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Phenotypic and DNA sequence data comparisons reveal three discrete species in the *Ceratocystis polonica* species complex

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Ceratocystis polonica and *C. laricicola* are two morphologically similar species that occur on conifers and reside in the *Ceratocystis coerulescens* species complex. They, however, represent two ecologically distinct entities. *C. polonica* causes blue stain on Norway spruce (*Picea abies*) and other spruce species (*Picea* spp.) in Eurasia and is associated with the bark beetles *Ips typographus*, *I. typographus japonicus*, *I. amitinus* and *I. duplicatus*. In contrast, *C. laricicola* lives in a symbiotic relationship with the bark beetles *Ips cembrae* and *I. subelongatus* that infest various larch species (*Larix* spp.). The objective of this study was to consider the phylogenetic relationships of *C. polonica* and *C. laricicola* and more specifically to determine the identity of Japanese isolates from both spruce and larch, based on sequences derived from the ITS regions of the rRNA operon, the β-tubulin gene and the HMG box of the MAT-2 gene. Isolates were also compared based on morphology and cultural characteristics. Comparisons of anamorph and teleomorph structures confirmed that *C. polonica* and *C. laricicola* are indistinguishable based on morphology. Both species had an optimal growth temperature of 25 °C. However, at temperatures between 31–33 °, *C. polonica* isolates grew slowly or not at all, while *C. laricicola* isolates grew more actively at these temperatures. Thus, a growth test at 32 ° can differentiate these species. Phylograms generated using parsimony for the three gene regions were strongly congruent. These showed three distinct clades supported by high bootstrap values. Two of the clades clearly separate *C. laricicola* from Europe and *C. polonica*, supporting the view that they represent two discrete taxa. A third clade included isolates obtained from galleries of *Ips subelongatus* on *Larix kaempferi* in Japan. This fungus clearly represents a discrete taxon that is closely related to, but distinct from *C. laricicola*, which is described here as *C. fujiensis* sp. nov.

INTRODUCTION

The genus *Ceratocystis* includes a number of important pathogens of both angiosperms and gymnosperms. Eleven species belonging to this genus can be grouped together in the *C. coerulescens* complex. These fungi share a similar morphology and are related based on DNA sequence data and isozyme profiles (Harrington *et al.* 1996, Harrington & Wingfield 1998, Witthuhn *et al.* 1998, 1999, 2000). Seven of these species (*C. coerulescens*, *C. pinicola*, *C. rufipenni*, *C. resinifera*, *C. laricicola*, *C. polonica*, *C. douglasii*) cause sap stain on conifers in the *Pinaceae* (Harrington *et al.* 1996, Harrington & Wingfield 1998). The remaining four species (*C. virescens*, *C. eucalypti*, *Thielaviopsis australis*, *T. neocaledoniae*) are pathogens or sap stain agents on hardwoods (Harrington *et al.* 1996, Paulin-Mahady, Harrington & McNew 2002). During the last decade, species in the *C. coerulescens* complex have been studied using several phenotypic and genotypic characters (Harrington *et al.* 1996, Harrington & McNew 1998, Harrington & Wingfield 1998, Witthuhn *et al.* 1998, 2000, Harrington *et al.* 2002). These studies

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have added considerable resolution to the taxonomy of nine species. However, *C. polonica* and *C. laricicola* are very similar and problems have been experienced in distinguishing them. *C. polonica* and *C. laricicola* are considered to be morphologically indistinguishable (Harrington & Wingfield 1998). Additionally, they have been reported to have identical ITS sequences (Witthuhn *et al.* 1999, 2000) and a low level of isozyme variation (Harrington *et al.* 1996). However, they represent two distinct ecological entities. *C. polonica* causes blue stain on Norway spruce (*Picea abies*) and is associated with the beetles *Ips typographus*, *I. amitinus* and *I. duplicatus* in Europe (Solheim 1986, Krokene & Solheim 1996, Kirisits 2001, 2004). In contrast, *C. laricicola* infests larch (*Larix decidua*) and lives in a mutualistic relationship with the bark beetle *I. cembrae* in Europe (Redfern *et al.* 1987, Stauffer *et al.* 2001, Kirisits 2001, 2004). The two species can cross, but perithecia formed in these interspecific crosses are not fertile or yield only low numbers of distorted ascospores that do not result in viable progeny (Harrington & McNew 1998, Harrington *et al.* 2002). Harrington *et al.* (2002) also showed differences between these two fungi based on DNA sequences from a portion of the MAT-2 idiomorph. In addition, Kirisits (2001) and Harrington *et al.* (2002) provided evidence of physiological specialization of *C. polonica* and *C. laricicola* on their respective host trees.

Inoculation tests with *C. polonica* and *C. laricicola* on their respective hosts have shown that both species probably play an important role in tree death following attack by their insect vectors (Christiansen 1985, Redfern *et al.* 1987, Krokene & Solheim 1998, Yamaoka *et al.* 1998, Kirisits & Offenthaler 2002). They are aggressive vascular stain pathogens causing intensive and extensive blue-stain in the sapwood of bark beetle-infested spruce and larch. This results in substantial economic losses, because markets pay much lower prices for blue-stained timber and wood products (Uzunovic *et al.* 1999, Kirisits 2001).

Both *C. polonica* and *C. laricicola* are known to occur in Japan. Yamaoka *et al.* (1998) studied the ophiostomatoid fungi associated with *I. cembrae* in Japan and found that *C. laricicola* was the only fungus in this niche that had the ability to kill Japanese larch (*L. kaempferi*). Similarly, *C. polonica* is recognized as an important fungal associate of *I. typographus japonicus*, an Asian subspecies of *I. typographus*, that infests Yezo (*P. jezoensis*) and Sachalin (*P. glehnii*) spruce in Japan (Yamaoka *et al.* 1997). Pathogenicity tests have shown that *C. polonica* displays high levels of virulence to Yezo spruce (Yamaoka, Takahashi & Iguchi 2000).

The objective of this study was to consider the phylogenetic relationships between *C. polonica* and *C. laricicola*, and more specifically to determine the identity of Japanese isolates from both spruce and larch, using multigene sequence data. This goal was achieved through the analysis of sequences derived from the ITS regions of the rRNA operon, part of the β-tubulin gene and the HMG box of the MAT-2 gene, using a large number of isolates from different regions in Europe and Japan. The fungi were also compared morphologically and in culture.

MATERIALS AND METHODS

Isolates

Isolates (50) of *Ceratocystis polonica* from Austria, the Czech Republic, France, Japan, Norway, Poland, and the UK were included in this study (Table 1). All of these isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and a subset has been deposited in the Japan collection of Microorganisms (JCM), the collection of the Department of Life and Environmental Sciences University of Tsukuba (YCC), and also in CBS (Centraalbureau voor Schimmelcultures, Utrecht). Dried reference specimens have been deposited in the National Collection of Fungi, Pretoria (PREM), and the National Science Museum (TNS), Tokyo.

