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The causal agent of anthracnose of *Rhododendron* in Sweden and Latvia

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Anthracnose caused by *Colletotrichum* is a severe foliar disease in rhododendron plantations in Sweden and Latvia. Isolates of this pathogen were collected and characterised based on morphological and molecular criteria. Out of 37 isolates examined, two with falcate spores were identified as *C. dematium*. The remainder of the isolates had straight cylindrical conidia, which were too variable to be reliably assigned to *C. gloeosporioides* (syn. *C. azaleae*) the reported causal agent of anthracnose of *Rhododendron* spp. These isolates were compared with the reference strains of *C. gloeosporioides*, with holo- and paratypes and with reference isolates of *C. acutatum*. The light and scanning electron microscopy examinations revealed that conidial shape was not a reliable criterion for separation of these two taxa and for the identification of our isolates. The two internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were PCR amplified and sequenced from all the rhododendron isolates and the reference strains, including the holo- and paratype materials. In addition, a segment of mitochondrial small subunit ribosomal DNA (mtSSU rDNA) and a fragment of a β-tubulin gene from several representatives of isolates of rhododendron as well as the reference strains were amplified and sequenced. The sizes of the amplified fragments among the isolates studied were heterogeneous only for mtSSU rDNA, which allowed separation of *C. acutatum* from *C. gloeosporioides*. Parsimony analysis of the individual and combined nucleotide sequence data sets were concordant and indicated that all isolates originating from rhododendrons belonged to *C. acutatum*. The grouping of the isolates was further supported by bootstrap analysis. Thus, *C. acutatum* was identified as the causal agent of anthracnose of *Rhododendron* in Sweden and Latvia. To our knowledge, this is the first report of *C. acutatum* and *C. dematium* on *Rhododendron*. This study is also the first to determine the genetic status of the holotype of *C. acutatum* and also one of the paratypes on the basis of ITS sequences in comparison with world-wide living isolates of this taxon.

INTRODUCTION

Rhododendron species are popular ornamentals that include both rhododendrons and azaleas. They have been cultivated for more than 200 years (Kondratovics 1979). These plants are affected by several foliar diseases including anthracnose, resulting in leaf spots and defoliation and often results in severe financial losses (Coyier & Roane 1986).

Anthracnose of *Rhododendron* was reported for the first time in detail in 1958 in Louisiana, USA, and the causal agent was assigned to *Colletotrichum azaleae*. The species was originally described on *Azalea viscosa* (syn. *R. viscosum*) from Florida (Statis & Plakidas 1958). This species was later considered to be synonymous with *C. gloeosporioides* (von Arx 1957, Farr *et al.* 1989). The occurrence of anthracnose has also been reported on a variety of species of *Rhododendron* from several other countries (Coyier & Roane 1986, Farr, Esteban & Palm 1996). Severe damage caused by the disease was observed by the authors in both Swedish and Latvian rhododendron plantations and nurseries. However, to our knowledge the identity of the causal agent has not been studied so far.

The species concept in the genus *Colletotrichum* has been widely studied, using spore and colony morphology, isozyme patterns and molecular characteristics, the latter including RFLP of mitochondrial DNA and nucleotide sequences from internally transcribed spacers (ITS1 and ITS2) and from domain 2 of the large subunit ribosomal rRNA genes (Smith & Black 1990, Bonde, Peterson & Maas 1991, Hodson, Mills & Brown 1993, Sheriff *et al.* 1994, Sreenivasaprasad *et al.* 1996, Freeman & Katan 1997, Johnston & Jones 1997). The ITS1 sequences were found to be informative for separating the species of *Colletotrichum* and it was used for phylogenetic analysis of these taxa (Sreenivasaprasad, Mills & Brown 1994, Sreenivasaprasad *et al.* 1996). For certain species, e.g. *C. coccodes*, the morphology, in particular conidial shape and size,

agrees with the molecular data, whereas for others, such as *C. gloeosporioides* and *C. acutatum*, isolates have displayed a wide range of ITS sequence variations, and the morphology has sometimes been not in accordance with the molecular groupings (Sreenivasaprasad *et al.* 1996, Yang & Sweetingham 1998). In the latter case, there has been a general acceptance amongst mycologists of broad species boundaries in these taxa in order to place all isolates with similar morphology from a wide range of hosts into a single species, despite genetic dissimilarities. In addition to the nuclear ribosomal genes and their spacers, sequences from other regions of the genome, for instance, the mitochondrial ribosomal DNA, and functional genes such as exons and introns of the β-tubulin gene have been used in the identification of fungi and for construction of fungal phylogeny (Glass & Donaldson 1995, O'Donnell, Cigelink & Nirenberg 1997). The importance of the use of sequence data of multiple loci for the true delineation of species and for robust phylogenetic inferences has been shown with several fungal pathogens (O'Donnell *et al.* 1997).

The main aim of this study was to identify and characterise the isolates of *Colletotrichum* associated with anthracnose of rhododendrons from Latvia and Sweden using morphology, colony characters and nucleotide sequence data from nuclear, mitochondrial and ribosomal genes and a fragment of the β-tubulin gene. The morphological and molecular data were authenticated by inclusion of holo- and paratype herbarium specimens and reference isolates obtained from different laboratories.

MATERIALS AND METHODS

Isolation and preservation

Fungal cultures were isolated from leaf and petiole lesions. Potato Dextrose Agar (PDA) plates were inoculated with five approximately 9 mm^2 large pieces of a surface sterilised tissue taken from the margin of healthy and diseased tissue, five plates per leaf. Reference strains were kindly provided by S. Freeman (Volcani Centre, Israel), B. J. Smith (USDA, USA), S. Sreenivasaprasad (HRI, UK), O. Constantinescu (UPSC, Sweden) and CABI Bioscience (IMI Egham, UK). The origin of the isolates used is shown in the Tables 1 and 2. All the isolates were maintained on PDA slants at 4 °C and as freeze-dried spore suspensions in 0.5 % skim milk. The strains are preserved in the Mycotec of the Unit and can be requested by anyone who has an interest in the work we report.

