–	–
Dumontinia tuberosa	Estonia		*Anemone nemorosa*	TU 109263			LT158412	KX090843	KX090897	–	KX090697
Elliottinia kerneri	Switzerland	Zurich	*Abies alba*	ZT Myc 66401	**CBS 149470**	single spore	**OP325832**	**OP325826**	**OP325820**	**OP346980**	**OP346974**
Encoelia furfuracea	Estonia		*Corylus avellana*	TAAM 165633			LT158416	KX090798	KX090850	KX090701	KX090653
Lambertella subrenispora	Japan		*Aster ageratoides* var. *ovata*	CUP JA 3663	CBS 811.85		MH861915	DQ470978	DQ471030	DQ470930	DQ471101
Lanzia luteovirescens	Estonia		*Acer platanoides*	TU 104450			LT158431	KX090814	–	KX090716	KX090666
Microstrobilinia castrans sp. nov.	Switzerland	Zurich	*Picea smithiana*	ZT Myc 66340[H]	**CBS 149465**	pollen cone	**OP325827**	**OP325825**	**OP325815**	**OP346975**	**OP346969**
Microstrobilinia castrans sp. nov.	Switzerland	Jura	*Picea abies*	ZT Myc 66346	**CBS 149466**	single spore	**OP325828**	**OP325822**	**OP325816**	**OP346976**	**OP346970**
Microstrobilinia castrans sp. nov.	Switzerland	Valais	*Picea abies*	ZT Myc 66349	**CBS 149467**	single spore	**OP325829**	**OP325821**	**OP325817**	**OP346977**	**OP346971**
Microstrobilinia castrans sp. nov.	Switzerland	Ticino	*Picea abies*	ZT Myc 66363	**CBS 149468**	single spore	**OP325830**	**OP325824**	**OP325818**	**OP346978**	**OP346972**
Microstrobilinia castrans sp. nov.	Switzerland	Grisons	*Picea omorika*	ZT Myc 66365	**CBS 149469**	single spore	**OP325831**	**OP325823**	**OP325819**	**OP346979**	**OP346973**
Monilinia laxa	Netherlands		–	OSC 100063	CBS 122031		–	AY544670	AY544714	DQ470889	DQ471057
Piceomphale bulgarioides	Estonia		*Picea abies*	TAAM 165289			LT158483	KX090797	KX090848	KX090700	–
Pycnopeziza sejournei	France		*Hedera helix*	JHP 11.054			LT158443	KX090827	KX090878	KX090726	KX090679
Rutstroemia firma	Estonia		*Alnus incana*	TU 104481			LT158450	KX090832	KX090881	KX090731	KX090684
Rutstroemia tiliacea	Germany		*Tilia* sp.	TAAM 132844			LT158423	KX090808	KX090860	KX090711	KX090661
Sclerencoelia fraxinicola	Germany		*Fraxinus excelsior*	HB 5714			LT158420	KX090805	KX090857	KX090708	KX090659
Sclerencoelia pruinosa	USA		*Populus tremuloides*	NY 02533482			LT158462	–	KX090888	KX090735	–
Sclerotinia sclerotiorum	Netherlands		*Linum usitatissimum*	NRRL 13681	CBS 499.50		MH856725	DQ470965	DQ471013	DQ470916	DQ471086
Trochila laurocerasi	Germany		*Prunus laurocerasus*	BPI 879818			LT158460	KX090835	KX090887	KX090734	KX090689

[H]=Holotype; CBS-numbers of strains isolates and GenBank accession numbers of sequences generated in the present study are shown in bold

Table 2 Growth media used for cultivation

Abbr	Growth medium	Formula
DMA	DiaMalt agar	15 g Plant Propagation Agar (Conda), 20 g DiaMalt (Hefe Schweiz AG), 1 l ddH_2O
DMA + S	DiaMalt agar with streptomycin	15 g Plant Propagation Agar (Conda), 20 g DiaMalt (Hefe Schweiz AG), 100 mg streptomycin (Sigma), 1 l ddH_2O
PDA	Potato dextrose agar	15 g PDA (Difco), 1 l ddH_2O
WA + S	Water agar with streptomycin	15 g Plant Propagation Agar (Conda), 100 mg streptomycin (Sigma), 1 l ddH_2O

placed on DMA + S plates and incubated at 20 °C for 2 weeks (Fig. S2). The identity of colonies emerging from them was determined by culture morphology and ITS sequencing.

Phenology

The observation of the pollen cones of the same *P. smithiana* tree mentioned above started at the WSL site on April 12, 2019. On May 2, 2019, ten freshly developed, immature pollen cones showing early symptoms of an infection were marked. At this time of the year, the fruiting bodies on old stromatized pollen cones had already sporulated. As a control, 10 healthy looking freshly developed pollen cones were marked as well. These cones were regularly observed throughout the year. The final inspection took place on December 16, 2019. Phenology data of the fungal development are supplemented by 158 field observations on three *Picea* species from about 80 collections sites at different altitudes and at different times of the year (Table S1).

DNA extraction, PCR amplification, and sequencing

About 1 cm^2 of mycelium was harvested from the edge of each fungal culture, lyophilized and ground to fine powder. DNA was extracted using the Kingfisher 96/Flex machine and kit (TermoFisher Scientific, LGC Genomics 169 GmbH, Berlin, Germany) according to the manufacturers' protocols.

PCRs were performed using the following primer pairs (annealing temperatures in brackets) and a standard PCR protocol with 35 cycles. ITS: ITS1/ITS4 (50 °C) (White et al. 1990); LSU: LR0R/LR6 (52 °C) (Rehner and Samuels 1994; Vilgalys and Hester 1990); SSU: NS1/NS4 (48 °C) (White et al. 1990); *TEF1*: EF1-983F/ EF1-2218R (55 °C) (Rehner and Buckley 2005); *RPB2*: fRPB2-5F/fRPB2-7cR (58 °C) (Liu et al. 1999). Additionally, a new primer pair for *RPB2* more specific to the *Sclerotiniaceae* was designed: RPB2-Scl-F: 5′-GGWCAAGCTTGTGGYTTGGT-3′ and RPB2-Scl-R: 5′-CGGATTGATAACAYTTACGAGGA-3′ (53 °C). DNA sequencing in two directions was carried out by Microsynth AG (Balgach, Switzerland) using the same forward and reverse primers as in the PCRs. Resulting sequences were deposited in GenBank (Table 1).

Comparison with sequences in databases

Similar published sequences were retrieved by BLAST searches (search option blastn, Altschul et al. 1990) by using the full-length ITS1-5.8S-ITS2 sequence or partial ITS1 and ITS2 sequences as queries against the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, date: 2022–03-24) and the UNITE database (Nilsson et al. 2019, https://unite.ut.ee, date: 2022–03-24). Furthermore, we checked for the presence of highly similar sequences in high-throughput sequencing data from environmental samples in the GlobalFungi database (Větrovský et al. 2020) (2022–03-24). To determine the taxonomic position of the species, the corresponding SSU-, LSU-, *RPB2*-, *TEF1*-sequences were also compared with accessions deposited in GenBank by BLAST searches.

Phylogenetic analyses

A combined SSU-ITS-LSU-*RPB2*-*TEF1*-alignment was created. The newly generated sequences were combined with sequences of 10 species of *Sclerotiniaceae* from GenBank that revealed the highest similarity to the query sequences. The data set was supplemented by 10 species of the sister families *Rutstroemiaceae* and *Cenangiaceae* following Pärtel et al. (2017) (Table 1). Lacking sequences were supplemented with unknown bases (N). All alignments were performed using MAFFT v.7.017 (Katoh and Standley 2013) without further manual optimizations. Ambiguously aligned regions within the resulting alignments were excluded from analyses with Gblocks v.0.91b (Castresana 2000). The final combined alignment used for phylogenetic analyses was deposited at TeeBASE (ID 29960).

Maximum likelihood (ML) phylogenetic analyses were performed by RAxML v. 8.2.11 (Stamatakis 2014) as implemented in Geneious prime 2021.1.1 (https://www.geneious.com), using the GTR GAMMA model with partitions according to the respective submatrices (SSU, ITS1, 5.8, ITS2, LSU, *RPB2*, and *TEF1*; 1st, 2nd, and 3rd positions of codons of *RPB2* and *TEF1* exons). Analyses were based on ML and 1000 rapid bootstrap replicates. Bayesian analyses were performed with MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001) as implemented in Geneious prime 2021.1.1 using *Encoelia furfuracea* as

outgroup. The analysis was run under the general time reversible (GTR) model, with gamma distribution rate variation across sites and a proportion of invariable sites. Two parallel Markov chain Monte Carlo (MCMC) runs with four chains and 1.1 million generations were performed and every 200th tree was sampled. The first 2000 trees were discarded (burn-in) before calculating posterior probabilities in the majority rule consensus trees. Posterior probability (PP) values equal to or greater than 0.95 were considered significant. In the same way, phylogenies of the individual gene regions and combinations of them (ITS-LSU vs *RPB2-TEF1*) were calculated and analyzed to reveal possible topological incongruences between them.