DNA sequence comparisons

Isolates were grown on 2% malt extract agar (MEA; 20 g malt extract, 15 g agar in 1 l deionised water; Biolab Diagnostics, Midrand, South Africa) for 14–21 d at room temperature before mycelium was collected and lyophilized. DNA extraction was achieved as previously described by Barnes *et al.* (2001). The ITS and the 5.8S regions of the ribosomal RNA operon were amplified from the DNA of 38 isolates (Table 1), using the primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′; White *et al.* 1990). DNA from a subset of isolates (26 and 22 isolates; Table 1) was used to amplify a portion of the β-tubulin gene and the MAT-2 HMG box, using the primers Bt1-a (5′ TTC CCC CGT CTC CAC TTC TTC ATG 3′)/Bt1-b (5′ GAC GAG ATC GTT CAT GTT GAA CTC 3′; Glass & Donaldson 1995); and COER2-1 (5′ GAC ACC AAG ACG TCA AAG CC 3′)/COER 2-2 (5′ GCT TTT CTT GTA AGT TTC AGC 3′; Witthuhn *et al.* 2000), respectively.

The 25 μl polymerase chain reaction (PCR) mixture included 0.2 mM of each dNTP; 0.4 μM of each primer; 1 × Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 1.25 U of Expand High Fidelity PCR system enzyme mix (Roche Molecular Biochemicals, Mannheim, Germany) and 5–10 ng of DNA template. The PCR amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 30 s at 94 °, 50 s at 58 ° and 2 min at 72 °. Final chain elongation was achieved at 72 ° for 5 min. PCR products were

Table 1. Isolates of *Ceratocystis* used in this study.

Isolates[a]	Species	Host (Plant/insect)	Origin/year of isolation	Collector(s)	Test/s[b]	Genbank accession nos[c]
CMW7152IF TA/18/5	*C. polonica*	*Picea abies*/*Ips typographus*	Austria, Tamsweg, 1998	*R. Grubelnik* *T. Kirisits*	I	AY233902
CMW7133IF KRB/5/6/1-SHT CBS 109251	*C. polonica*	*P. abies*/*I. typographus*	Austria, Kreisbach, 1998	*T. Kirisits*	I,M,G,MO	AY233903, AY233958
CMW7143IF IA/2/5-SHT CBS 109245	*C. polonica*	*P. abies*/*I. amitinus*	Austria, Radurschltal, 1997	*T. Kirisits*	I,G,MO	AY233904
CMW7146 IF PR/25/5-SHT CBS 109249	*C. polonica*	*P. abies*/*I. typographus*	Austria, Prinzersdorf, 1997	*R. Grubelnik* *T. Kirisits*	T,M	AY233938, AY233962
CMW7149 IF OS/30/5	*C. polonica*	*P. abies*/*I. typographus*	Austria, Ossiach, 1997	*R. Grubelnik* *T. Kirisits*	I,T	AY233905, AY233937
CMW5026 IF EW/1/2/3/8	*C. polonica*	*P. abies*/*I. typographus*	Austria, Ehrwald, 1997	*T. Kirisits*	I,T,M	AY233907, AY233932, AY233959
CMW7138 IF KRB/5/6/1-SHT/RE 2/12-5	*C. polonica*	*P. abies*/*I. typographus*	Austria, Kreisbach, 1998	*T. Kirisits*	I,T	AY233892, AY233931
CMW7754 IF N/43/H10 CBS 109256	*C. polonica*	*P. abies*/*I. typographus*	Austria, Naßwald, 1993	*T. Kirisits*	I,T	AY233900, AY233940
CMW7151 IF KB/19/3	*C. polonica*	*P. abies*/*I. typographus*	Austria, Karlsbach, 1997	*R. Grubelnik* *T. Kirisits*	I	AY233896
CMW8845 IF II/3/3/7 CBS 109266	*C. polonica*	*Pinus sylvestris*/ *Tomicus minor*	Austria, Horn, 1998	*T. Kirisits*	I,T,M	AY233890, AY233926, AY233956
CMW2272 JCM 9370 YCC-115	*C. polonica*	*Picea jezoensis*/ *Ips typographus japonicus*	Japan, Hokkaido, 1990	*Y. Yamaoka*	I,T,MO	AY233893, AY233934
CMW2284	*C. polonica*	*P. jezoensis*/ *I. typographus japonicus*	Japan, Hokkaido, 1989	*Y. Yamaoka*	I,T,M,G,MO	AY233894, AY233935, AY233964
CMW2285	*C. polonica*	*P. jezoensis*/ *I. typographus japonicus*	Japan, Hokkaido, 1990	*Y. Yamaoka*	G	
CMW2286	*C. polonica*	*P. jezoensis*/ *I. typographus japonicus*	Japan, Hokkaido, 1990	*Y. Yamaoka*	I,M	AY233895, AY233963
CMW2210	*C. polonica*	*P. jezoensis*/*I. typographus japonicus*	Japan, Hokkaido, 1990	*Y. Yamaoka*	I,T,M	AY233897, AY233936, AY233960
CMW10522 CBS133.38	*C. polonica*	*P. abies*/*I. typographus*	Poland, NA[d], 1938	*W. Siemaszko*	I,T	AY233898, AY233933
CMW7748 IF SUM/VI/3/1	*C. polonica*	*P. abies*/*I. typographus*	Czech Republic, Sumava, 2001	*T. Kirisits* *R. Jakus*	I,T	AY233899, AY233939
CMW8831 IF France/1	*C. polonica*	*P. abies*/*I. typographus*	France, Haguenau, NA	*F. Lieutier*	M	AY233953
CMW8830	*C. polonica*	*P. abies*/*I. typographus*	Norway, Ås, 1992	*T. Kirisits* *H. Solheim*	I,T,M,MO	AY233889, AY233929, AY233955
CMW8873 NISK90-120/181	*C. polonica*	*P. abies*/*I. typographus*	Norway, Nord-Trøndelag, 1990	*H. Solheim*	I,T,M	AY233888, AY233928, AY233954
CMW1164	*C. polonica*	*P. abies*/*I. typographus*	Norway, NA	*H. Solheim*	I,M	AY233901, AY233961
CMW1165	*C. polonica*	*P. abies*/*I. typographus*	Norway, NA	*H. Solheim*	I	AY233906
CMW8874 NISK93-208/115	*C. polonica*	*P. abies*/*I. typographus*	Norway, Ås, 1993	*H. Solheim*	I,T,M	AY233891, AY233927, AY233957
CMW8091 NISK80-53/7D	*C. polonica*	*P. abies*/*I. typographus*	Norway, Akershus, 1980	*H. Solheim*	G	

Table 1. (*Cont.*)