Pathogenicity tests

The pathogenicity of the strains isolated from rhododendrons was confirmed, fulfilling Koch's postulates using a detached leaf assay (Hohryakov 1976). Two-year-old leaves of the rhododendron cultivar 'Nova Zembla' were used. Inoculum was obtained from fungal colonies, grown on solid modified Mathur's medium (Tu 1985) for 7 d at 21 ° in the darkness. Conidia were harvested and dispersed in sterile water and the concentration adjusted to 1×10^5 spores ml^{-1}. Leaf disks 20 mm diam were cut from the leaves with a cork borer and placed on moistened filter paper in Petri dishes under sterile conditions. Each disc was inoculated with two 20 µl droplets of conidial suspension and incubated at room temperature (22 ± 2 °). The appearance of typical anthracnose lesions was recorded 10 days after inoculation. Pieces of infected tissue were transferred onto PDA and pure *Colletotrichum* cultures were recovered to satisfy Koch's postulates. The assay was carried out twice.

Morphological examination

For morphological examination, Petri plates with PDA were inoculated with mycelial discs, 5 mm diam, taken from the margin of the young growing colonies. Growth rate was determined from the colonies grown on PDA in darkness for 7 d at 21 °, with two replicates for each strain. Size and shape of conidia were recorded from the colonies grown on malt extract agar (MA) under the same conditions as mentioned above. Poorly sporulating cultures were placed under Black light (Phillips, TL20W/08) for 3 d prior to examination.

For electron microscopy, dilute suspensions of conidia were made up by adding approximately 0.1 ml of distilled water directly to the fungal colonies on MA. Aliquots were then removed from the cultures and pipetted directly onto 0.22 µm millipore filters and allowed to dry overnight in a dessicator. In the case of dried culture herbarium specimens, small pieces of samples, which contained conidia were taken using a needle and suspended in distilled water. From this, aliquots were then taken and placed onto filter paper. Small areas of the filters were then removed and mounted on scanning electron microscope (SEM) stubs using double-sided cellotape. Samples were then coated with Pt/Au using and Agar High Resolution Sputter coater and finally examined in a Hitachi 4500 Field Emission-SEM operated at 15 kV. Images were digitalized using a Quartz PCL system.

Molecular characterisation

Mycelial plugs of single-spore cultures were transferred in Petri dishes with 20 ml of liquid Glucose Yeast-extract Medium (Mugnai, Bridge & Evans 1989). The fungi were grown for 5 d and mycelia harvested, lyophilised and ground to powder.

Total genomic DNA was extracted from 100 mg powdered mycelium in 1.5-ml ependorf tube following the protocol of Reader & Broda (1985). DNA extraction from the dried herbarium material of only the holotype (IMI 117617) and one of the paratypes (IMI 117619) of *C. acutatum* was performed as described by Cubero *et*

Table 1. Origin of the living cultures of *Colletotrichum* used.

Isolate	Identified/ received as	Geographical origin	Host plant	GenBank accession numbers		
				ITS	mtSSU	β-tubulin
S1	*Colletotrichum acutatum*	Sofiero, Sweden	*Rhododendron catawbiense*	AF411709		
S2	*C. acutatum*	Sofiero, Sweden	*Rh.* × *orbiculare*	AF411719	AF411742	AF411758
S3	*C. acutatum*	Sofiero, Sweden	*Rh.* × *orbiculare*	AF411726	AF411743	AF411759
S4	*C. acutatum*	Sofiero, Sweden	*Rh. japonicum*	AF411727		
S5	*C. acutatum*	Sofiero, Sweden	*Rh. catawbiense*	AF411728		
S6	*C. acutatum*	Sofiero, Sweden	*Rh. hybr.* 'Blue Peter'	AF411729		
S7	*C. acutatum*	Sofiero, Sweden	*Rh. calophytum*	AF411730	AF411744	AF411760
S8	*C. acutatum*	Sofiero, Sweden	*Rh. japonicum*	AF411731		
S9	*C. acutatum*	Sofiero, Sweden	*Rh. japonicum*	AF411732		
S10	*C. acutatum*	Sofiero, Sweden	*Rh. orbiculare*	AF411710		
S11	*C. acutatum*	Sofiero, Sweden	*Rh. callophytum*	AF411711		
S12	*C. acutatum*	Sofiero, Sweden	*Rh. insigne*	AF411712		
S13	*C. acutatum*	Sofiero, Sweden	*Rh. catawbiense*	AF411713		
S14	*C. acutatum*	Sofiero, Sweden	*Rh. brachycarpum*	AF411714		
S16	*C. acutatum*	Sofiero, Sweden	*Rh. japonicum*	AF411715		
S17	*C. acutatum*	Sofiero, Sweden	*Rh. luteum*	AF411716	AF411741	AF411757
S18	*C. acutatum*	Sofiero, Sweden	*Rh. hybr.* 'Cunningham's White'	AF411717		
S19	*C. acutatum*	Sofiero, Sweden	*Rh. degronianum*	AF411718		
S20	*C. acutatum*	Sofiero, Sweden	*Rh. brachycarpum*	AF411720		
S21	*C. acutatum*	Sofiero, Sweden	*Rh. orbiculare*	AF411721		
S22	*C. acutatum*	Sofiero, Sweden	*Rh. luteum*	AF411722		
S23	*C. acutatum*	Sofiero, Sweden	*Rh. luteum*	AF411723		
S24	*C. acutatum*	Sofiero, Sweden	*Rh. brachycarpum*	AF411724		
S25	*C. acutatum*	Sofiero, Sweden	*Rh. degronianum*	AF411725		
P1	*C. acutatum*	Pålsjö, Sweden	*Rh.* × *catawbiense*	AF411708		
G1	*C. acutatum*	Gothenburg, Sweden	*Rh. ponticum*	AF411697		
G2	*C. acutatum*	Gothenburg, Sweden	*Rh. ponticum*	AF411698		
G4	*C. acutatum*	Gothenburg, Sweden	*Rh. ponticum*	AF411699		
L1	*C. acutatum*	Babite, Latvia	*Rh.* × *brachycarpum*	AF411702	AF411738	AF411754
L2	*C. acutatum*	Babite, Latvia	*Rh.* × *brachycarpum*	AF411703		
L3	*C. acutatum*	Babite, Latvia	*Rh.* × *catawbiense*	AF411704		
L4	*C. acutatum*	Babite, Latvia	*Rh.* × *catawbiense*	AF411705		
L5	*C. acutatum*	Babite, Latvia	*Rh.* × *catawbiense*	AF411706		
L6	*C. acutatum*	Babite, Latvia	*Rh.* × *catawbiense*	AF411707		
397	*C. acutatum*	USA	*Fragaria* × *ananassa*	AF411765	AF411734	AF411749
NI90	*C. acutatum*	UK	*F.* × *ananassa*	AF411766	AF411739	AF411755
Nantana A	*C. acutatum*	USA	*Vitis vinifera*	AF411772		
Clemson SF21	*C. acutatum*	USA	*Prunus persicus*	AF411768	AF411776	AF411751
TUT-5954	*C. acutatum*	Israel	*F.* × *ananassa*	AF411794*	AF411746	AF411762
S15	*C. dematium*	Sofiero, Sweden	*Rh.* × *catawbiense*	AF411770	AF411740	AF411756
G3	*C. dematium*	Gothenburg, Sweden	*Rh.* × *catawbiense*	AF411773	AF411737	AF411753
AVO 37-4B	*C. gloeosporioides*	Israel	*Persea americana*	AF411792*	AF411735	AF411750
231	*C. gloeosporioides*	USA	*F.* × *ananassa*	AF411764	AF411733	AF411748
Fl BB	*C. gloeosporioides*	USA	*Vaccinium myrtillus*	AF411769	AF411736	AF411752
TN3	*C. gloeosporioides*	USA	*F.* × *ananassa*	AF411774	AF411745	AF411761
UPSC 2866	*Glomerella cingulata*	Uppsala, Sweden	*Salix* sp.	AF411775	AF411747	AF411763
CF63	*C. fragariae*	USA	*F.* × *ananassa*	AF411767		
La26	*C. fragariae*	USA	*F.* × *ananassa*	AF411771		