Morphology

One hundred fifty-eight specimens (Table S1) were studied. Measurements and photos were performed with a Zeiss Discovery V8 stereo lens equipped with a Zeiss Axiocam 506 digital camera and a Zeiss Axio Scope A1 microscope equipped with a Axiocam 208 color digital camera and Zen 2.3 software (Carl Zeiss Microscopy GmbH, Germany). Sections of fresh material were made by hand; thin sections of dried apothecia and stromatized pollen cones were made using a hand microtome. The preparations were investigated in water, 2.5% and 5% potassium hydroxide solution (KOH), lactic acid (with and without cotton blue) or Congo red. The iodine reaction was tested with Melzer's reagent (MLZ) and Lugol's solution (IKI), with and without KOH-pretreatment (Baral 1987; Baral et al. 2020). Asci ($n = 20$) and spores ($n = 50$) from fresh, sporulating apothecia, therefore considered to be alive, each of five collections representing the hosts *Picea smithiana* (ZT Myc 66340, ZT Myc 66345), *P. abies* (ZT Myc 66346, ZT Myc 66349), and *P. omorika* (ZT Myc 66365) were measured in water. In addition, 10 asci and 20 ascospores each were measured in 5% KOH from the same specimens after they were air-dried for 6–12 month. These asci and ascospores were considered to be dead. Mean value and length width (L/W) ratio were calculated. Results were given as minimum–mean–maximum value.

Cultural characteristics of the ex-type culture (CBS 149465) were studied using three different growth media (two without and one with antibiotics, Table 2). Mycelial plugs (3 × 3 mm) cut out from the margin of an actively growing DMA + S culture were placed in the center of each of two 9 cm PDA, DMA, and DMA + S plates. These 6 subcultures were incubated at room temperature (20 °C) in the dark. The culture morphology was observed, and their radial growth was measured (in mm) after 3, 7, 14, and 21 days. The shortest and the longest distance from the center were measured (Table 3).

Table 3 Radial growth of mycelia on different media at 22 °C in darkness

Days of incubation	3	7	14	21
PDA	6–11 mm	18–25 mm	23–29 mm	29–37 mm
DMA	6–13 mm	14–18 mm	23–34 mm	32–45 mm
DMA + S	5–6 mm	10–17 mm	16–25 mm	36–42 mm

Nucleus staining

Hand sections of fresh and air-dried apothecia were carefully heated in 5% KOH and then washed twice in tap water. The sections were placed in 4′,6-diamidino-2-phenylindole (DAPI) (AppliChem, Darmstadt Germany) solution (10 µg/1 ml H_2O) on a microscope slide. The preparation was covered with a cover glass and squeezed gently until individual asci were released. Nuclei of asci and spores were immediately counted using a ZEISS Axio Vert.A1 inverted phase fluorescence microscope (365 nm filter) and the camera/software system as specified earlier.

Note: In a pre-test of the DAPI staining without KOH treatment, the cell content of young asci showed strong fluorescence that did not allow the counting of nuclei. The fluorescence of the cell contents was also observed without DAPI staining in fresh tap water.

Results

Fungal isolations from different sources

The 500 single spore isolates and 20 isolates obtained from stromatized pollen cones of the 5 representative samples showed no remarkable differences in culture morphology. The ten ITS sequences obtained from ascospores and stromatized cone tissues were identical. They significantly differed from ITS sequences of similar related species in the NBCI data bank (see below). No other fungi were isolated from the stromatized pollen cones. No colonies emerged from control samples (Fig. S2a). Tissue pieces from cones showing early symptoms generated 56 colonies (90%), while no colonies emerged from 4 samples. The 56 colonies represented one single morphotype (Fig. S2b). One isolate per cone was selected and their identity with the new fungus was confirmed by ITS sequencing. No other fungal species was isolated from the fresh pollen cones.

Phenology

On April 12, 2019, all buds of pollen cones of the *P. smithiana* at WSL site were still closed and covered by bud scales. On April 25, buds started to burst. The exposed pollen cones

showed no symptoms. The first brown discolorations and deformations appeared on April 30 on approx. 5% of the fresh pollen cones. The ten healthy pollen cones marked on May 2 opened by stretching their axis and released pollen grains from early May to early June. All of them had fallen off by December. The ten symptomatic pollen cones marked on May 2 remained closed and did not stretch or stretched only partially on one side with the pollen grains remaining in the cones. Apothecia were formed on nine of the marked cones during the summer while one cone had fallen off in December. The 158 field observations revealed a similar phenology depending on weather and altitude above sea level (Table S1, taxonomy part).

Comparison with sequences in databases

The five representative samples (Table 1) from the different host trees and geographical locations had identical sequences in all five gene regions examined. The BLAST search in the NCBI database and the analysis of its output revealed that the fungus belongs to the *Sclerotiniaceae*. All sequences analyzed had a high percentage of identity with the corresponding sequences of *Sclerotinia sclerotiorum* (Lib.) de Bary, type species of the genus *Sclerotinia* and the *Sclerotiniaceae*: SSU > 99%, LSU > 95%, ITS > 92%, *TEF1* > 95%, *RPB2* > 85%. Similarly, high percentages of identity were found with other members of this family. BLAST searches for similar ITS sequences in the UNITE database identified entries with a maximum sequence similarity of 95.5%, most of them belonging to the species hypothesis cluster *Sclerotiniaceae* sp./SH1518609.08FU with a distribution in Europe and North America (Kõljalg et al. 2021). A search for environmental genetic variants in GlobalFungi further identified two records of a ITS2 sequence 100% identical to the newly obtained ITS sequences. They were obtained from two air samples taken by a high-volume PM10 sampler at the Puy de Dôme meteorological station (1465 m asl.), Auvergne, France on April 26, and May 10, 2017 (Tignat-Perrier et al. 2020).

Phylogenetic analyses (Fig. 1)

Phylogenies inferred from individual loci had similar topologies (data not shown). The maximum likelihood and Bayesian phylogenetic analyses of the combined SSU-ITS-LSU-*RPB2*-*TEF1*-alignment resulted in trees with matching topologies. The new fungus appeared in a well-supported clade of the *Sclerotiniaceae*. It is placed in sister position to the main sub-clade of *Sclerotiniaceae* containing *Sclerotinia sclerotiorum*, *Botrytis cinerea* Pers., *Ciboria caucus* (Rebent.) Fuckel, *Dumontinia tuberosa* (Bull.) L.M. Kohn, *Elliottinia kerneri*, *Monilinia laxa* (Aderh. & Ruhland) Honey, *Sclerencoelia fraxinicola* Baral & Pärtel and *Sclerencoelia pruinosa* (Ellis & Everh.) Pärtel & Baral. *Pycnopeziza sejournei* (Boud.) Whetzel & W.L. White and *Ciboria viridifusca* (Fuckel) Höhn. appeared in more basal positions in the *Sclerotiniaceae*. The species of *Rutstroemiaceae* form the sister clade to the *Sclerotiniaceae*.

Taxonomy

***Microstrobilinia* Beenken & Andr. Gross, gen. nov.**

MycoBank 845510

Etymology: The genus name refers to the substrate: "*microstrobilus*" means "small cone" in Greek and is the botanical term for the male pollen cone of conifers.

Diagnosis: Member of *Sclerotiniaceae* Whetzel (1945) emend. Holst-Jensen et al. (1997) (*Helotiales*, *Leotiomycetes*), different from all other members of the *Sclerotiniaceae* in 4-spored, cylindrical asci with a short stipe and an iodine negative apex without apical apparatus, mature ascospores containing 16 nuclei, parasitizing pollen cones of *Picea* spp.

Type species: *Microstrobilinia castrans* Beenken & Andr. Gross

***Microstrobilinia* castrans Beenken & Andr. Gross, spec. nov.**

MycoBank 845511

(Figs. 2, 3, 4, 5, 6, 7, and 8).

Etymology: The species name refers to the fact that the fungus prevents the male reproduction of its host: "*castrans*" is Latin for castrating/emasculating.

Short diagnosis: Apothecia brown, short stipitate, desiccation-tolerant, 1–5 mm in diameter, growing on stromatized pollen cones of *Picea* spp.; ectal excipulum of brown textura globulosa-angularis; asci cylindric, av. 108 × 14.6 µm when alive, iodine negative without visible apical apparatus, 4-spored, ascospores citriform, and almond-shaped, av. 25 × 13 µm when alive, containing 16 nuclei when mature.

Holotype: Switzerland, Canton Zurich, Birmensdorf, arboretum of the Swiss Federal Research Institute WSL, on pollen cones of *Picea smithiana*, solitary tree in a park, 47.36047 N, 8.45633 E, 555 m asl. May 17, 2018, leg. A. Gross, ZT Myc 66340; ex type culture CBS 149465.

Additional specimens examined are given in Table S1.