Isolates[a]	Species	Host (Plant/insect)	Origin/year of isolation	Collector(s)	Test/s[b]	Genbank accession nos[c]
CMW8092 NISK82-77/1	*C. polonica*	*P. abies*/*I. typographus*	Norway, Akershus, 1982	*H. Solheim*	G	
CMW4513	*C. laricicola*	*Larix decidua*/*Ips cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,M	AY233917, AY233968
CMW4522	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I	AY233908
CMW4556	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,T	AY233909, AY233944
CMW4562	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,G,MO	AY233914
CMW4540	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,T,M	AY233910, AY233947, AY233966
CMW4546	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,T,M	AY233911, AY233945, AY233970
CMW3214	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,G,MO	AY233913
CMW1016	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, NA	*D. B. Redfern*	I,T	AY233915, AY233946
CMW1017	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, NA	*D. B. Redfern*	I	AY233920
CMW3221	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	I,T,MO	AY233921, AY233943
CMW3220	*C. laricicola*	*L. decidua*/*I. cembrae*	Scotland, Atholl, 1997	*T. Kirisits* *M. J. Wingfield* *D. B. Redfern*	G	
CMW7760 IF SA/II/2/1/ 5-SHT CBS 109260	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Seetaler Alpen, 1998	*T. Kirisits*	I,T,M,MO	AY233918, AY233942, AY233969
CMW7759 IF SA/I/2/3/ 5-SHT CBS 109262	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Seetaler Alpen, 1998	*T. Kirisits*	I,T,M,MO	AY233919, AY233941, AY233965
CMW4356 IF GL/2/1/3	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Glein, 1997	*T. Kirisits*	I	AY233916
CMW5064 IF GL/3/2/1	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Glein, 1997	*T. Kirisits*	I	AY233912
CMW7756 IF 3/1/1/5-SHT CBS 109257	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Kindberg, 1995	*T. Kirisits*	G	
CMW7761 IF SA/II/3/2/5-SHT CBS 109261	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Seetaler Alpen, 1998	*T. Kirisits*	G	
CMW8832 IF 3/2/2/4	*C. laricicola*	*L. decidua*/*I. cembrae*	Austria, Kindberg, 1995	*T. Kirisits*	G	

CMW1965 PREM57515	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	I,T,M,G,MO	AY233922, AY233950, AY233972
CMW1969	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	I,T,M, MO	AY233923, AY233951, AY233967
CMW1952 YCC-286 PREM57516 TNS-F-11162	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	I,T,M,G,MO	AY233924, AY233949, AY233971
CMW1955 PREM57513 TNS-F-11161	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	T,M,G, MO	AY233948, AY233973
CMW1953	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	G	
CMW1954 YCC-285 JCM 9810 PREM57514	*C. fujiensis*	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	G,MO	
CMW1970	*C. fujiensis*.	*L. kaempferi/I. subelongatus*	Japan, Fujiyoshida, 1992	*M. J. Wingfield Y. Yamaoka*	G	
CMW3273	*C. pinicola*	*Picea abies*	Norway, NA, 1994	*H. Solheim*	I,T,M	AY233925, AY233952, AY233974

[a] All isolates are maintained in the collection of fungus cultures (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria. CBS, Centraalbureau voor Schimmelcultures, Utrecht IF, Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, BOKU – University of Natural Resources and Applied Life Sciences, Vienna JCM, Japan Collection of Microorganisms, Wako, Saitama; YCC, Laboratory of Plant Pathology and Mycology, Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572; PREM, National Collection of Fungi, Pretoria NISK, Norwegian Forest Research Institute, Ås; and TNS, National Science Museum (TNS), Tokyo.

[b] Tests conducted on various isolates: I, ITS sequence comparisons; T, β-tubulin sequence comparisons; M, Mat-2 HMG box sequence comparisons; G, growth comparisons in culture; and MO, morphological comparisons.

[c] GenBank accession nos are given in the same order as in the test column.

[d] NA, data not available.

visualized using UV-light after separation on a 1.5% agarose gel with ethidium bromide. The products were then purified using the High pure PCR product purification kit (Roche Molecular Biochemicals) for direct sequencing using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, CA). Sequencing reactions were analysed on an ABI Prism 377 DNA sequencer. Sequences were aligned using the program Sequence Navigator version 1.0.1 (Applied Biosystems). The alignment was analysed using PAUP software version 3.1.1. (Swofford 1998). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection was used. Gaps were treated as fifth character and confidence intervals using 1000 bootstrap replicates were calculated. *C. pinicola* (CMW 3273) was used as an outgroup in the analysis and was treated as monophyletic sister group. A partition-homogeneity test was used to evaluate the combinability of the sequence data for the ITS, β-tubulin gene and the MAT-2 HMG box. Finally, a multigene phylogenetic tree was generated following the PAUP options described above.

Morphology and cultural characteristics

Five isolates of *Ceratocystis polonica* and *C. laricicola*, as well as five strains isolated from ascospores obtained from perithecia occurring along the gallery walls of larch bark beetles infesting *Larix kaempferi* in Japan (Table 1) were chosen for detailed morphological comparisons. These isolates were grown on 2% MEA at 25 ° in the dark. Colour descriptions were made using the colour charts of Rayner (1970). Fungal structures were mounted in lactophenol and measurements of perithecia (base diameter, neck length and width, length of ostiolar and ornamental hyphae on the perithecial bases), ascospores and conidia (length and width) were made using a Zeiss Axioskop light microscope (Carl Zeiss, Heidenheim, Germany). 50 measurements per isolate were made for each character and means, standard deviations, as well as minimum and maximum measurements were computed.

Growth of six isolates of each species (Table 1) was determined at nine temperatures i.e. 10, 15, 20, 25, 30, 31, 32, 33 and 34 °. Mycelial plugs (4 mm diam) were removed from actively growing margins of colonies and placed at the centers of 9 cm diam plastic Petri dishes. Growth was determined after 8 d using four replicate plates at each temperature. Two diameter measurements at right angles to each other were made for each plate. Differences in growth between isolates of *C. polonica*, *C. laricicola* and the *Ceratocystis* species from *L. kaempferi* in Japan were analysed for each temperature stage between 10 and 30 ° by using one-way ANOVA and Tukey post-hoc pairwise comparisons (SYSTAT, SPSS, Chicago, ILL).

RESULTS

DNA sequence comparisons

The partition-homogeneity test indicated that the sequence data for three genes studied could be combined ($P = 1.0$). Alignment of the ITS1, 5.8S and ITS2, β-tubulin gene and the MAT-2 HMG box sequences, yielded a data set of 1280 characters. Parsimony analysis showed that 1177 characters were constant and there were 46 parsimony-informative characters. Three major clades emerged from the analysed combined data (Fig. 1). All of the *Ceratocystis polonica* isolates from Europe and Japan grouped together, and only slight differences (one or two bases) were found between them. The *C. laricicola* isolates grouped in a clade that differed by 30 changes from the *C. polonica* clade (Fig. 1). Furthermore, a clear phylogenetic separation was found between the European isolates of *C. laricicola* and those from larch in Japan. The Japanese isolates were grouped in a distinct clade separated by more than 20 changes compared to the isolates of *C. laricicola* from Europe. Separation of these three clades was supported by bootstrap values higher than 96%. This phylogenetic tree had a length of 117 steps and was defined by a CI of 0.92 and RI of 0.94 (Fig. 1). Phylograms produced using individual sequence data from each genome region had similar topologies to that obtained in the combined tree. In the three phylograms generated, bootstrap analysis supported the separation of all the isolates in the same three clades that were obtained in the combined analysis. Alignment of the ITS sequences from 38 isolates yielded a data set of 555 characters. Parsimony analysis showed that 16 characters were parsimony-informative and two most parsimonious trees (CI 0.93; RI 0.97; length 58 steps) were generated (Fig. 2). These trees had nine changes between isolates of *C. polonica* and *C. laricicola* from Europe, while ten changes were observed between the Japanese isolates obtained from larch and the European *C. laricicola* isolates.