* Freeman *et al.* (2000).

al. (1998) using about 16 mm² piece of the dried agar containing fungal material. The ITS regions and 5.8S were amplified by PCR using primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). In the case of old herbarium specimens a semi-nested PCR was performed on the product of the first amplification in which primer ITS1F was replaced by ITS1 (White *et al.* 1990). The PCR reaction was carried out using PE AmpliTaq PCR kit (PE Applied Biosystems AB), according to the manufacturer's instructions. Amplification was carried out in Perkin Elmer PCR thermocycler, at 1 cycle of 96 ° for 3 min, and 35 cycles consisting of denaturation step at 96 ° for 75 s, annealing at 50 ° for 75 s, extension at 72 ° for 2 min followed by a 10 min extension step at 72 °. The amplified fragments were electrophoresed in 1.5 % LE agarose (Promega, USA) gel, stained in Tris–Borate–EDTA buffer containing 0.5 μl ml^{-1} of ethidium bromide and visualised under UV light. The amplified products were purified with QiaGen PCR Purification

Table 2. The type material of *Colletotrichum acutatum* (Simmonds 1968).

Isolate	Geographical origin	Host plant	GenBenk accession number, ITS
IMI 117617 Holotype	Australia, Ormiston	*Carica papaya*	AF411700
IMI 117618 Paratype	Australia, Ormiston	*C. papaya*	
IMI 117619 Paratype (pink type)	Australia, Ormiston	*C. papaya*	AF411701
IMI 117620 Paratype (pink type)	Australia, Ormiston	*C. papaya*	
IMI 117622 Paratype	Australia, Brisbane	*Capsicum* sp.	
IMI 117623 Paratype	Australia, Nambour	*Delphinium* sp.	

Kit (QiaGen, Crawley, UK) and direct sequencing was performed using the same primer set and PE Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems AB) following the manufacturer's instructions. Sequencing gels were run by the Centre for Genomic Research at Karolinska Institute, Stockholm, Sweden.

Fragments from mtSSU rDNA and β-tubulin genes were PCR amplified using primer pairs MS1/MS2 and Bt1-A/Bt1-B, respectively (White *et al*. 1990, Glass & Donaldson 1995). PCR, electrophoresis and sequencing conditions were the same as those described above for the ITS region. All the primers used in the work were synthesised by LifeTech Corporation (UK). The mtSSU rDNA and β-tubulin regions were sequenced only for the *Rhododendron* isolates that appeared to be the most divergent in their ITS sequences as well as for the reference strains of *C. acutatum* and *C. gloeosporioides*.

Phylogenetic analysis

Sequences were initially aligned using the DNAStar computer software package (Lasergene, Madison, WI) and the alignments were improved manually. Parsimony analysis was carried out on the aligned sequences of each of the three loci separately and in combination using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b1 for Macintosh (Swofford 1993). Most of the parsimonious trees were constructed using the heuristic search strategy with 1000 random addition sequences when alignment gaps were treated as missing data. Only a few phylogenetically informative gaps in the ITS region were treated as the fifth character. *Fusarium redolens* was selected as the outgroup (Tehler *et al*. 2000) and the trees were rooted with the sequence data of homologous regions, extracted from GenBank with the accession numbers AF008562 for ITS, U34507 for mtSSU and U34478 for the β-tubulin trees. The robustness of the internal branches of the trees was estimated by bootstrap analysis using 1000 replications in heuristic search with stepwise addition of random with 5 replicates. The bootstrap majority-rule (> 50 %) consensus trees were obtained.