Description: *Apothecia* scattered on stromatized pollen cones of *Picea* spp.: primordial apothecia single or in groups of up to 3 in the middle of cone scale near the scale axis, black, spherical, 0.2–0.5 mm in diam., opening at apex; excipulum pinkish violet turning bluish blackish in KOH (reaction reversible in acid), external excipulum with globose cells in outer layer, inner part hyalin, primordial hymenium containing paraphyses only. Mature apothecia 1–5 mm in diam., desiccation-tolerant, slightly cupulate to

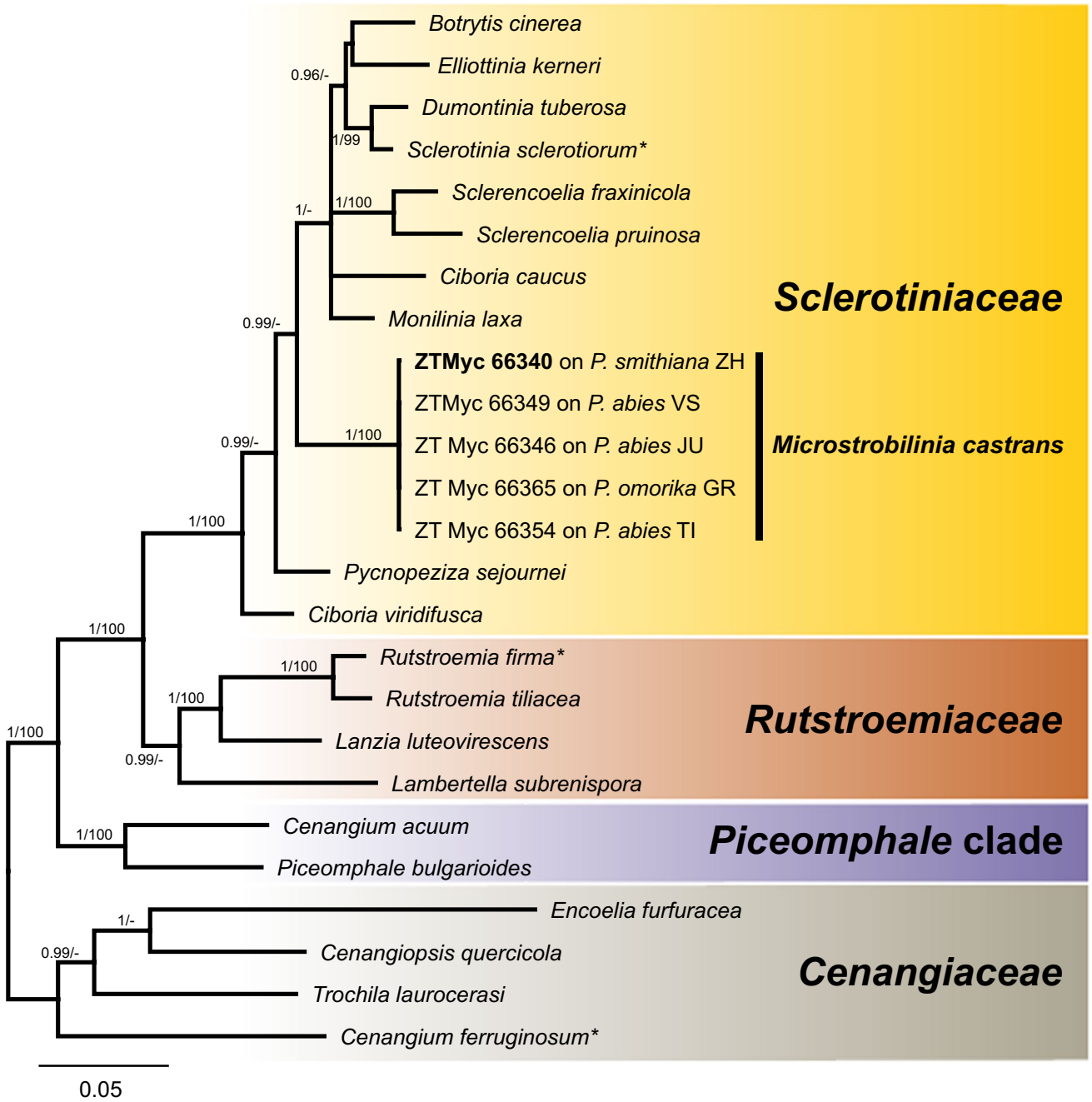
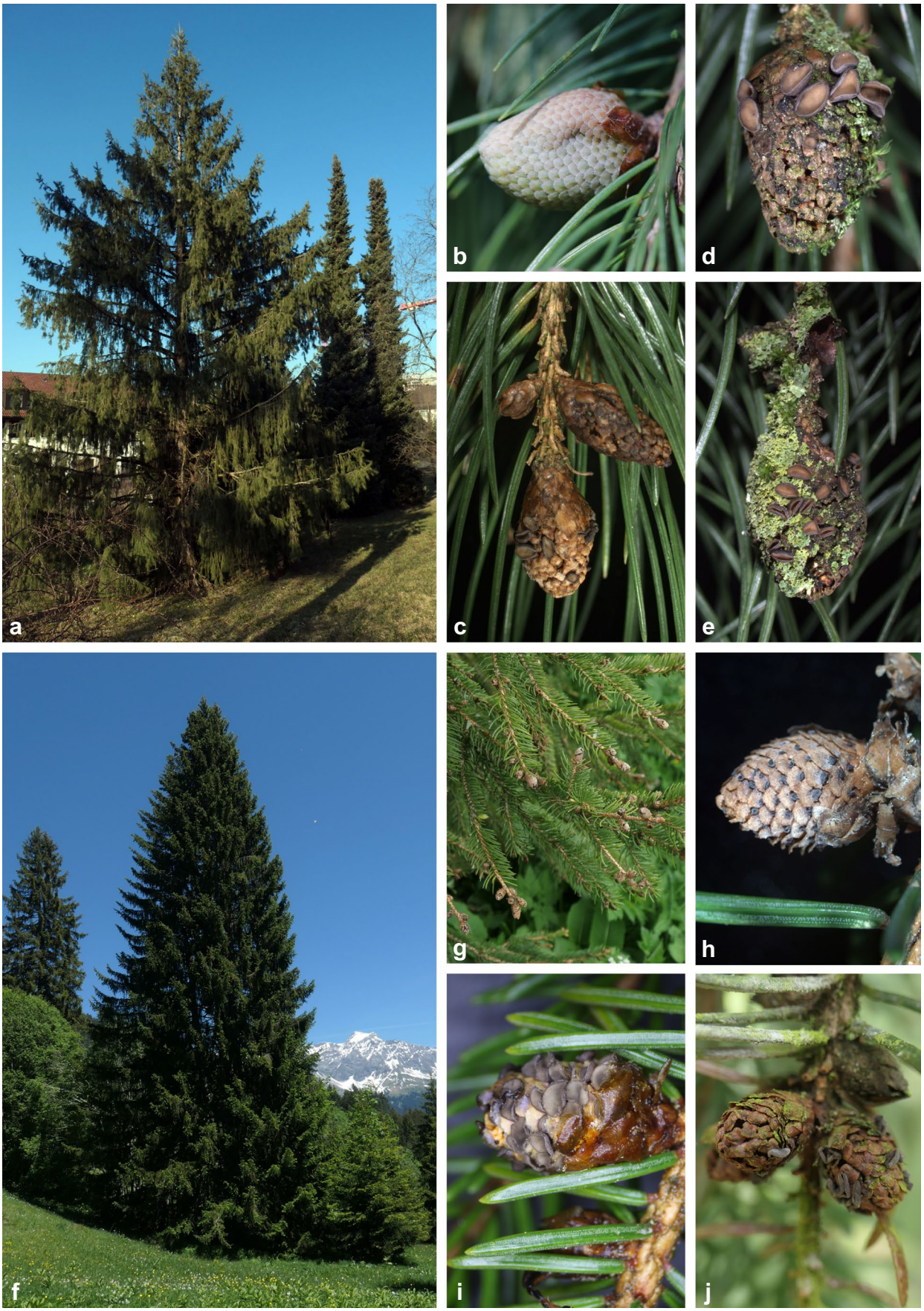


Fig. 1 Majority rule consensus tree from Bayesian analyses recovered from combined ITS-LSU-SSU-*RPB2-TEF1* sequences. *Microstrobilinia castrans* is inferred as a member of the *Sclerotiniaceae*. RAxML bootstrap support > 50%/Bayesian posterior probabilities > 0.95 are given at nodes. The type of *M. castrans* is highlighted in bold. Type species of the families are marked with an asterisk

flat at maturity and when hydrated, shell-like folded when dry, flanks 200–260 m thick, stipitate, stipe up to 0.5 mm long, ca. 0.5 mm wide; hymenium cream to light brown when young, grayish when old, margin white to light brown ciliated; outside dark brown to black, white-pruinose when young, bare and rough to wrinkled when old.