Morphology and cultural characteristics

All the isolates examined (Table 2), had dark perithecia with round bases (125–)150–263(–302) μm diam and long necks (373–)508–1102(–1402) μm tall, characteristic of the genus *Ceratocystis* (Table 2). They had ascospores with sheaths wider at the sides than at the ends, in top view. The anamorph states had cylindrical conidia typical of *Thielaviopsis* spp. Lengths of hyphae on the bases of perithecia were characteristically longer in *C. polonica* with (306–)463–763(–967) μm in comparison to those of *C. laricicola*, which measured (116–)173–319 (–385) μm, and the Japanese isolates from larch, (244–)324–498(–667) μm (Table 2). However, as these values overlap, they were insufficient to enable a clear separation of the isolates. The same was true for lengths of ostiolar hyphae, which were shorter in *C. laricicola* from Europe (14.5–)18–28.5(–37) μm, than in the Japanese isolates (23.5–)29–52(–70.5) μm or in *C. polonica* (26–)32.5–59(–69) μm. Measurements for these structures also overlapped and were not useful for species delimitation. Between 10–30 °, isolates of *C. polonica*, as well as those of *C. laricicola* from Europe and Japan, showed similar growth-temperature profiles and had a growth optimum of 25 ° (Fig. 3). At all temperatures, *C. polonica* grew significantly slower than *C. laricicola* from Europe. The isolates from larch in Japan were intermediate between the two former groups of isolates, except at 30 °, where *C. polonica* grew faster. Major differences between the groups of isolates, especially between *C. polonica* and *C. laricicola* from Europe and Japan, were observed above 30 ° (Table 3). *C. polonica* isolates did not grow or grew only very slowly, at 31 ° and 32 °. This was in contrast to *C. laricicola* isolates from Europe and Japan that were able to grow at these temperatures. In the case of the latter fungi, 33–34 ° was the uppermost limit for growth (Table 3).

TAXONOMY

Despite their morphological similarity, phylogenetic comparisons between isolates of *Ceratocystis polonica* and *C. laricicola* show clearly that they can be distinguished from each other. This is consistent with their distinctive ecologies and there is little question that they represent discrete taxonomic entities. Likewise, Japanese and European isolates of *C. laricicola* were shown, in robust phylogenetic comparisons, to be distinct from each other. We, therefore, describe the Japanese fungus as new species.

Ceratocystis fujiensis M. J. Wingf., Yamaoka & Marin, **sp. nov.**

Etym.: Referring to the origin of the isolates from Mount Fuji, Japan.

Coloniae crescunt celeriter in agaro cum 2% extracto malti, incremento optime ad 25 °, in octo diebus diametrum medium 8.5 cm attingentes; supra 33 ° non crescunt. Mycelium aereum juventute fusce olivaceo-griseum, senectute laete olivaceo-griseum. Superficies inversa coloniae juventute fusce griseo-olivacea, sed in culturis veteris olivaceo-nigra. Perithecia superficialia, basibus fuscis globosis (132–)156–254(–294) μm diametro; hyphis basalibus fuscis (247–)307–502(–646) μm ornata. Colla (380–)493–1097(–1398) μm longa, recta vel subcurvata, basin versus latiora (31–)33–54(–64) μm apice (9–)13.5–21(–30) μm. Hyphae ostiolares divergentes, hyalinae, aseptatae, (24.5–)32.5–51(–70) μm longae, vaginis exclusis. Ascosporae unicellulares e hyalina, (3–)3.5–5.5(–6.5) μm × (1–)1.5–2(–3) μm. Vaginae ascosporarum translucentes, (0.5–)1–1.5(–2) μm, e summo visae in lateribus latiores quam in extremis. Conidiophorae plerumque adsunt, lateraliter e hyphis vegetativis exorientes, mononemata, (110–)152–294(–403) μm, multicellulares, cum cellula terminali phialidica conidiogena (29–)40–75(–86) μm longa. Conidia cylindrica vel oblonga, hyalina, (8–)9.5–15(–20) × (3–)4–6(–8.5) μm.

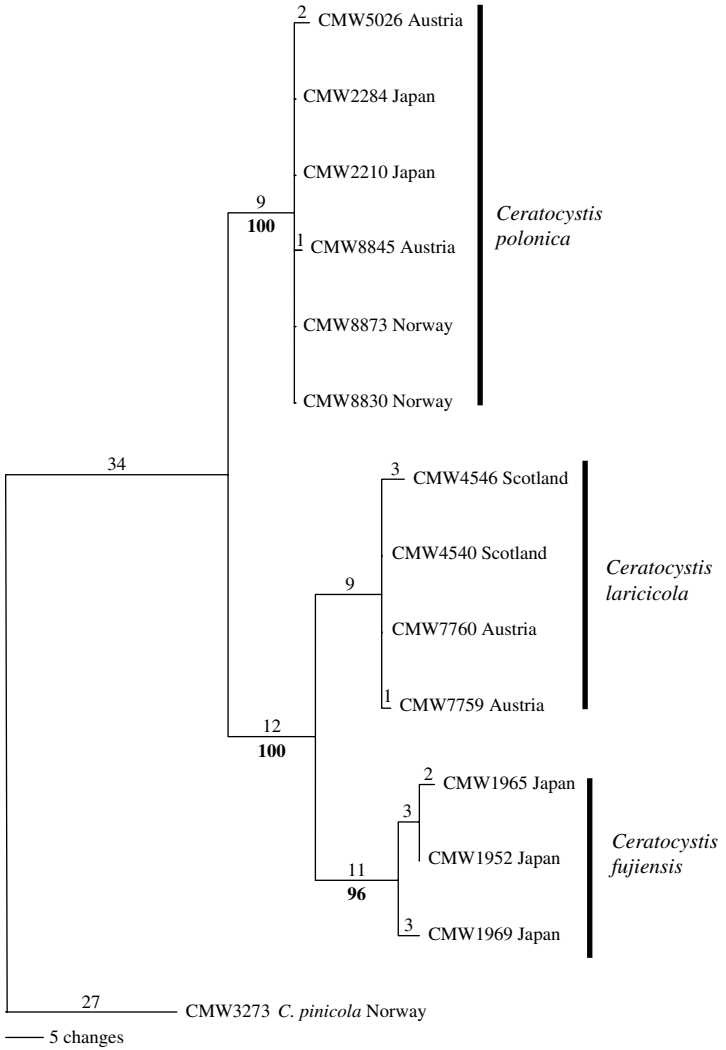

Fig. 1. One of the two most parsimonious trees generated for a combination of ITS, β-tubulin and MAT-2 HMG box sequence data. Branch lengths are shown above and bootstrap values below the branches. CI = 0.92, RI = 0.94, Tree length = 117, *P* value = 1.0. *Ceratocytsis pinicola* was used as outgroup to root the tree.