RESULTS AND DISCUSSION

Isolation and pathogenicity tests

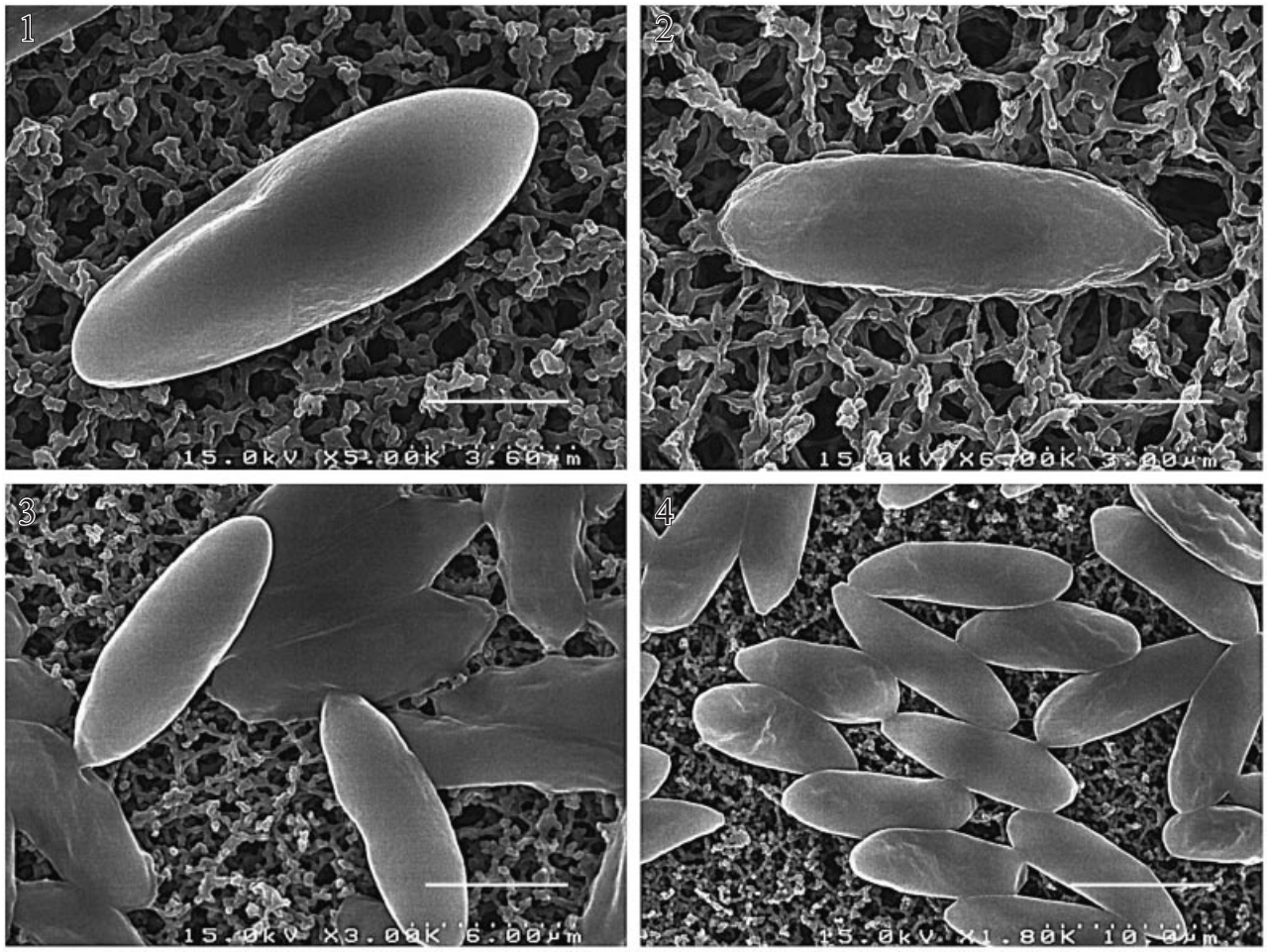
Thirty-six isolates of *Colletotrichum* were detained from leaves, and one from petioles, of rhododendrons and azalea displaying anthracnose symptoms in Sweden and Latvia (Table 1). Pathogenicity tests indicated that 35 isolates of this collection were able to cause the disease on *Rhododendron* leaves and produced lesions greater than 4 mm in diameter. One of the two isolates with falcate conidia, S15, failed to produce any symptoms.

Morphological examination

Spore shape and colony growth characteristics

Thirty-four rhododendron isolates produced straight conidia and only two produced falcate ones. The colonies of the straight-spored isolates originating from rhododendrons were initially white and became grey in colour after 3 d. However, no pigment diffused into the agar. Conidia in majority of the straight-spored rhododendron isolates as well as the reference strains of *C. acutatum* were produced in a slimy orange-pink matrix. Only in two isolates from Latvia, L1 and L2, yellowish white conidial masses were observed. Isolates S2, L3, L4, L5 and L6 produced very few conidia. Colonies of *C. acutatum* reference strains and straight-spored isolates from rhododendrons were 20–52 mm diam after 7 d growth while isolates of *C. gloeosporioides* reached 63.4–69 mm. The slower growth rate of *C. acutatum* compared to *C. gloeosporioides* has been considered as a characteristic feature of the former species by several workers (Simmonds 1965, Sutton 1980, Bernstein *et al*. 1995, Lardner *et al*. 1999). Two strains (G3 and S15) producing falcate spores and distinctly different colony appearance with a red pigmentation which diffused into agar, were also slowly growing (17–23 mm diam after 7 d). These isolates were identified as *C. dematium* and this identification was confirmed by J. E. M. Mordue and E. Punithalingam (CABI Bioscience, Egham, UK). To our knowledge, this is the first record of occurrence of this species on *Rhododendron*.