Ectal excipulum 35–70 µm thick, dark brown; outermost cell layer composed of globose cells 4–16 µm in diam. (textura globulosa), walls brown, approximately 0.3 µm thick, smooth; inner part composed of 2–3 (–4) rows of globose to rectangular cells (textura globulosa-angularis-prismatica), cells vertically oriented, 5–25 × 4–12 µm, walls thin, light brown; on the surface of flanks with scattered groups of globose to irregularly shaped cells partly with red-brown content turning bluish to blackish violet in KOH, reaction reversible in acid, with bundles of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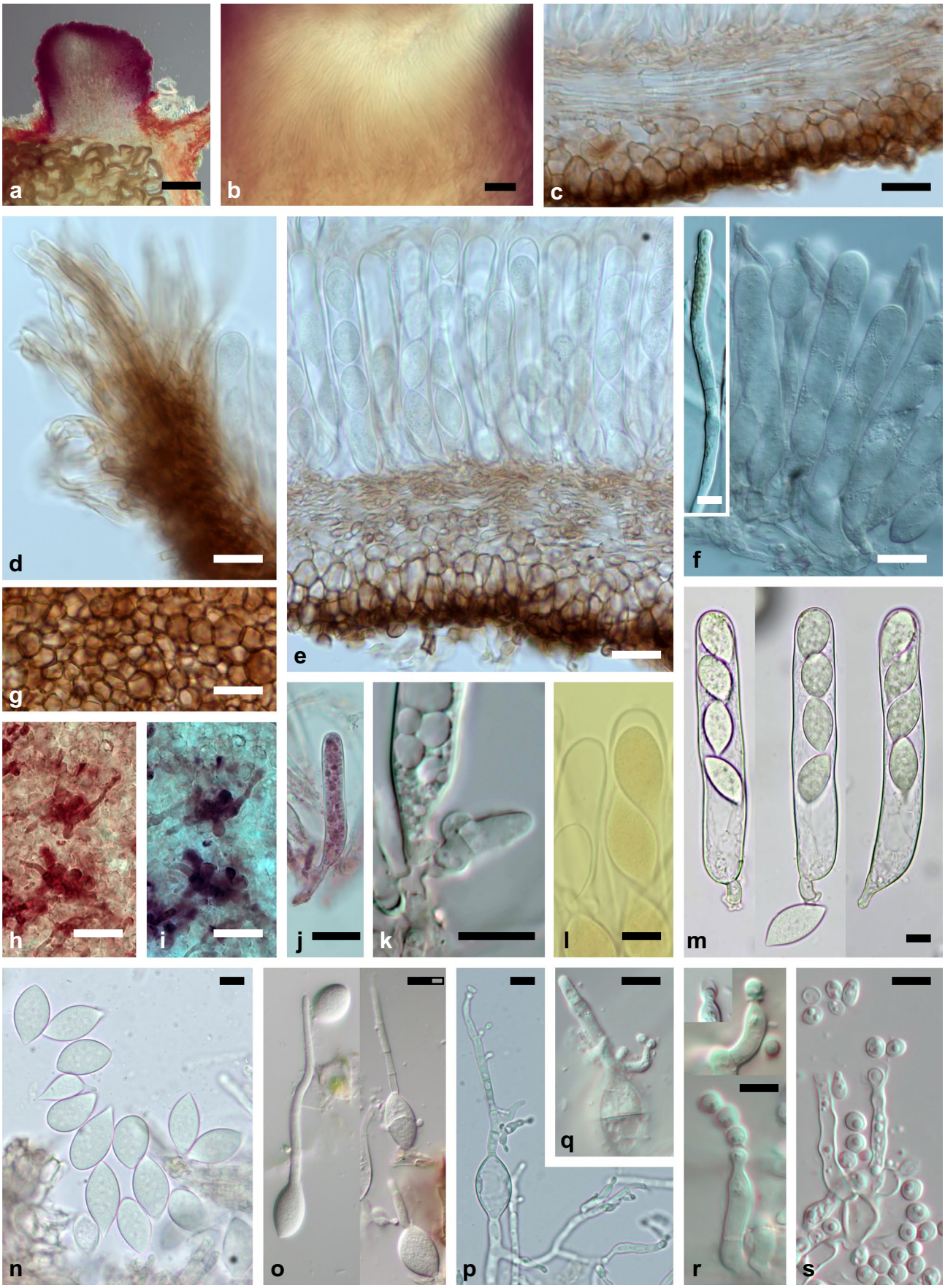
◂**Fig. 2** *Microstrobilinia castrans*: **a**–**e** On *Picea smithiana*: **a** *P. smithiana* tree at type location, two *P. omorika* trees in the background; **b** fresh infected pollen cone showing first symptoms like deformation and brown discoloration; **c** infected pollen cone with apothecia in the first year after infection; **d**, **e** several years old pollen cones overgrown with mosses and lichens with fresh apothecia and remnants of last year's apothecia. **f**–**i** On *Picea abies*: **f** *P. abies* at a typical habitat in the Swiss Alps; **g** infested pollen cones on twigs; **h** pollen cone with small immature apothecia; **i** pollen cone with mature apothecia. **j** Pollen cones of *P. omorika* with dry, mature apothecia

hyaline hyphal hairs of approximately 20–50 × 2–5 µm; at margin with colorless to light brown, multiseptate, flexuous hyphal hairs, up to 100 µm long, 3–6 µm wide, with thin, smooth walls. *Medullary excipulum* 30–50 µm thick, composed of parallel running hyphae (textura porrecta), hyphae 3–5 µm wide, walls incrusted with small brown granules. *Subhymenium* 10–20 µm thick, light brown, composed of 2–3 µm wide, interwoven hyphae (textura intricata). *Hymenium* sometimes with a pinkish to reddish brown pigment turning blue to blackish-violet in KOH, reaction reversible in acid. *Asci* broadly cylindrical with hemispheric apex, basis abruptly narrowing into a short cylindrical stipe arising with two septa from non- or very small-perforated croziers, 97–108.1–130 µm long (90–95.5–110 µm long without stipe) and 12–14.6–18 µm wide when alive, 77–88.5–97 × 11–12.3–14 µm when dead; stipe 4–8.4–20 µm long and 3–4.2–6 µm wide; wall all over 1 µm thick, without apical apparatus, inamyloid, dehiscing by apical split; 4-spored (rarely 6-spored); sometimes asci with violet content in water turning blackish-blue in

Fig. 3 *Microstrobilinia castrans*, ontogeny of apothecia: **a** Black primordium in center of a cone scale in early summer; **b** immature apothecium in summer; **c** mature apothecia in autumn with gray velvet outer surface; **d** dehydrated mature apothecia with black smooth outer surface in next spring; **e**, **f** hydrated mature apothecia in next spring; **g** apothecium in side-view showing the stipe; **h** dissected pollen cone scales with apothecium. **a–d** from *P. abies*; **e, f, h** from *P. smithiana*; **g** from *P. omorika*. Scale bars: **a**, **b** = 0.25 mm; **c**, **d**, **g** = 0.5 mm; **e**, **f**, **h** = 1 mm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◂**Fig. 4** *Microstrobilinia castrans*, morphology of apothecia and anamorph: **a**, **b** Primordium in longitudinal section (in water): **a** Primordium with pink-purple excipulum on a cone scale with digested pollen at bottom; **b** primordial hymenium. **c**–**e** Apothecium in section (in lactic acid): **c** excipulum in radial cut; **d** margin with hyphal hairs; **e** tangential cut; **f** hymenium with immature asci and paraphyses (in water), insert: single paraphyse. **g**–**i** Ectal excipulum: **g** in plan view; **h**, **i** cells with red-brown content in water (**h**) turning blackish violet in KOH (**i**). **j**–**m** Asci: **j** young ascus with violet content (in water); **k** croziers at the base of young asci (in water); **l** ascus tips in Melzer's reagent; **m** three mature asci with short stipes (in water). **n**–**q** Ascospores (in water): **n** free ascospores; **o** germinating ascospores, one with a median septum. **p** Multiple branched hyphae with phialides arising from an ascospore; **q** septate ascospore forming a phialide at its germ hypha. **r**, **s** Phialides producing microconidia endogenously (in water): **r** from ascospores, **s** from culture. Scale bars: **a** = 100 µm; **b**–**i** = 20 µm; **j**–**q** = 10 µm; **f** insert**, r**, **s** = 5 µm. **a**, **b**, **n**–**r** from *P. abies*; **c**–**m** from *P. smithiana*

KOH. *Ascospores* obliquely 1-seriate, filling two-thirds of mature, fully turgescent asci, nearly the whole ascus when unripe or dead, hyaline, non-septate, broadly navicular to citriform, pointed at both ends, almond-shaped at the ascus tip, rarely ovoid or ellipsoid, (17–) 21–25.5–31.5 (–33.5) × 8.5–13.1–17.5 µm, L/W ratio 1.3–2–3.3 when alive, 17–23.6–27 × 10–11.8–14 µm, L/W ratio 1.5–2–2.5 when dead (citriform ascospores with two extended ends usually longer than almond-shaped or ovoid spores), with smooth walls, up to 0.5 (–1) µm thick, thinner at pointed ends, with granular content and 0.5–1.5 µm large guttules, with 16 nuclei at maturity; discharged ascospores rarely with one medium septum; discharged ascospores germinating at their ends in overmature apothecia, germ tubes 2–5 µm wide, forming the anamorph (see below). *Paraphyses* of same length as asci, cylindrical, 2–3 µm wide, mostly simple, sometimes branched, tips sometimes slightly capitate, 3–4 (–5) µm wide, multi-septate, walls colorless, content of terminal cells with small, colorless to light brown, 0.5–1.5 µm large guttules in water, disappearing in KOH.

Anamorph emerging from discharged, germinating ascospores that form multiply branched hyphae 3–5 µm wide, terminating in 6–17 µm long and 3–4 µm wide phialides showing distinct collarettes, producing globose to ovoid, guttulate, 2–3.5 × 1.5–2 µm large microconidia.