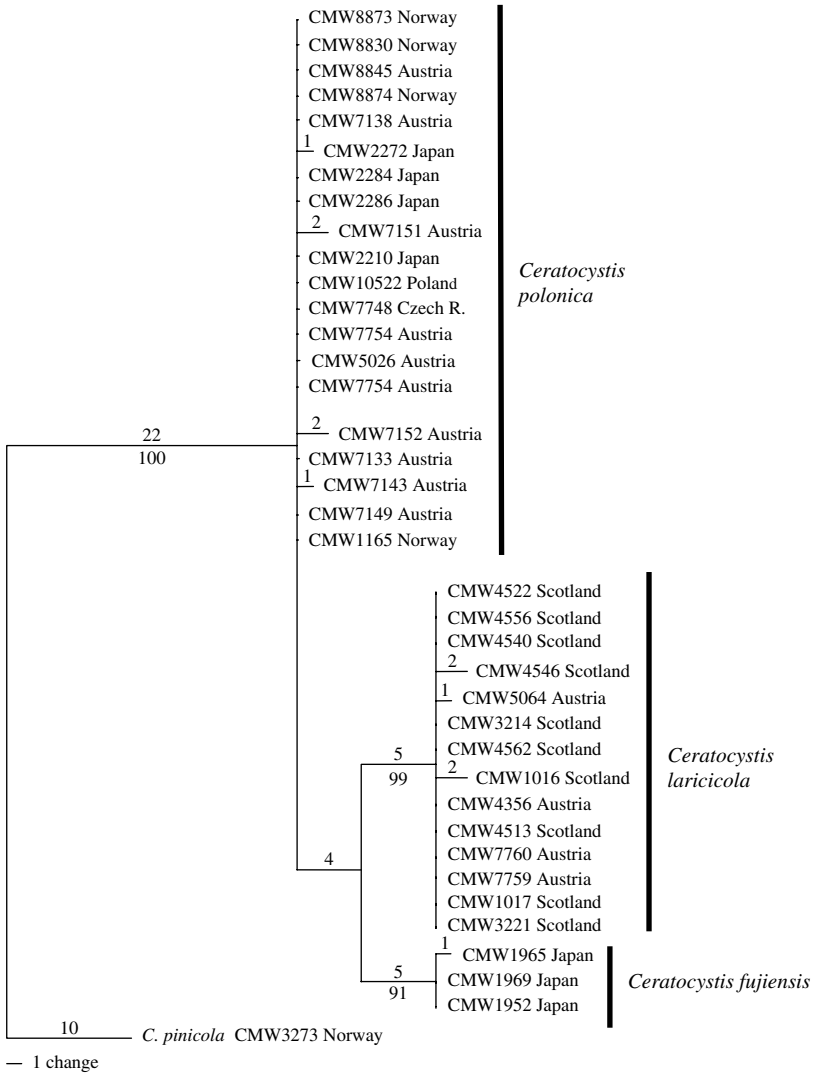

Fig. 2. One of two most parsimonious trees produced from DNA sequence data of the ITS region from isolates of *Ceratocystis polonica s. lat.* from Eurasia. Branch lengths are shown above and bootstrap values below the branches. CI = 0.93, RI = 0.97, Length = 58. *C. pinicola* was used as outgroup to root the tree.

Table 2. Comparison of morphological characteristics of *Ceratocystis laricicola*, *C. fujiensis*, and *C. polonica* from Eurasia[a].

Character	*C. laricicola*	*C. fujiensis*	*C. polonica*
Perithecial base width	(125–)151–242(–302)	(129–)172–263(–298)	(128–)150–211(–261)
Neck length	(449–)564–853(–1048)	(373–)508–1102(–1402)	(420–)525–742(–814)
Neck width at base	(25–)31–45(–54.5)	(30–)36.5–52(–61)	(21–)30–41(–45)
Neck width at tip	(10.5–)13–18.5(–21)	(9–)11.5–23(–32)	(10–)13.5–22(–27)
Ostiolar hyphal length	(14.5–)18–28.5(–37)	(23.5–)29–52(–70.5)	(26–)32.5–59.5(–69)
Basal hyphal length	(116–)173–319(–385)	(244–)324–498(–667)	(306–)463–763(–967)
Ascospore breadth	(1–)1.5–2(–2.5)	(1–)1.5–2(–3)	(1.5–)1.5–2.5(–5)
Ascospore length	(3–)4–5(–5)	(3–)4–6(–6.5)	(3–)3–5.5(–6)
Ascospore sheath	(0.5–)0.5–1.1(–1.5)	(0.5–)1–1.5(–2)	(0.5–)1–1.5(–2)
Conidia length	(6–)8–14.5(–21)	(7.5–)10–17(–23)	(7–)9–14.5(–18)
Conidia breadth	(2–)3.5–5(–6.5)	(3–)4–6.5(–8.5)	(2–)3–4.5(–6)

[a] Measurements are in μm and presented as (minimum value −) average minus standard deviation – standard deviation plus average (–maximum value), of 50 measurements for each character in each of five isolates per species. Isolates used to generate these measurements were: *C. laricicola* (CMW3214, CMW3221, CMW4562, CMW7760, CMW7759), *C. fujiensis* (CMW1952, CMW1954, CMW1955, CMW1965, CMW1969), and *C. polonica* (CMW2272, CMW2284, CMW7133, CMW7143, CMW8830).

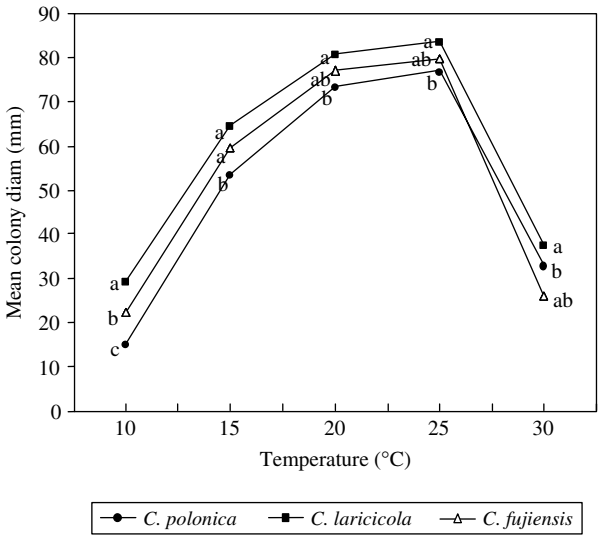

Fig. 3. Mean growth of *C. polonica*, *C. laricicola* and *C. fujiensis* on 2% malt extract agar after 8 d at 10, 15, 20, 25 and 30 °C. For each species, values represent the average colony diam of six isolates (Table 1), with four replicates per isolate. At each temperature of incubation, mean values followed by the same letter do not differ significantly from each other ($P < 0.05$).

Table 3. Growth of *Ceratocystis polonica*, *C. laricicola* and *C. fujiensis* on 2% malt extract agar after eight days of incubation between 31 and 34 °C.

	Colony diam after 8 d (mm)[a]			
Species and isolates	31 °C	32 °C	33 °C	34 °C
C. laricicola	**2.9**	**1.8**	**0.2**	**0.1**
CMW3214	2.1	1.1	0.5	0
CMW3220	1.3	1.1	0.5	0
CMW4562	2.5	2	0.5	0.1
CMW7756	3.2	1.6	0	0
CMW7761	3.9	2.9	0	0
CMW8832	4.3	2.2	0	0
C. fujiensis	**1.2**	**0.7**	**0.4**	**0.1**
CMW1955	1.8	1.1	0.4	0
CMW1952	0.7	0.5	0	0
CMW1965	1.6	0.9	0.6	0
CMW1953	1	0.5	0	0
CMW1954	1	0.8	0.6	0
CMW1970	1	0.4	0.5	0.5
C. polonica	**0.8**	**0.1**	**0**	**0**
CMW2285	1.7	0.3	0	0
CMW2284	1.1	0.2	0	0
CMW8092	0	0	0	0
CMW8091	0	0	0	0
CMW7143	1.7	0	0	0
CMW7133	0.4	0	0	0

[a] Measurements represent average colony diam for six isolates of each species, with four replicate plates and readings per isolate. Values in **bold** represent overall averages.