Within the straight-spored rhododendron isolates of *Colletotrichum* as well as among the reference strains and the holo- and paratype material of *C. acutatum*, conidial shapes and dimensions varied tremendously. Recent studies have shown that the high level of variation in conidial shape limits the reliability of this character for the separation of this species from *C. gloeosporioides* (Buddie *et al*. 1999, Sreenivasaprasad & Brown, pers. comm.). Although the small size, variability in length and pointed ends of conidia were

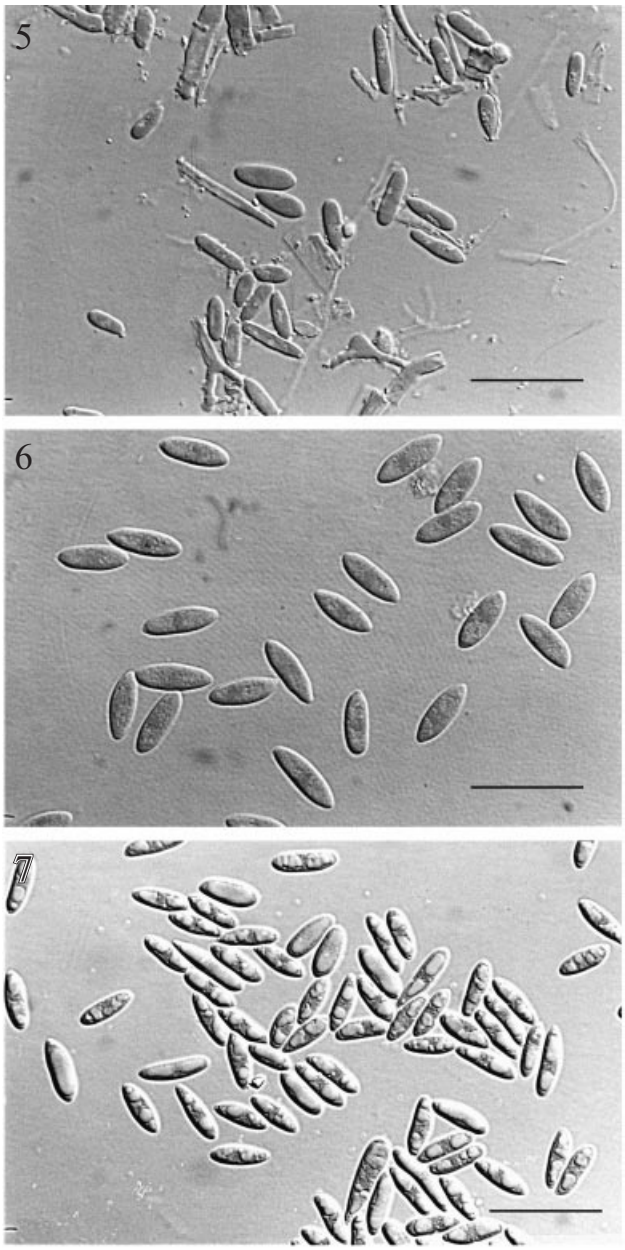


Figs 1–4. SEM of conidia of *Colletotrichum* species. **Fig. 1.** *C. gloeosporioides* (isolate 315). Bar = 3.6 μm. **Fig. 2.** *C. acutatum* (IMI 117617-holotype). Bar = 3 μm. **Fig. 3.** *C. acutatum* (IMI 117619-paratype). Bar = 6 μm. **Fig. 4.** *C. acutatum* from rhododendron (isolate S16). Bar = 10 μm.

described as the diagnostic features of *C. acutatum* by Simmonds (1965) this was not clearly evident even in the figures provided for this taxon in his description. Among six dried agar-cultured specimens as holotype and paratypes of *C. acutatum* deposited in IMI by Simmonds, are two specimens distinguished as 'Pink Type' from the holotype, and three other paratypes which all have grey coloured colonies (Simmonds 1965). Our examination of these materials has shown that the conidial shape in the 'Pink Type' specimens is mostly elliptic with both ends pointed, different from those in the holotype and other grey-colony paratypes in which the conidia were much more variable in the shape, mostly cylindrical, narrowed toward base with both rounded ends similar to those from *C. gloeosporioides*, or one round end and acute in the base (Figs 2–7). A similar conidial shape was found in all the straight-spored isolates from rhododendron and some of the reference isolates of this taxon (Figs 1–7). None of our isolates from rhododendron produced elliptical conidia with acute ends. The conidial dimensions in *C. acutatum* also seem to be highly variable compared to those found in *C. gloeosporioides*. Simmonds reported the mean size as '8.3–14.4 × 2.5–4.0 μm' in his description of *C. acutatum*, although he mentioned the inconsistent production of larger conidia in some colonies (Simmonds 1965). Our examination showed that conidia in the holotype were smaller (10 × 3.5 μm) than those measured from paratype materials (11–15. 5 × 3.5–4 μm). The size of conidia in rhododendron isolates and reference isolates of *C. acutatum* ranged 10–22.5 × 3–5.5 μm. These measurements were undertaken with 7 d-old colonies and were consistently obtained despite sub-culturing. Conidia of *C. gloeosporioides* showed less variation with the size of 14–18 × 4–5.5 μm. The overlaps of conidial sizes among isolates of these two taxa have already been pointed out while the larger conidial dimensions have also commonly been reported for *C. gloeosporioides* (Simmonds 1965, Mordue 1971, Dyko & Mordue 1979, Walker, Nikandrow & Millar 1991).

Molecular characterisation

The PCR amplification using primer pair ITS1F/ITS4 resulted in a single fragment, approximately 570 bp, in all isolates from *Rhododendron* and other reference isolates and herbarium specimens studied. Electro-



Figs 5–7. LM of conidia of *Colletotrichum acutatum*. **Fig. 5.** IMI 117617 (holotype). **Fig. 6.** IMI 117619 (paratype). **Fig. 7.** From *Rhododendron* (isolate S16). Bars = 20 μm.

phoresis of the ITS products of the straight-spored rhododendron isolates in agarose gel did not reveal any size variation among each other and they were identical in size to those of *Colletotrichum acutatum* reference isolates, but were different from *C. gloeosporioides* and *C. dematium* isolates. The ITS products of the latter two taxa were similar in size on electrophoresis gels and were about 550 bp long.