Recently infected pollen cones in spring (March to April) without apothecia, yellow to light brown, normally ovoid shaped or showing deformations, 0.7–1.3 × 0.5–0.7 cm on *P. abies* and *P. omorika*, 1.1–2.0 × 0.7–1.3 cm on *P. smithiana*, compact, not stretching, not releasing their pollen; inside with white, cottony mycelium, hyphae 1–2 µm thick, hyalin, between and within cone axis, scales, and pollen sacs, penetrating and growing within pollen grains. *Infected pollen cones in summer and autumn* with spherical primordia (May to July) or cup shaped, immature apothecia (August to October), light brown; cone axis dark brown to black, stromatized, with hyphae growing within the partly dissolved plant tissue; pollen grains adhering to each other due to mycelium spreading in pollen sacs, penetrated and later completely filled by hyphae consisting of short, inflated, 7–16 × 4–8 µm large cells, with one to two large drops 3–6 µm in diam per cell. *Infected pollen cones in spring of second year* with mature apothecia, gray to dark brown; fully stromatized inside; stroma replacing most of the plant tissue of the cone axis and scales; stroma rind not clearly differentiated, 10–20 µm thick, dark-brown, composed of collapsed and brown hyphae; medulla light, hyaline, hyphae fused, with 1–2.5 (–3.5) µm thick walls, with (1–) 2–4 (–5) µm wide lumen, arranged parallelly (textura porrecta) along the remaining central part of the cone axis and along the scales, incorporating remaining red brown plant cells and tracheids; pollen grains often empty. *Infected pollen cones in summer of second and following years* dark brown to black, with old apothecia and new primordia, remnants of the old apothecia remaining until autumn; stromatized pollen cones may remain on trees for several years.

Culture characteristics: Colonies on PDA consisting of superficially formed, tomentose aerial mycelium, dark gray in center, margin irregularly lobed, white; media turning dark brown to black in center. Colonies on DMA and DMA + S consisting of submerged hyphae, surface velvet with sparse aerial mycelium, dark gray to black in center, margin irregularly lobed, white; media turning dark brown to black in the center, with blackish brown spots at the margin. Colony growth rate identical on PDA and DMA, initially more slowly spreading on DMA + S, all colonies about the same diam. after 21 days (Table 3).

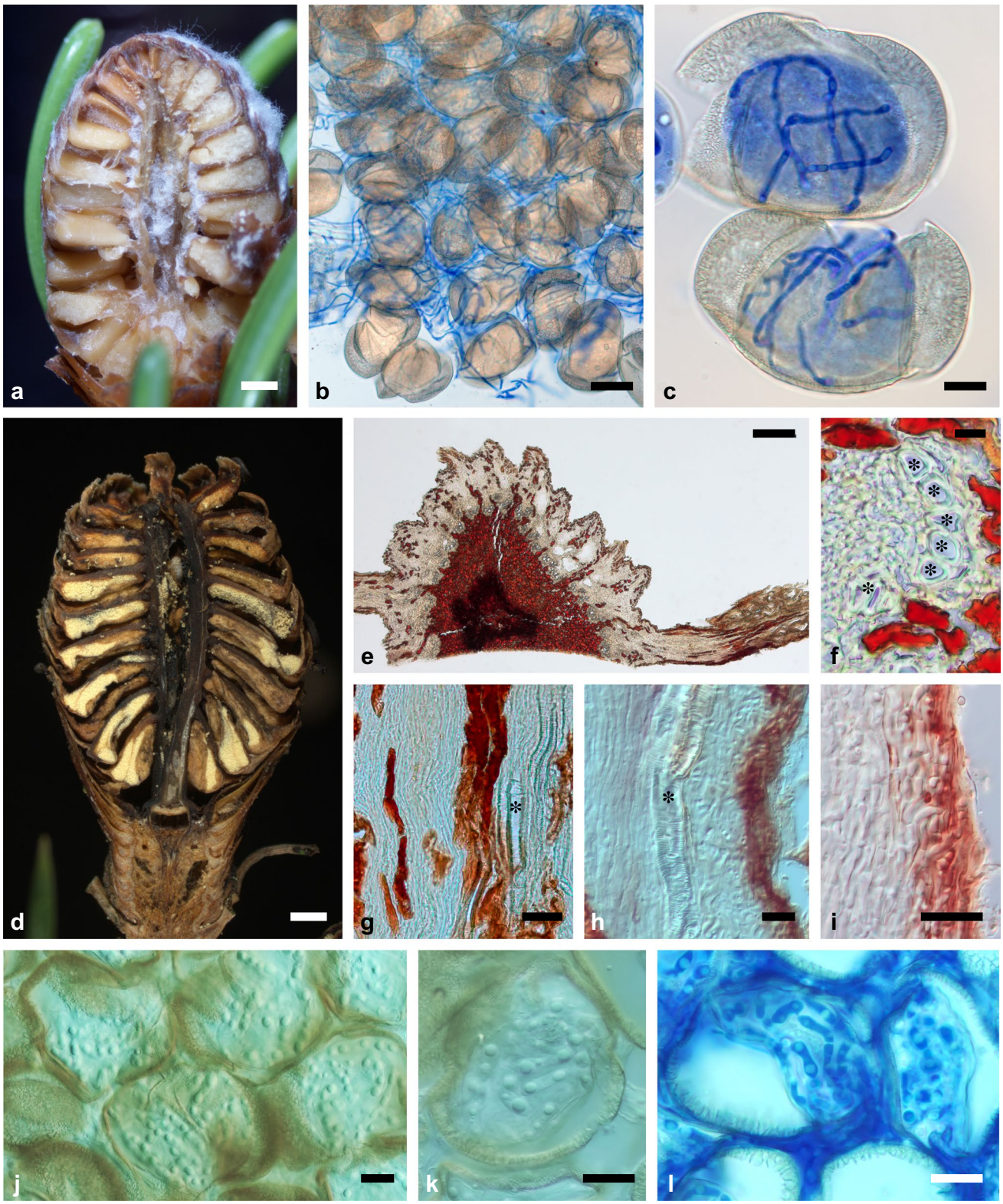
Myrioconium-like sporodochia sporadically formed on agar surface, with small slime droplets, 0.5–1 mm in diam., with a basal, nearly pseudoparenchymatous centrum of short, highly branched hyphae consisting of 5–10 × 3–6 µm large cells, phialides 6–15 µm × 2–3 µm, with distinct collarettes; microconidia globose to broadly ovoid, guttulate, 2–3 × 1.5–3 µm.

Hosts, habitats, and distribution: In Europe, on native *Picea abies* in mountain regions of Switzerland (Jura, Prealps and Alps), Germany (Black Forest, Prealps and Alps), France (Auvergne) and Italy (north-eastern Alps), 850–1900 m asl., on solitary trees in meadows or forest edges and interior; on cultivated *Picea omorika* in urban areas of Switzerland, (540–) 890–1260 m asl.; on cultivated *Picea smithiana* in urban areas of Switzerland, 380–560 m asl.

Notes: The apothecia on the large stromatized pollen cones of *P. smithiana* tend to be larger (up to 5 mm in diam.), while they are smaller on *P. abies* and *P. omorika* (up to 4 mm at max.). No other morphological differences were found between the collections on the different spruce species. Old stromatized cones, which still produced apothecia, are often overgrown with algae, lichens, and mosses.

From this observation we conclude that infected pollen cones remain attached to the trees and produce apothecia probably for several years. The large pollen cones of *P. smithiana* remain attached much longer (often more than three years) than the small ones of the other two spruce species (probably 2 years at most).

The length of asci depends strongly on their maturity. Immature asci are shorter in autumn to early spring than in late spring.



◂**Fig. 5** *Microstrobilinia castrans*, morphology of stromatized pollen cones: **a**–**c** Fresh infected pollen cone of *P. abies*: **a** Longitudinal section, white mycelium on axis, scales, and pollen sacs; **b** Dense hyphal network around and in the pollen grains; **c** Two pollen grains with fungal hyphae inside. **d**–**l** Older, completely stromatized pollen cone on *P. smithina*: **d** longitudinal section, scales, and axis of the cone dark brown to black, pollen sacs yellow. **e**–**i** Microtome sections, fungal stroma whitish with a darker rind, remnants of plant cells red brown, plant tracheids marked by asterisks; **e** cross-section through a pollen cone, on the right cut scale with pollen sac; **f** cross-section through the fungal stroma close to the cone axis. **g**, **h** Longitudinal sections of the cone axis: **g** central part with parallel running hyphae; **h** outer area with brown rind; **i** rind with brown colored hyphae. **j**–**l** Sections of infected pollen sacs: **j**, **k** agglomerated pollen grains filled with fungal hyphae; **l** hyphae between and in pollen grains. Scale bars: **a**, **d** = 1 mm; **b**, **g** = 50 µm; **c**, **f**, **h**–**l** = 20 µm; **e** = 100 µm; **b**, **c**, **l** stained with cotton blue; **e**–**k** fixed in lactic acid

During autumn to early spring spores fill almost the entire length of asci while in late spring, when the mature asci are fully stretched and turgescent, their spores only fill the upper two-thirds of the asci. The size of mature spores, however, does not change noticeably over the winter but is relatively variable due to their shape (Fig. 4n). The shape of the spores depends mainly on their position in the ascus. In most cases, the apical spore is almond-shaped with the rounded end orientated upwards, the two middle ones are pointed at both ends, and the fourth bottom spore usually has a pointed and a somewhat blunter end, so that they fill the young ascus optimally (Fig. 4f, l, m).