Typus: **Japan**: *Yamanashi*: Fujiyoshida, foot of Mount Fuji, isolated from ascospores obtained from a perithecium occurring in a gallery of *Ips subelongatus* on *Larix kaempferi*, July 1992, *M. J. Wingfield & Y. Yamaoka* (TNS-F-11161 – holotypus; PREM 57513 – isotypus). [Dried specimens of culture JCM 9810, also known as CMW 1955 and YCC 285.]

Colonies fast growing on 2% malt extract agar, with optimum growth at 25 ° and maximum at 33 °, reaching after eight days an average of 2.9 cm diam at 10 °, 5.9 cm diameter at 15 °, 6.7 cm diam at 20 °, 8.5 cm diam at 25 °, 2.2 cm diam at 30 °, 1.8 cm diam at 31 °, 1.1 cm diam at 32 ° and no growth at 33 ° and above. Aerial mycelium olivaceous grey (23′′′′b), turning to pale olivaceous grey (23′′′′f) when old. Reverse side of colony dark grayish olive (21′′′k) when young but olivaceous black (23′′′′m) in old cultures. *Perithecia* produced superficially, especially at the edges of the colony after two weeks, with bases dark and globose, (132–)156–254(–294) μm diam; ornamented by dark basal hyphae, (247–)307–502(–646) μm (Fig. 4A, C). Necks long (380–)493–1097(–1398) μm, straight to slightly curved, wider at the base (31–)33–54(–64) μm, than at the apex (9–)13.5–21(–30) μm (Fig. 4A). Ostiolar hyphae divergent, hyaline, aseptate, (24.5–)32.5–51(–70) μm long (Fig. 4B). *Ascospores* unicellular, hyaline, oblong, (3–)3.5–5.5(–6.5) μm long and (1–)1.5–2(–3) μm wide, excluding sheaths (Fig. 4D).

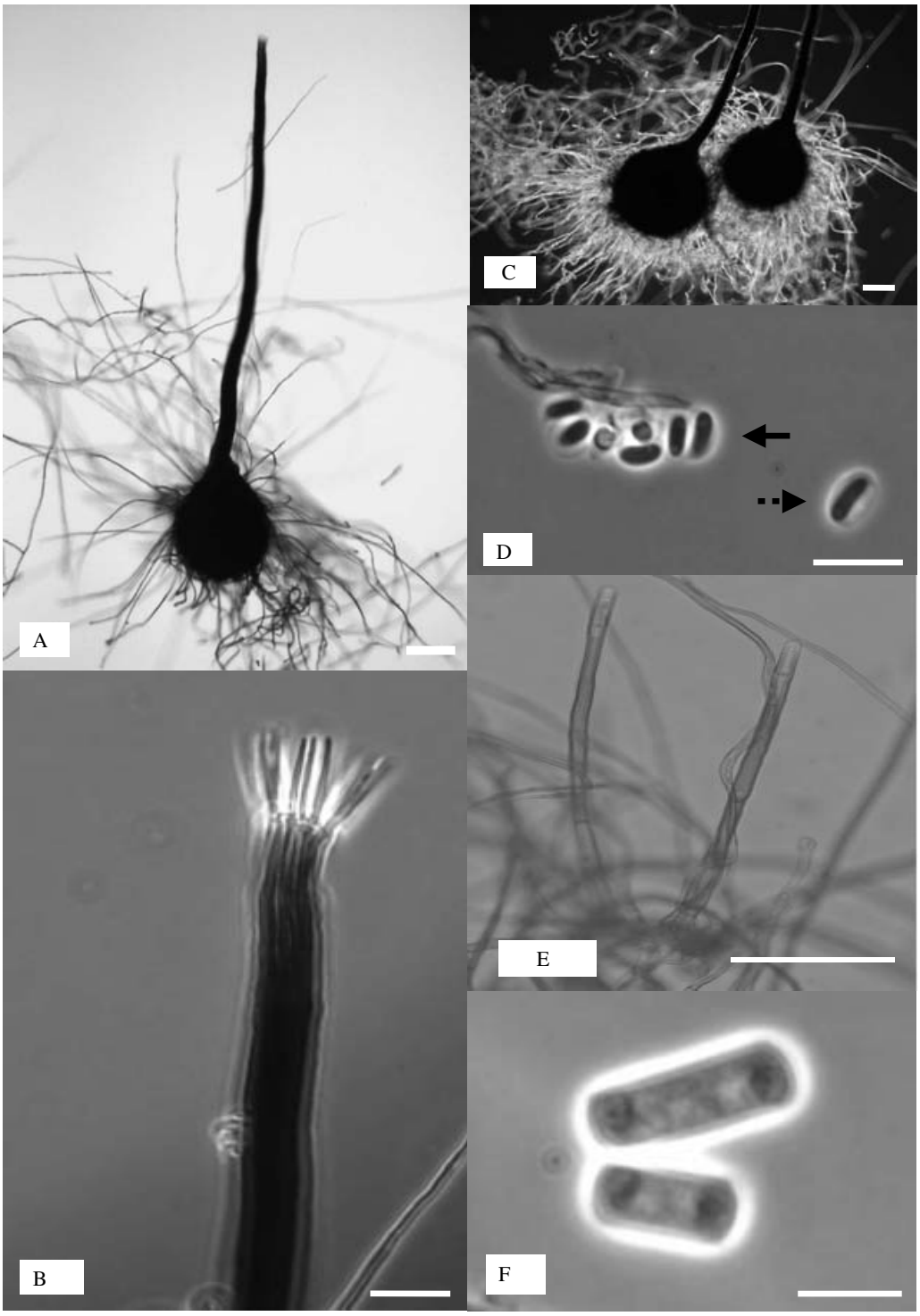

Fig. 4. Morphological characteristics of *Ceratocystis fujiensis* (PREM57516, CMW1965). A. Perithecium. Bar: 100 μm. B. Ostiolar hyphae at apex of perithecial neck. Bar: 20 μm. C. Perithecial bases showing hyphal ornamentation. Bar: 100 μm. D. Ascospores in side view (solid arrow) and top view (arrow), showing translucent sheaths wider at the sides than at the ends. Bar: 10 μm. E. *Thielaviopsis* conidiophores with phialidic conidiogenous cells. Bar: 50 μm. F. Cylindrical conidia. Bars: A, C = 100 μm; E = 50 μm; B = 20 μm; and D, F = 10 μm.

Ascospore sheaths translucent, (0.5–)1–1.5(–2) μm, wider at the sides than at the ends in top view (Fig. 4D). *Conidiophores* usually present, rising laterally from vegetative hyphae, mononematous, (110–)152–294(–403) μm, multicellular, with a terminal phialidic conidiogenous cell, (29–)40–75(–86) μm long (Fig. 4E). *Conidia* cylindrical or oblong, hyaline, (8–)9.5–15(–20) × (3–)4–6(–8.5) μm (Fig. 4F).

Additional specimens examined: **Japan**: *Yamanashi*: Fujiyoshida, foot of Mount Fuji, isolated from ascospores obtained from perithecia occurring along gallery walls of *Ips subelongatus* on *Larix kaempferi*, *July 1992*, M. J. Wingfield and Y. Yamaoka PREM 57515

(CMW1965), PREM 57514 (CMW1954), PREM 57516, TNS-F-11162 (CMW1952; YCC 286).