The aligned nucleotide sequences of the ITS fragments from all the isolates studied were compared with those of 18 *Colletotrichum* species which were available in the GenBank. The total size of the ITS regions including the 5.8S rRNA gene of the isolates studied varied from 481 to 491 bp. This variation was due to the size divergence in ITS1, which ranged from 171 to 181 bp, while ITS2 and 5.8S were consistently 152 and 158 bp, respectively, in all the isolates examined. ITS1 in the *C. acutatum* reference isolates and straight-spored rhododendron isolates was from 178 to 181 bp while it ranged from 171 to 173 in *C. gloeosporioides* isolates. Up to 4.1 % nucleotide divergence was observed in the ITS1 among the rhododendron isolates while 15 of these isolates contained an identical ITS region. In comparison with the ITS sequence obtained from the reference strains examined in this study and those from the GenBank, all the straight-spored rhododendron isolates showed higher level of dissimilarity to *C. gloeosporioides* (11.0–15.5 %) than to *C. acutatum* (0–9.5 %) in the ITS1. The ITS1 and ITS2 regions of both falcate-spored isolates identified as *C. dematium* were identical with the size of 171 and 152 bp, respectively. The level of nucleotide sequence variation among studied isolates of these three taxa is shown in Table 3.

Comparison of the 5.8S sequence data revealed that *C. acutatum* differed from *C. gloeosporioides* in nucleotide position 135 of this region. Similar variation was observed when sequences of seven other species of *Colletotrichum*, two of *Glomerella* and eleven other species of ascomycetes randomly chosen from EMBL were aligned with *C. acutatum* sequences. The presence of adenine (A) in this position in only *C. acutatum* appeared to be characteristic for this species since other taxa compared had guanine (G) in this position. The 5.8S sequences were all identical in straight-spored *Colletotrichum* isolates from rhododendron and reference strains and type materials of *C. acutatum*.

Phylogenetic analysis based on ITS1, ITS2 and 5.8S regions clearly detected 3 major clades with 100 % bootstrapped values, representing the three species of *C. acutatum*, *C. gloeosporioides* and *C. dematium* (Fig. 8). All straight-spored isolates from rhododendron fall into the *C. acutatum* clade. Two isolates, 397 and NI90, previously reported as two highly divergent isolates of *C. acutatum* in ITS regions by Sreenivasaprasad *et al.* (1996), grouped separately with a rather low bootstrap support but higher than 50 %. Both the examined holo- and paratype specimens, despite their different morphological appearances clustered with 397 and several others reference strains while the majority of straight-spored rhododendron isolates grouped with NI90. Six of the straight-spored isolates from rhododendron represented a new ITS population, which grouped in a distinct cluster with a bootstrap value of 98 %. None of the *Colletotrichum* isolates from rhododendron grouped with *C. gloeosporioides*. Two of the *C. fragariae* isolates used in the analysis came together with the *C. gloeosporioides* reference isolates, supporting earlier observations of Sreenivasaprasad *et al.* (1996) (Fig. 8).

Amplification of a portion of the mtSSU rDNA was successfully carried out using primer pair MS1/MS2 from the *Colletotrichum* isolates studied. The amplification of this fragment and a part of the β-tubulin gene,

Table 3. Percent of divergence among isolates of different species of *Colletotrichum* within the studied regions (%).

Region	Species	*C. gloeosporioides*	*C. acutatum*	*C. dematium*
ITS1	*C. gloeosporioides*	0.0–3.0		
	C. acutatum	10.8–15.5	0.0–9.6	
	C. dematium	10.8–12.3	10.8–14.7	0.0
5.8S	*C. gloeosporioides*	0.0		
	C. acutatum	1.3	0.0	
	C. dematium	0.0	1.3	0.0
ITS2	*C. gloeosporioides*	0.0–2.0		
	C. acutatum	11.5–15.4	0.0–3.8	
	C. dematium	7.0–10.9	10.8–14.9	0.0
β-tubulin	*C. gloeosporioides*	0.0–3.5		
	C. acutatum	8.0–10.7	0.0–7.9	
	C. dematium	9.2–14.3	7.3–15.7	14.3
mtSSU	*C. gloeosporioides*	0.0–0.2		
	C. acutatum	7.0–7.8	0.0–2.2	
	C. dematium	6.6–6.9	3.5–5.2	0.0

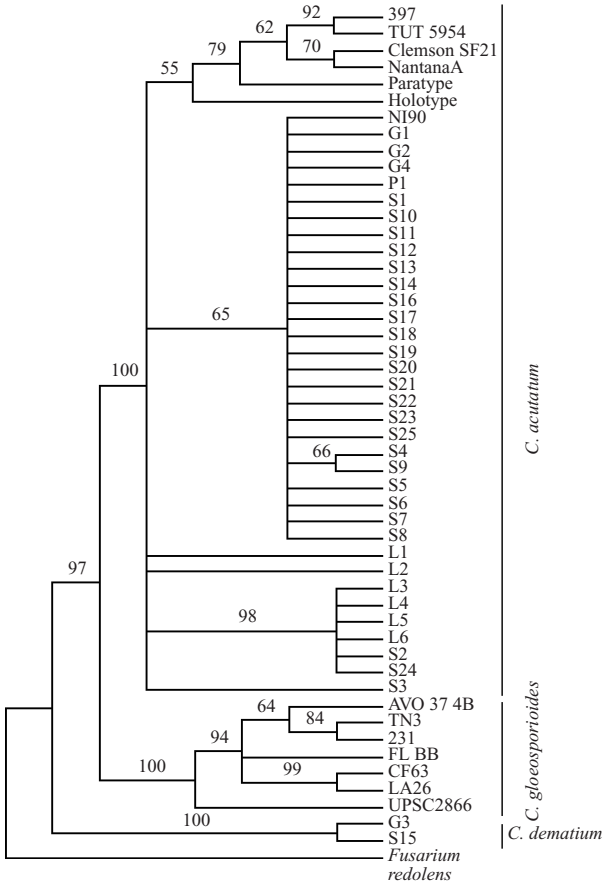


Fig. 8. Phylogenetic tree of all the studied *Colletotrichum* isolates based on ITS sequence data. The numbers on each branch correspond to the bootstrap value.