The infested pollen cones were collected mainly from low-hanging branches of solitary or small groups of spruce trees on meadows and at forest edges, where they were easily accessible. They were often found on the shade side of the trees. Occasionally they were also found in upper crowns of fallen trees within the forest. Lowland records are almost only known from the exotic *P. smithiana.* All examined trees of this species were infested with *M. castrans* (in total 12 trees), apart from those that were still too young to flower. In the lowlands, *M. castrans* was found only twice on *P. omorika* planted in the immediate vicinity of infested *P. smithiana* trees. All other seven collections from *P. omorika* came from trees planted in urban areas in mountainous regions above 890 m asl. All 133 findings from the native *P. abies* came from its natural distribution range between 850 and 1900 m asl. In lower regions, *P. abies* was never found to be infested even if it grew directly next to infested *P. smithiana* trees. *Microstrobilinia castrans* was not found on any other spruce species planted in the arboreta, gardens, and parks visited. It was very common in some places but in other places with an apparently similar habitat, it was very rare or could not be found at all despite an intensive search. It is also noteworthy that of several spruces standing next to each other, often only one or a few were infested. Whether there is a difference in susceptibility between the tree species and/or individuals remains up to further investigations.

Possibilities of confusion: Only the pyrenomycete *Gemmamyces piceae* (Borthw.) Casagr. (*Melanommataceae*, *Pleosporales*, *Dothideomycetes*), the causal agent of spruce bud blight disease, can cause similar symptoms on pollen cones (own observations). In contrast to the cup-shaped apothecia of *M. castrans*, *G. piceae* produces spherical black perithecia and conidiomata (Černý et al. 2016; Jaklitsch and Voglmayr 2017). Thus, their fruiting bodies are not to be confused. Furthermore, the cones affected by *G. piceae* are not stromatized. Its hyphae do not grow into the pollen grains like those of *M. castrans*. The pollen remains powdery and is not agglomerated as in the case of an infection with *M. castrans*. Sometimes, both species were found together on one twig, but not on the same cone.

Ascospore genesis (Fig. 8): Starting with one nucleus per ascus, the three nuclear divisions occur in the common cytoplasm of the ascus resulting in eight nuclei arranged in four pairs. Afterwards, one spore forms around each pair. This leads to four ascospores per ascus. Then, the nuclei start to divide within the spores three times in succession

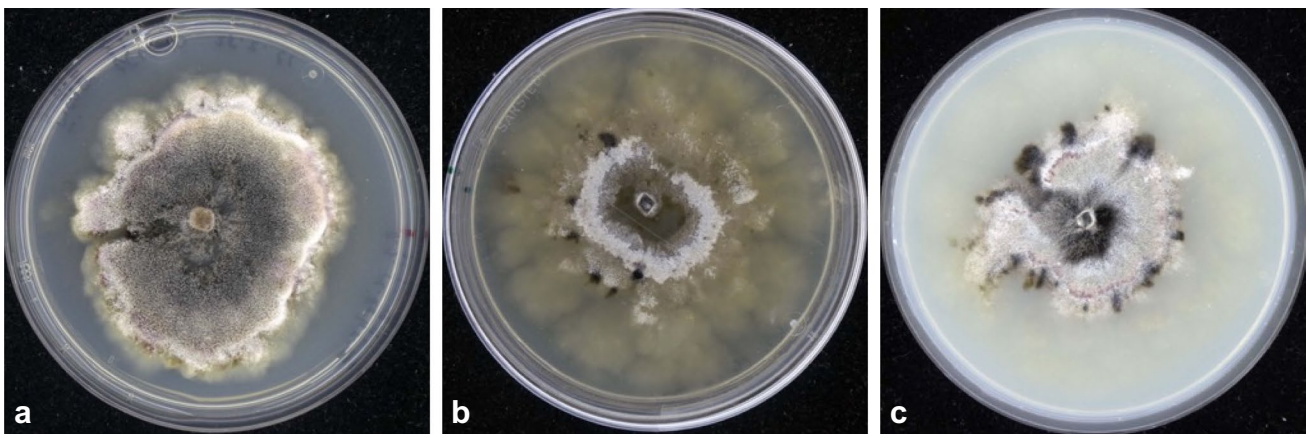


Fig. 6 Colony morphology of *Microstrobilinia castrans* on different media after 21 days incubation in darkness at 22 °C: **a** on PDA; **b** on DMA; **c** on DMA + S; petri dishes 9 cm in diam

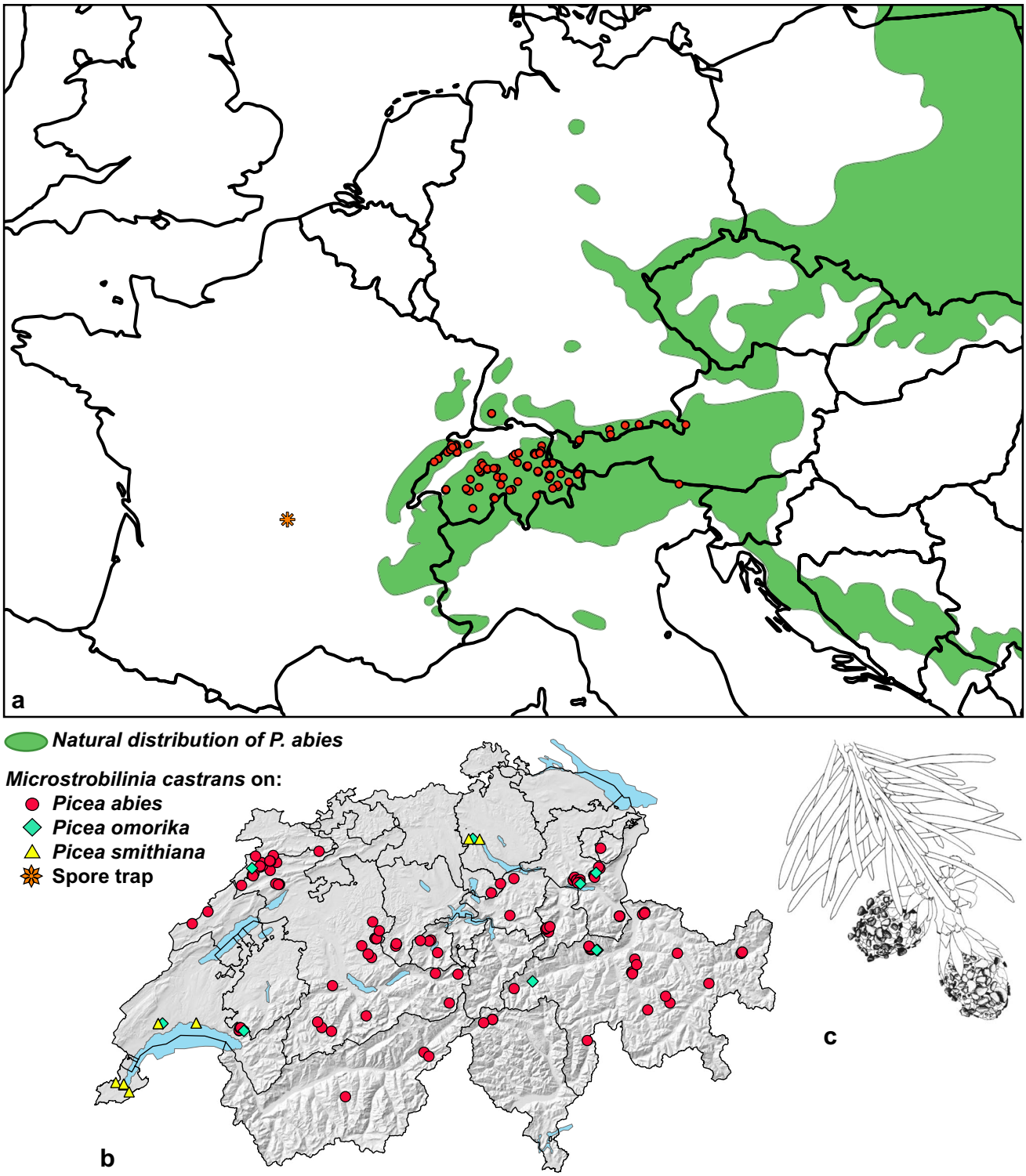


Fig. 7 Distribution of *Microstrobilinia castrans*: **a** Collection points of *M. castrans* within the natural distribution areas of *Picea abies* in Central Europe; asterisk indicates position of spore trap in France (Tignat-Perrier et al. 2020). **b** Collections points of *M. castrans* from *Picea abies*, *P. omorika*, and *P. smithiana* in Switzerland. **c** Apothecia of *M. castrans* on two pollen cones of *P. smithiana*