DISCUSSION

In this study we have shown, based on comparisons of DNA sequences of three genome regions, that *Ceratocystis polonica* and *C. laricicola* represent two distinct species in the *C. coerulescens* species complex. This is despite the fact that they appear to be morphologically identical (Harrington & Wingfield 1998), but is consistent with their discrete ecologies. Identical ITS sequences (Witthuhn *et al.* 1998) and similar isozyme profiles (Harrington *et al.* 1996), have in the past led to questions relating to the similarities or differences between *C. polonica* and *C. laricicola*. Results of this study, and those of Harrington *et al.* (2002), provide robust justification for treating *C. polonica* and *C. laricicola* as distinct. They are, however, closely related sibling species that have apparently undergone speciation relatively recently. ITS sequence data produced in this study revealed at least nine changes in the nucleotide sequence between *C. polonica* and *C. laricicola*. However, this is in contrast to the results of Witthuhn *et al.* (1998), who showed identical ITS sequences for these species. Their study included only one isolate of each species, and their sequences were generated using the more laborious manual sequencing method, which is prone to band compression particularly in regions with a high G+C content. We were fortunate to be able to obtain a large number of isolates, enabling us to produce a robust comparison supported by high bootstrap values.

C. polonica and *C. laricicola* could be distinguished from each by differences in the partial sequence of two protein coding genes, β-tubulin and the MAT-2 HMG box. Phylogenetic analysis of both regions separated isolates belonging to each species in two well-supported clades and combination of the three DNA regions considered, allowed the unequivocal distinction of both species. This is consistent with Harrington *et al.* (2002), who used DNA sequences of the MAT-2 idiomorph and sexual interfertility to compare isolates of the two species. In addition, Kirisits (2001) and Harrington *et al.* (2002) used reciprocal inoculation experiments to compare these fungi. These studies have all shown that *C. polonica* is preferentially pathogenic on spruce, and that *C. laricicola* is specialized to infect larch. Growth studies in culture allowed the differentiation of isolates of *C. polonica* and *C. laricicola*. *C. polonica* grew very slowly or not at all at 32 ° and above. In contrast, *C. laricicola* isolates grew relatively moderately at this temperature. This simple phenotypic characteristic can now be used for rapid diagnostic purposes to discriminate between the two species. A similar growth test at 32 ° has been recommended by Brasier & Stephens (1993) to discriminate between isolates of *Ophiostoma piceae* and *O. quercus*. This test is currently used (Harrington *et al.* 2001) as an effective tool to identify isolates of the latter two sibling species and of other species in the *O. piceae* species complex.

C. polonica isolates from Europe and Japan shared the same sequences in the three DNA regions studied. This was irrespective of their different hosts and vectors. The name *C. polonica* is clearly appropriate for both Japaneses and European isolates, and the name *C. jezoensis* provided by Aoshima (1965; cited by Yamaoka *et al.* 1997) for the Japanese isolates does not have phylogenetic support.

The conspecificity of European and Japanese isolates of *C. polonica* is in agreement with the genetic similarity found for the insect vectors of this fungus in Europe and Asia. Stauffer & Lakatos (2000) studied the phylogenetic relationships between the European (*IPS typographus*) and Japanese (*I. typographus japonicus*) eight-spined spruce bark beetles, using the mitochondrial cytochrome oxidase I (COI) gene and determined that the insects in Europe and Asia differed by only 1.71 % in the sequences of this gene. They concluded that these beetle species should be treated as conspecific, which is also consistent with our findings for the primary fungal associate of *I. typographus*.

Isolates of *C. laricicola* from Europe and Japan resided in two discrete phylogenetic lineages, based on sequence comparisons for three genes. The results show that the fungus from Japan previously known as *C. laricicola* represents a distinct species, and this is also supported by genetic differences between the insect vectors of these two fungi. Stauffer *et al.* (2001) studied the phylogenetic relationships between the European and Asian populations of *I. cembrae*, based on DNA sequences of the cytochrome I gene (COI) and a non-coding region between COI and $tRNA_{LEU}$ of the mitochondrial genome. These authors found that the European populations differed by 4.3 % from the Asian populations. They thus concluded that *I. cembrae* represents two taxa, and suggested that the former name *I. subelongatus* (Motschulsky 1860) should be used for the beetle infesting larch in Japan. Thus, *C. fujiensis* described below should be recognized as the primary fungal associate of *I. subelongatus*, and not of *I. cembrae*.

Recognition of *C. fujiensis* as a discrete taxon is also supported by previous studies. Harrington *et al.* (2002) showed, using MAT-2 idiomorph sequences, that the Japanese isolates from larch had slightly different sequences than the European isolates of *C. laricicola*. In addition, these authors were not able to produce perithecia in sexual crosses between European or Russian isolates of *C. laricicola* with isolates obtained from larch trees in Japan. Distinction of the two species is also supported by the other fungi associated with *I. subelongatus* in Japan (van der Westhuizen *et al.* 1995, Yamaoka *et al.* 1998, Jacobs & Wingfield 2001) which are different to those belonging to the guild of fungi associated with *I. cembrae* in Europe (Stauffer *et al.* 2001, Jacobs & Wingfield

2001, Kirisits 2001, 2004, Jacobs, Kirisits & Wingfield 2003).

C. laricicola and *C. fujiensis* therefore represent phylogenetically and ecologically discrete species. However, they are closely related and virtually impossible to distinguish morphologically. This is perhaps not surprising as the more distantly related *C. polonica* and *C. laricicola* have also been considered to be morphologically identical (Harrington & Wingfield 1998). In terms of non-DNA based differences, we consider the hosts, vectors and geographical occurrence of the fungi as sufficient to distinguish them. The smaller lengths of hyphae on the bases and ostioles of perithecia of *C. laricicola* provided some distinction between these two species, but a large number of measurements are required to avoid the overlapping values found for these characters.

Based on the evidence of host specialization of *C. polonica* and *C. laricicola* (Kirisits 2001, Harrington *et al.* 2002) it is possible that *C. laricicola* and *C. fujiensis* have become specialized on the respective larch species in Europe and Asia, with which they are associated. This intriguing hypothesis deserves further study.

C. polonica, *C. laricicola* and *C. fujiensis*, together with their bark beetle vectors, represent a substantial quarantine threat to forestry in the Northern Hemisphere. This is especially true for North America, where neither of these insects or their fungal associates are established. Stauffer *et al.* (2001) suggest that great care should be taken to avoid the introduction of these organisms into areas where they are not already present. This is especially because little is known of their ecological behaviour outside their natural range, and because likely hosts are present in other regions. For example, *C. polonica* has been shown to be pathogenic to three North American spruce species (*Picea sitchensis*, *P. glauca* and *P. mariana*), as well as to Douglas-fir (*Pseudotsuga menziesii*); (Christiansen & Solheim 1990, 1994). Our study provides cultural and molecular tools that can be used to identify these fungal species. These should aid in the avoidence of the movement of these pathogens and their vectors to regions where they are not already present. They might also be used to unambiguously detect the fungi in the case of accidental introductions into exotic environments.

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REFERENCES

Aoshima, K. (1965) *Studies on wood-staining fungi of Japan*. PhD thesis, University of Tokyo.