failed in the type specimens, perhaps because of the quantity or quality of the DNA extracted from these old herbarium specimens. The mtSSU rDNA fragments appeared as a single band for each isolate with significant size variations among the isolates. The sequence comparison of this region revealed that four *C. gloeosporioides* isolates were identical in this region, with the size of 407 bp, which was shorter than the ones obtained from representative *C. acutatum* and straight-spored rhododendron isolates. This region varied from 452 to 481 bp among both the reference *C. acutatum* and the rhododendron isolates examined. However, all the sequenced straight-spored isolates originating from rhododendron and one of the *C. acutatum* isolates (NI90) were identical in this region (Table 3). Three other reference isolates of *C. acutatum* (397, TUT 5954 and Clemson SF21) were similar to each other and different from the rest of the isolates included in this analysis in the possession of the longest PCR products. Sequence comparison revealed that the length divergence in this region between and within these taxa was only due to a highly variable region close to the 3′ end of the amplified fragment. The sequence alignment of this polymorphic region is shown on the Fig. 9.

Phylogenetic analysis inferred from this region was concordant with that of the ITS regions. Straight-spored rhododendron isolates grouped with the *C. acutatum* clade, distinct from *C. gloeosporioides* and *C. dematium*, with 100 % stability assessed by bootstrapping (data not shown). The situation of the two highly divergent ITS isolates, 397 and NI90, in the gene tree obtained from this locus confirmed the heterogeneous genetic identities of these two *C. acutatum* populations. Similar, representative straight-spored isolates from rhododendron grouped with NI90 within the clade of *C. acutatum*. *C. dematium* showed a closer genetic relationship based on this region to *C. acutatum* than *C. gloeosporioides*. The examination of mtSSU rRNA gene sequences in this study indicates the potential usefulness of this locus in distinguishing species for phylogenetic studies of *Colletotrichum*.

Amplification of the β-tubulin gene resulted in products, which were visualised as similar sized fragments on gel electrophoresis with approximately 540 bp in length for all the isolates studied. This fragment was sequenced from the 15 isolates selected and the nucleotide sequences were aligned and compared with the relevant region from *Neurospora crassa*, extracted

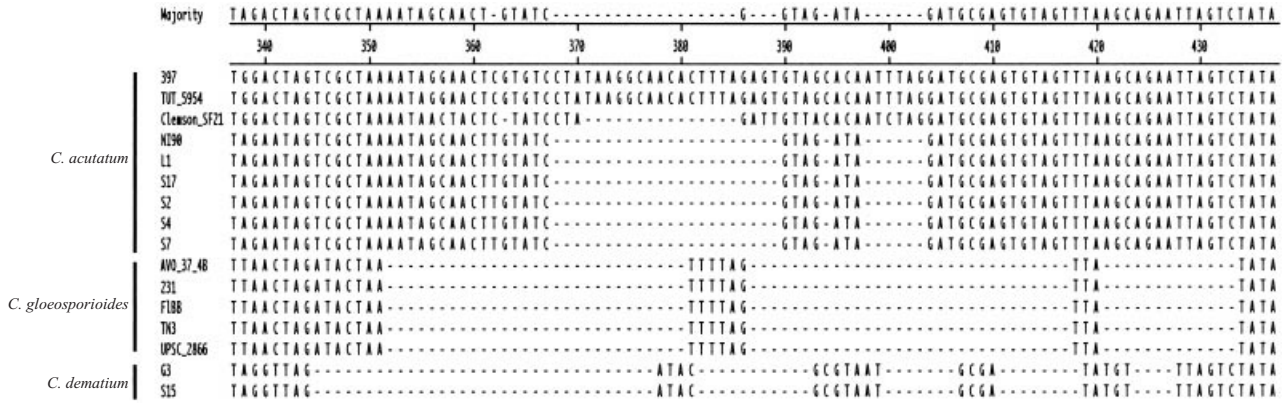


Fig. 9. Nucleotide alignment of DNA sequences in a fragment of mtSSU region.

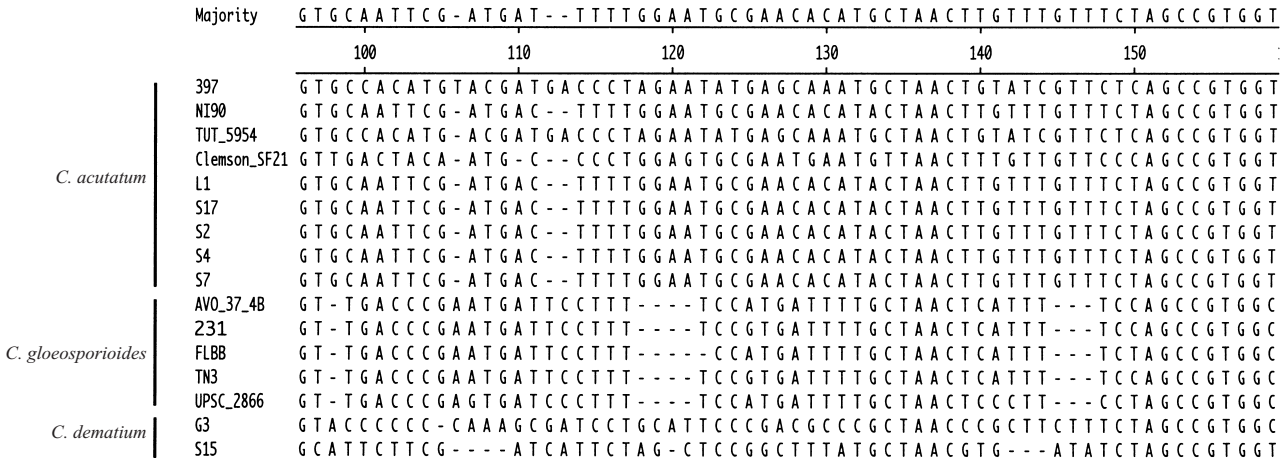


Fig. 10. Nucleotide alignment of DNA sequences in a fragment of the β-tubulin gene.