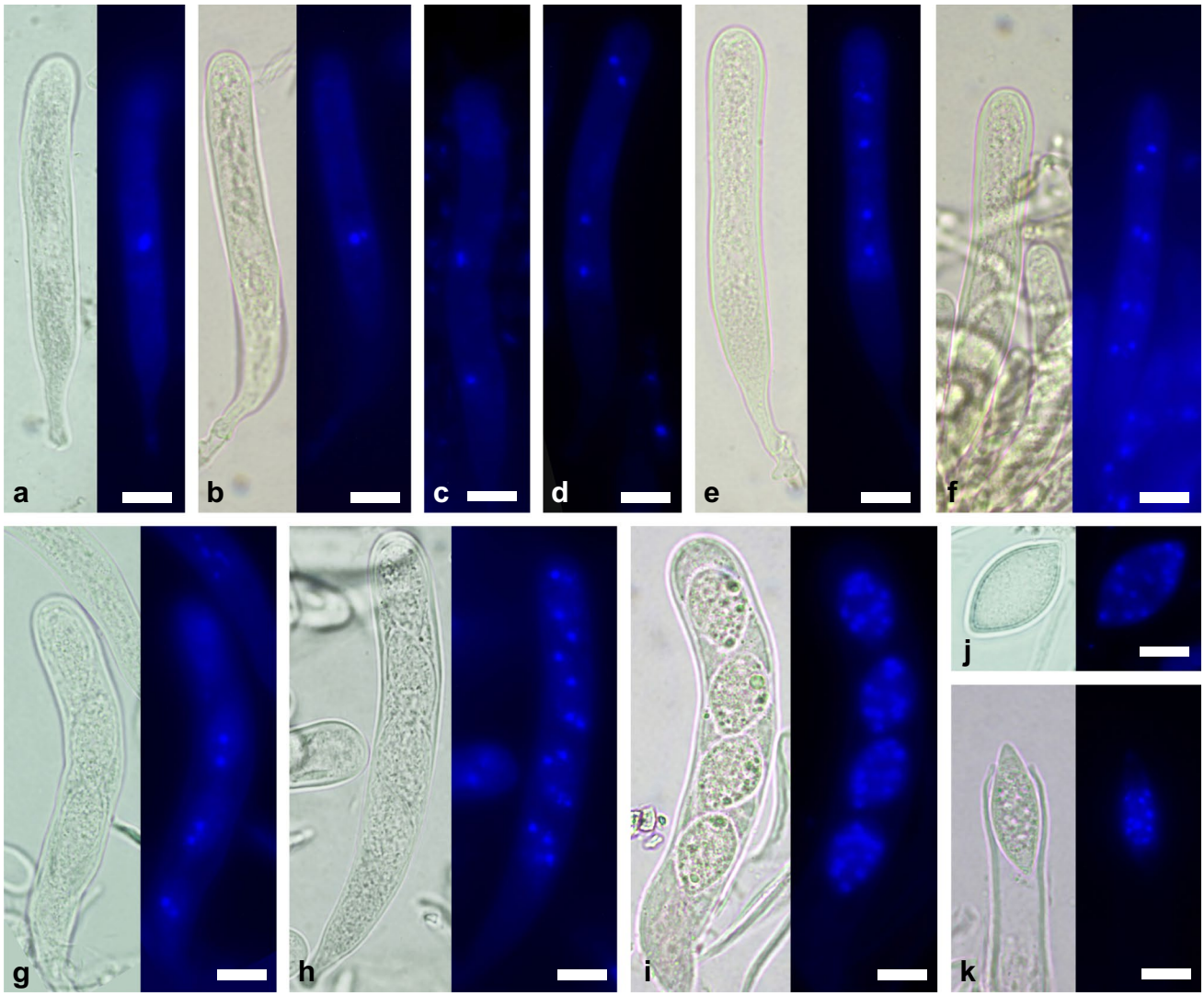


Fig. 8 *Microstrobilinia castrans*, ascospore, and nucleus genesis: **a**–**f** Young asci showing the nucleus divisions from 1 to 8-nuclei state: **a** 1-nucleate ascus; **b** two nuclei shortly after their division; **c** Two nuclei that have migrated apart; **d, e** four nuclei spreading in the ascus; **f** eight nuclei, in four pairs of two nuclei close together, before spore formation is visible. **g**–**k** Ascospore genesis: **g** one ascospore formed around each pair of nuclei, resulting in four binucleate ascospores per ascus; **h** immature ascus with four 4-nucleate spores; **i** immature ascus with four 16-nucleate spores; **j** discharged mature ascospore with 16 nuclei; **k** ascospore with 16 nuclei just leaving the ascus through a split at the ascus tip. Samples stained with DAPI; on the left side: transmitted light microscopy, on the right side: fluorescence microscopy with 365 nm filter (not always all nuclei in focus). All Scale bars = 10 µm; **a**–**i** Fresh material from *P. abies* collected in late autumn (ZT Myc 66411); **j**, **k** Dried material from *P. smithiana* collected in spring (ZT Myc 66409)

until there are 16 nuclei per spore. This already happens in late autumn. In the following spring, almost only spores with 16 nuclei were observed in ripe apothecia.

Discussion

Phylogeny vs. morphology

Our molecular phylogenetic analysis (Fig. 1), which correspond well with that of Pärtel et al. (2017, Fig. 1), classified *M. castrans* with strong support in the *Sclerotiniaceae* (*Helotiales*, *Leotiomycetes*). The *Sclerotiniaceae* was established by Whetzel (1945) to accommodate a group of fungi in the *Helotiales* (*Leotiomycetes*) producing apothecial ascomata arising from stromata, specifically with inoperculate asci, ellipsoid ascospores, and globose microconidia. Holst-Jensen et al. (1997) emended the family based on a molecular phylogenetic analysis of rDNA sequence data. They split off the species with simple stromata and grouped them in the newly created *Rutstroemiaceae*. The *Sclerotiniaceae* were narrowed to species with

distinct sclerotia or stromata that are well differentiated in a rind and a medulla. Currently, the family consists of 31 genera, 10 of which are monotypic (Wijayawardene et al. 2020). Characteristics of the asci and spores distinguish *M. castrans* from any other member of the *Sclerotiniaceae*. The typical inoperculate ascus of the *Sclerotiniaceae* has a subtruncate apex and a distinct iodine positive (rarely iodine negative) apical apparatus with an annulus. Its middle part is cylindrical and contains 8 (rarely 4) elliptical spores, and its basis is long tapering (e.g., Verkley 1998; Whetzel 1945; Pärtel et al. 2017). The ascus of *M. castrans* differs significantly from this *Sclerotinia*-type, as it has no apical apparatus and a base that narrows abruptly to a short stipe. The apex of the ascus is round and has the same wall thickness as the ascus sides. Thus, no differentiation in the structure of the apex is visible in light microscopy. Even after treatment with KOH, there was no reaction with any of the iodine reagents. Iodine-negative ascal tips also exist in other genera, such as *Sclerencoelia*, but their apical ascus walls show differentiation in thickness, shape, and structure, and open by a pore (Pärtel et al. 2017 called them "reduced *Sclerotinia*-type"). The ascus of *M. castrans* opens with a split that rips open the wall at the tip when the spores are mature. In the molecular phylogeny, *M. castrans* appears among species having inoperculate asci in our phylogeny. Thus, we assumed that *M. castrans* lost the ascus apical apparatus secondarily. This could be interpreted as an adaptation to its microhabitat. Sherwood (1981) assumed that several corticolous and lignicolous discomycetes that grow in tree crowns "lost all traces of an active spore discharge mechanism," since gravity and wind is sufficient for spore dispersal. Verkley (1995) also describes "a strong regression in the structure of the apical apparatus" of *Cenangium ferruginosum* Fr. (*Cenangiaceae*, *Helotiales*) that causes the ascus to open by ripping at the tip. *Cenangium ferruginosum* occurs on the bark of dead branches and twigs attached to pine trees and thus has a comparable microhabitat as *M. castrans*. Other examples of asci without a visible apical apparatus are found in *Mycosphaerangium* and *Neomelanconium*. Both genera also belong to the *Cenangiaceae* and form apothecia growing on air-exposed parts of their host trees (Voglmayr et al. 2020). Like the mentioned examples from the *Sclerotiniaceae* (Pärtel et al. 2017) and *Cenangiaceae* (Voglmayr et al. 2020), the apothecia of *M. castrans* show further adaptation to the air-exposed habitat in treetops. They are desiccation-tolerant (poikilohydric) and can respond to the changing humidity on site. They close when they dry out within a few hours and revive quickly when they get wet again. Dry apothecia ejected spores after rehydration that were still viable, as shown in the production of single spore cultures (see above). This desiccation-tolerance is also shown by the fact that vital looking asci and ascospores can be found in air-dried specimens kept at room temperature for 6 months.

Microsrobilinia castrans differs from other members of *Sclerotiniaceae* in their number of ascospores per ascus and number of nuclei per spore. Four-spored asci also occur in other genera of the family: *Kohninia linnaeicola* Holst-Jensen, Vrålstad & T. Schumach. has 4-nucleate spores (Holst-Jensen et al. 2004)., *Sclerotinia gIaciaIis* Frank Graf & T. Schumach. has 1 (–2) nuclei per spores (Graf and Schumacher 1995), and the nucleation of *S. tetraspora* Holst-Jensen & T. Schumach. is variable and can reach from two to four to eight nuclei per ascospore (Holst-Jensen and Schumacher 1994). However, the 4-spored asci of these species are of the *Sclerotinia*-type and their ascospores are ellipsoidal (Holst-Jensen et al. 2004; Graf and Schumacher 1995; Holst-Jensen and Schumacher 1994).