Barnes, I., Roux, J., Wingfield, M. J., Coetzee, M. P. A. & Wingfield, B. D. (2001) Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and Histone sequences. *Plant Disease* **85**: 317–321.

Brasier, C. M. & Stephens, T. M. (1993) Temperature-growth responses distinguish the OPC and OPH sibling species within *Ophiostoma piceae*. *Mycological Research* **97**: 1416–1418.

Christiansen, E. (1985) *Ceratocystis polonica* inoculated in Norway spruce: blue-staining in relation to inoculum density, resinosis and tree growth. *European Journal of Forest Pathology* **15**: 160–167.

Christiansen, E. & Solheim, H. (1990) The bark beetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas-fir. *European Journal of Forest Pathology* **20**: 436–446.

Christiansen, E. & Solheim, H. (1994) Pathogenicity of five species of *Ophiostoma* fungi to Douglas-fir. *Meddelelser fra Skogforsk* **47**: 1–12.

Glass, N. L. & Donaldson, G. C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Harrington, T. C. & McNew, D. L. (1998) Partial interfertility among the *Ceratocystis* species on conifers. *Fungal Genetics and Biology* **25**: 44–53.

Harrington, T. C., McNew, D. L., Steimel, J., Hofstra, D. & Farrell, R. (2001) Phylogeny and taxonomy of the *Ophiostoma piceae* complex and the Dutch elm disease fungi. *Mycologia* **93**: 111–136.

Harrington, T. C., Pashenova, N. V., McNew, D. L., Steimel, J. & Konstantinov, M. Y. (2002) Species delimitation and host specialization of *Ceratocystis laricicola* and *C. polonica* to larch and spruce. *Plant Disease* **86**: 418–422.

Harrington, T. C., Steimel, J., Wingfield, M. J. & Kile, G. A. (1996) Isozyme variation and species delimitation in the *Ceratocystis coerulescens* complex. *Mycologia* **88**: 104–113.

Harrington, T. C. & Wingfield, M. J. (1998) The *Ceratocystis* species on conifers. *Canadian Journal of Botany* **76**: 1446–1457.

Jacobs, K., Kirisits, T. & Wingfield, M. J. (2003) Taxonomic re-evaluation of three related species of *Graphium*, based on morphology, ecology and phylogeny. *Mycologia* **95**: 714–727.

Jacobs, K. & Wingfield, M. J. (2001) *Leptographium Species: tree pathogens, insect associates, and agents of blue-stain*. American Phytopathological Society Press, St. Paul, MN.

Kirisits, T. (2001) Studies on the association of ophiostomatoid fungi with bark beetles in Austria with special emphasis on *Ips typographus* and *Ips cembrae* and their associated fungi *Ceratocystis polonica* and *Ceratocystis laricicola*. PhD thesis, BOKU, University of Natural Resources and Applied Life Sciences, Vienna.

Kirisits, T. (2004) Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In *Bark and Wood Boring Insects in Living Trees in Europe: a synthesis* (F. Lieutier, K. R. Day, J. C. Grégoire & H. Evans, eds): 181–235. Kluwer Academic Publishers, Dordrecht.

Kirisits, T. & Offenthaler, I. (2002) Xylem sap flow of Norway spruce after inoculation with the blue-stain fungus *Ceratocystis polonica*. *Plant Pathology* **51**: 359–364.

Krokene, P. & Solheim, H. (1996) Fungal associates of five bark beetle species colonizing Norway spruce. *Canadian Journal of Forest Research* **26**: 2115–2122.

Krokene, P. & Solheim, H. (1998) Phytopathogenicity of four blue-stain fungi associated with aggressive and nonaggressive bark beetles. *Phytopathology* **88**: 39–44.

Motschulsky, J. S. (1860) *Insectes du Japon. Etudes Entomologiques* **9**: 19–20.

Paulin-Mahady, A. E., Harrington, T. C. & McNew, D. L. (2002) Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocystis*. *Mycologia* **94**: 62–72.

Rayner, R. W. (1970) *A Mycological Colour Chart*. Commonwealth Mycological Institute, Kew.

Redfern, D. B., Stoakley, J. T., Steele, H. & Minter, D. W. (1987) Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**: 467–480.

Solheim, H. (1986) Species of Ophiostomataceae isolated from *Picea abies* infested by the bark beetle *Ips typographus*. *Nordic Journal of Botany* **6**: 199–207.

Stauffer, C., Kirisits, T., Nussbaumer, C., Pavlin, R. & Wingfield, M. J. (2001) Phylogenetic relationships between the European and Asian eight spined larch bark beetle populations (*Coleoptera*, *Scolytidae*) inferred from DNA sequences and fungal associates. *European Journal of Entomology* **98**: 99–105.

Stauffer, C. & Lakatos, F. (2000) *Ips typographus* f. *japonicus* Niijima (*Col.*, *Scolytidae*): a genetical analysis by allozymes and mitochondrial sequence data. In *Forests and Society: the role of research, XXI IUFRO World Congress 7–12 August 2000, Kuala Lumpur, Malaysia* (R. Jandl, M. Devall, M. Khorchidi, E. Schimpf, G. Wolfrum & B. Krishnapillay, eds): 397. Pramaju Sdn. Bhd, Kuala Lumpur.

Swofford, D. L. (1998) *PAUP*: phylogenetic analysis using parsimony* (**and other methods*). Version 4. Sinauer Associates, Sunderland, MA.

Uzunovic, A., Yang, D. Q., Gagné, P., Breuil, C., Bernier, L., Byrne, A., Gignac, M. & Kim, S. H. (1999) Fungi that cause sap stain in Canadian softwoods. *Canadian Journal of Microbiology* **45**: 914–922.

van der Westhuizen, K., Wingfield, M. J., Yamaoka, Y., Kemp, G. H. J. & Crous, P. W. (1995) A new species of *Ophiostoma* with a *Leptographium* anamorph from larch in Japan. *Mycological Research* **99**: 1334–1338.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal rRNA genes for phylogenetics. In *PCR Protocols: a guide to methods and applications* (A. M. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. Academic Press, San Diego.

Witthuhn, R. C., Harrington, T. C., Steimel, J. P., Wingfield, B. D. & Wingfield, M. J. (2000) Comparison of isozymes, rRNA spacer regions and MAT-2 DNA sequences as phylogenetic characters in the analysis of the *Ceratocystis coerulescens* complex. *Mycologia* **92**: 447–452.

Witthuhn, R. C., Wingfield, B. D., Wingfield, M. J. & Harrington, T. C. (1999) PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **103**: 743–749.

Witthuhn, R. C., Wingfield, B. D., Wingfield, M. J., Wolfaardt, M. & Harrington, T. C. (1998) Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96–101.

Yamaoka, Y., Wingfield, M. J., Takahashi, I. & Solheim, H. (1997) Ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Japan. *Mycological Research* **101**: 1215–1227.

Yamaoka, Y., Wingfield, M. J., Ohsawa, M. & Kuroda, Y. (1998) Ophiostomatoid fungi associated with *Ips cembrae* in Japan and their pathogenicity to Japanese larch. *Mycoscience* **39**: 367–378.

Yamaoka, Y., Takahashi, I. & Iguchi, K. (2000) Virulence of ophiostomatoid fungi associated with the spruce bark beetle *Ips typographus* f. *japonicus* in Yezo spruce. *Journal of Forest Research* **5**: 87–94.

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