from GenBank (accession number M13630). This comparison showed that the amplified region in *Colletotrichum* isolates contained the regions of protein-coding sequences which flank an intron already described in the β-tubulin gene of *N. crassa* (Orbach, Porro & Janofsky 1986). The sequence data obtained from representative isolates of both *C. gloeosporioides* and *C. acutatum* and rhododendron isolates did not reveal any significant size variation between and within the isolates. Within the taxa, the variation was accumulated mostly in the intron region, while between taxa, the sequences diverged in both intron and exons. However, no sequence variation was observed among the straight-spored rhododendron isolates. Just as for ITS and mtSSU, the NI90 isolate had showed an identical sequence in this region to that of the straight-spored rhododendron isolates. Isolates 397, TUT 5954 and Clemson SF21 were distinctly different, showing up to 6.6% sequence dissimilarity with NI90 and the straight-spored rhododendron isolates, a similar percentage of dissimilarity was present in the ITS and mtSSU regions. Despite this dissimilarity, all the isolates mentioned above were distinctly different in this region from both the *C. gloeosporioides* and *C. dematium* reference isolates (Fig. 10). Variation within the nucleotide sequences of this fragment for five *C. gloeosporioides* reference isolates was the lowest – up to 3.5%. Surprisingly, two isolates of *C. dematium* showed a high variation in this region (14.3%), although they were 100% identical in both ITS and mtSSU (Table 3). This could be due to the presence of another divergent copy of the β-tubulin gene, as it was observed in *Colletotrichum gloeosporiodes* f. sp. *aeschynomene* (Buhr & Dickman 1993), and which could have been competitively amplified in the PCR.

Apart the position of the two isolates of *C. dematium*, the gene topology obtained from this fragment of β-tubulin gene was in agreement with those of ITS and mtSSU, although the *C. acutatum* clade was not supported by a high bootstrap value (only 54%). Representative straight-spored isolates from rhododendron grouped with NI90 (data not shown).

A robust phylogenetic tree was generated by the parsimony analysis of the combined nucleotide sequences of three loci including ITS regions and 5.8S, mtSSU rDNA and the segment of the β-tubulin gene. Out of 1473 total characters, 174 were parsimonious-informative and only one most-parsimonious tree was forthcoming. The three species of *Colletotrichum* were clustered into three major clades with the bootstrap

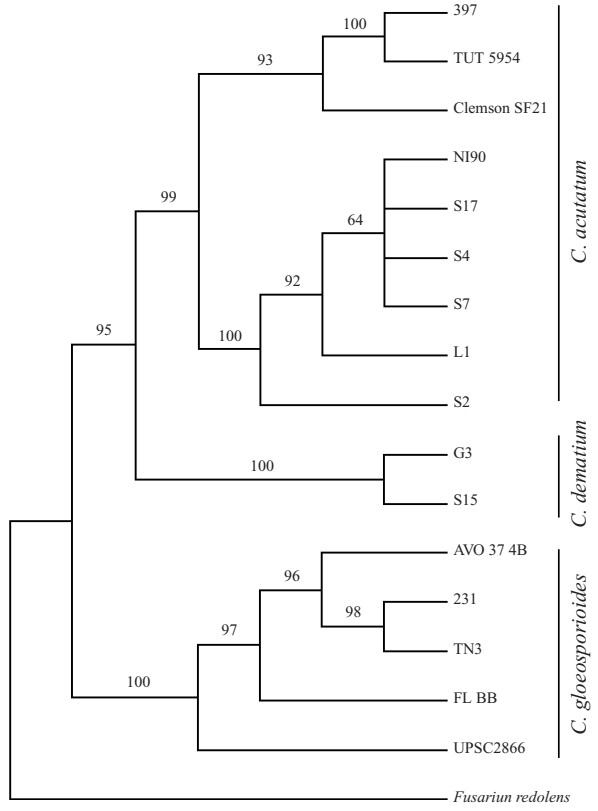


Fig. 11. Phylogenetic tree based on the data set combining sequence data of the ITS, mtSSU and β-tubulin gene for the representative strains of three species of *Colletotrichum*. The numbers on each branch correspond to the bootstrap values.

value of 99–100 % (Fig. 11). Representatives of straight-spored isolates from rhododendron grouped with isolate NI90 into a cluster with the support of 100 % distinct from three other reference isolates of *C. acutatum*, 397, TUT 5954 and Clemson SF21. The high genetic divergence observed in this study for these two populations of *C. acutatum* would support the previous suggestion for dividing *C. acutatum* into two species (Sreenivasaprasad *et al*. 1996).

All the evidence presented here confirms that *Colletotrichum* isolates with straight conidia responsible for the rhododendron anthracnose in Latvia and Sweden belong to *C. acutatum*. A recent study has also shown that *C. acutatum* was one of the causal agents of anthracnose of olive trees in Spain (Martin & Garcia-Figueres 1999). In addition, on the basis of ITS sequences and other molecular markers the isolates causing anthracnose of almond in Israel which originally were considered to be *C. gloeosporioides* clearly grouped with *C. acutatum* (Freeman *et al*. 2000). None of the isolates of *Colletotrichum* originating from rhododendrons in the United States, where this disease was reported once by Statis & Plakidas (1958), were examined in our work. We do not have data on the type material of *C. azaleae*, since no fructification was found on the holotype material of this taxon by these authors. Comparison of isolates of European and North American populations of *Colletotrichum* originating from *Rhododendron* species would be an interesting extension of this work.

In this study, the usefulness of other loci than ITS regions in the identification of species and subspecific populations of *Colletotrichum* was shown by analysing sequence data obtained from the fragments of mitochondrial rRNA and the β-tubulin gene. To our knowledge, this is the first study of the genetic status of the holotype and one of the paratype specimens of *C. acutatum*.

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