Four-spored asci can develop in two ways. Either four spores are formed per ascus, or eight spores are formed, of which four degenerate (e.g., Raju 2008; Raju and Perkins 2000). The ascospore genesis of the mentioned four-spored species of the *Sclerotiniaceae* were not described in detail (Graf and Schumacher 1995; Holst-Jensen and Schumacher 1994; Holst-Jensen et al. 2004; Kohn 1979). We used DAPI staining of the nuclei to clarify ascospore genesis in *M. castrans* (Fig. 8). Up to the 8-nucleus stage of the ascus, the development in *M. castrans* proceeds according to the textbook (e.g., Webster and Weber 2007). Afterwards, the process continues in a different way as spores are formed around nucleus pairs and not around individual nuclei. A similar genesis of four-spored asci has been described in detail for *Coniochaeta tetraspora* Cain (*Coniochaetaceae*, *Coniochaetales*), *Neurospora tetrasperma* Shear & B.O. Dodge (*Sordariaceae*, *Sordariales*), and *Podospora anserina* (Rabenh.) Niessl (*Podosporaceae*, *Sordariales*) (Raju and Perkins 2000; Raju 2008; Thompson-Coffe and Zickler 1994). In these species, it could be shown that each of the four ascospores encloses nuclei of opposite mating types. These species are therefore called pseudo-homothallic fungi (Grognet and Silar 2015; Raju and Perkins 2000; Raju 2008; Thompson-Coffe and Zickler 1994). Whether *M. castrans* is a pseudo-homothallic fungus needs further investigation. Multinucleate ascospores are also found in species of the *Sclerotiniaceae* with 8-spored asci (Kohn 1979). *Sclerotinia sclerotiorum* has 2 nuclei, and *S. minor* Jagger and *S. trifoliorum* Erikss. have 4 nuclei per ascospore (Wong and Willetts 1979; Kohn 1979). Two to 4 (6) nuclei per ascospore have been reported for *Dumontinia tuberosa* (Kohn 1979). *Ciboria latipes* Holst-Jensen & T. Schumach. has binucleate ascospores (Holst-Jensen and Schumacher 1994). Further multinucleate ascospores are known from fungi with 8-spored asci such as in the genus *Morchella* (*Morchellaceae*, *Pezizales*), which can have 20 or more nuclei per ascospore (Du et al. 2020). The pH-dependent color reaction

of excipulum cells, which is particularly distinct in the primordia, and in the hymenium is also a remarkable character of *M. castrans*.

Other features of the new species fit well into the morphological concept of the *Sclerotiniaceae* as newly defined by Holst-Jensen et al. (1997) and confirmed by Pärtel et al. (2017). Its apothecia arise from stromata that are differentiated into a rind and a medulla covering host tissue. Microconidial anamorphs similar to those found in *M. castrans* have been described for other genera of the *Sclerotiniaceae*. In *Sclerotinia*, conidiophores are located in the hymenium (Kohn 1979; Senn-Irlet and Peter 2016). The sporodochia of *Myriosclerotinia* (Schumacher and Kohn 1985) and *Septotinia* (Whetzel 1937) are formed on infected host tissue. In *Sclerencoelia*, microconidia are formed directly by ascospores or phialides formed by hyphae shortly after ascospore germination (Pärtel et al. 2017; Juzwik and Hinds 1984) as in *M. castrans*. *Myrioconium*-like sporodochia formed in culture have been described from several genera of *Sclerotiniaceae* (Kohn 1979; Schumacher and Kohn 1985; Juzwik and Hinds 1984; Whetzel 1937). The microconidia of *M. castrans* are formed late in spring when the infection of the new pollen cones has already occurred. Therefore, we assume that they have the function of spermatia.

Ecologically, the new species is also characterized by unique features. It is the only known fungal species that parasitizes the pollen cones of spruce trees by infecting pollen grains. The only other species of the *Sclerotiniaceae* that also attacks pollen cones of conifers is *Elliottinia kerneri* occurring on fir trees. This species differs from *M. castrans* not only in the host plant but also in producing spherical sclerotia that replace the pollen cones (Kohn 1979; Senn-Irlet et al. 2021). According to our phylogenetic analysis, *E. kerneri* is not closely related to the new species but to *Botrytis*, *Dumontia*, and *Sclerotinia*, which also produce tuberous sclerotia (Whetzel 1945). Consequently, the infection of conifer pollen cones may have evolved in the *Sclerotiniaceae* independently. Various other groups of fungi use free pollen grains as occasional or main nutrition source (Hutchison and Barron 1997; Wurzbacher et al. 2014). Some of them, such as the species of the genera *Retiarius* (Magyar et al. 2017a; Oliver 1978) and *Mycoceros* (Magyar et al. 2017b), both anamorphic *Orbiliomycetes*, are specialized to catch and parasitize free, wind-borne pollen grains (see also Baral et al. 2020, p. 145). However, we could not find any other example of a fungus that parasitizes pollen grains already inside the male cones of conifers.

In summary, the characteristics of *M. castrans* that are aberrant for *Sclerotiniaceae* are obviously adaptations to its special lifestyle. Other discomycetes from similar microhabitats in tree crowns show similar adaptations, although they belong to different genera or families (Pärtel et al. 2017; Voglmayr et al. 2020). Sherwood (1981) therefore speaks of "convergent evolution in discomycetes from bark and wood."

All this justifies the erection of the new genus *Microstrobilinia* in *Sclerotiniaceae* with the one species *M. castrans* for this exceptional fungus.

Host preferences

The altitudinal host preferences of *M. castrans* are remarkable: below 600 m asl., it occurred mainly on the non-native *P. smithiana* and above 800 m asl. on *P. abies* and *P. omorika*. At the same elevation, the three tree species flower at the same time (own observations in Aubonne, Birmensdorf and Geneva). The size of the pollen cones is the most striking difference between these species and could thus explain this altitudinal distribution pattern. *Picea smithiana* has much larger stromatized pollen cones that can store more nutrients and humidity than those of the other two species. Thus, the pollen cones of *P. smithiana* can persist for more than three years on the tree, those of the other two species only up to 2 years. Therefore, *M. castrans* might survive unfavorable periods or even whole years better on pollen cones of *P. smithiana* than on pollen cones of *P. abies* and *P. omorika* at similar altitudes. These features could be advantageous for the fungus in the comparably dry microclimates of urban habitats at lower altitudes. At higher elevations with higher humidity, the fungus can obviously grow and reproduce on the smaller pollen cones of *P. abies* and *P. omorika*. This would also fit with the observation that *M. castrans* was only found once on *P. omorika* at the WSL site in spring 2018, but not after the extremely dry summers of 2018 and 2019. On *P. smithiana*, on the other hand, apothecia were consistently observed, but almost only on old pollen cones. Freshly infected cones were very rare or absent from 2020 onwards. However, this explanatory hypothesis still needs to be verified by more and longer observations.

We never observed that all pollen cones of a tree were infected, but usually only very few. Thus, *M. castrans* most probably does not significantly reduce the fertility and reproduction ability of *Picea* spp.

Origin and distribution

Recently, some new undescribed species of fungi have appeared in European forests, and it is not always certain whether they are indigenous or have been introduced. *Cryphonectria carpinicola* D. Rigling, T. Cech, Cornejo & Beenken, which causes bark canker on hornbeam (Cornejo et al. 2021), and *Petrakia liobae* Beenken, Andr. Gross & Queloz, which causes necroses on beech leaves (Beenken et al. 2020), are such examples. While the former might have been confused with similar looking *Cryphonectria* and *Nectria* species from Europe, the conspicuous symptoms caused by *P. liobae* may not have been overlooked in the past.

The origin of *M. castrans* is unclear as well. In our opinion, it is highly unlikely that this conspicuous fungus could have been overlooked over the last centuries (e.g., Kujala 1950; Schmidt-Vogt 1989; Butin 2011; Farr and Rossman 2022), especially, if the species would always have been as common and widespread as it is today. Its consistent occurrence on the exotic *P. smithiana* could also indicate a non-native origin of *M. castrans* and that it could be a neomycet in Europe. Only more data on the European and worldwide distribution and population genetics of the fungus will probably help clarifying the origin of the new genus and species. In particular, the Asian native range of *P. smithiana* should be considered. For Europe, we expect that *M. castrans* can be found throughout the Alps and other mountain regions, e.g., in France, Austria, and Slovenia. I might also be present in more northern and north-eastern parts of the *Picea abies* distribution range. The use of new techniques like spore traps and metabarcoding might also be useful in unraveling the current distribution range of *M. castrans*. Like this, we detected *M. castrans* in France via the GlobalFungi database of environmental sequences. This genetic detection seems plausible, as the used spore trap was placed at a typical habitat of *M. castrans*, a mountain meadow surrounded by mixed forests with spruce, and sampled during a period when the fungus sporulates (Tignat-Perrier et al. 2020). Several recent studies have explored the use of spore sampling for fungal biodiversity monitoring (e.g., Abrego et al. 2018; Castaño et al. 2019; Ovaskainen et al. 2020; Tignat-Perrier et al. 2020; Redondo et al. 2020) and pathogen surveillance (Van der Heyden et al. 2021). Global and long-term monitoring projects are therefore important, as are initiatives like GlobalFungi, allowing an effective search for deposited DNA sequences (Baldrian et al. 2022). However, to understand the ecology and function of the species, the classical work of field mycologists and morphologists is still equally important and needed.

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Author contribution L. Beenken wrote the main part of the manuscript and performed the morphological and phylogenetic analyses. S. Stroheker and V. Dubach isolated the fungus from individual spores and pollen cones, cultivated it, and executed the experiment on the recently infected pollen cones. They were also responsible for the phenological observations at the WSL. V. Dubach drew Fig. 7c. M. Schlegel had the idea to search for the fungus in the GlobalFungi database and carried it out. V. Queloz helped designing the study and writing the text. A. Gross was the first to discover the new fungus and determined after preliminary molecular and morphological investigations and consultation with ascomycete specialists that the fungus is a new species. He initiated and designed the experiments and genetic investigation, including the design of the new primer pair. Furthermore, he gave significant input to unravel the biology of the species. All authors wrote their paragraphs, helped with sampling the new fungus, and proofread the final manuscript.

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Declarations

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