Ceratocystis bhutanensis sp. nov., associated with the bark beetle *Ips schmutzenhoferi* on *Picea spinulosa* in Bhutan

Marelize van Wyk[1*], Jolanda Roux[1], Irene Barnes[2], Brenda D. Wingfield[2], Dal Bahadur Chhetri[3], Thomas Kirisits[4] and Michael J. Wingfield[1]

[1]*Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002;* [2]*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, 0002;* [3]*Renewable Natural Resources Research Centre (RNR-RC), Yusipang, Council of Research & Extension, Ministry of Agriculture, P.O. Box No. 212, Thimphu, Bhutan;* [4]*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, Hasenauerstrasse 38, A-1190, Vienna Austria*

[*]*Correspondence*: Marelize van Wyk, marelize.vanwyk@fabi.up.ac.za

Abstract: The Eastern Himalayan spruce bark beetle, *Ips schmutzenhoferi*, is a serious pest of *Picea spinulosa* and *Pinus wallichiana* in Bhutan. A study to identify the ophiostomatoid fungi associated with this bark beetle resulted in the isolation of a *Ceratocystis* sp. from *I. schmutzenhoferi*, collected from galleries on *P. spinulosa.* Morphological characteristics and comparisons of DNA sequence data were used to identify this fungus. Based on morphology, the *Ceratocystis* sp. from Bhutan resembled *C. moniliformis* and *C. moniliformopsis*, but was distinct from these species, both in micro-morphological characteristics, growth at different temperatures, as well as in the odour that it produces in culture. Comparisons of DNA sequences for the ITS regions of the rDNA operon, β-tubulin and elongation factor 1-α genes, confirmed that this fungus represents a taxon distinct from all other species of *Ceratocystis*. Based on morphological characteristics, comparisons of DNA sequence data and its unique ecology, we provide the name *C. bhutanensis* sp. nov. for it.

Taxonomic novelty: *Ceratocystis bhutanensis* M. van Wyk, M.J. Wingf. & T. Kirisits sp. nov.
Key words: Bark beetles, blue-stain, *Ceratocystis*, *Ips schmutzenhoferi*, ophiostomatoid fungi, *Picea spinulosa*, Scolytidae.

INTRODUCTION

The Kingdom of Bhutan is renowned for its intact forest resources, which are of immense socio-economic and ecological importance for this Himalayan country. Sixty-four percent of Bhutan is covered by forests (FAO 1999, 2001). Conifer forests form the natural vegetation in most parts of the mountainous areas at elevations above 1800 m a.s.l. (FAO 1999). Eastern Himalayan spruce (*Picea spinulosa* (Griffith) A. Henry) and Himalayan blue pine (*Pinus wallichiana* Jackson) are important tree species in these forests, forming either pure stands or stands of mixed species, together with other conifers and occasionally also hardwoods.

Bark beetles (Coleoptera: Scolytidae) are amongst the most damaging agents affecting conifer forests, worldwide. Some of the most aggressive of these insects are species of *Ips* de Geer (Postner 1974, Wood & Bright 1992). The best known of these is the eight-spined European spruce bark beetle, *I. typographus* L., which can cause extensive mortality of Norway spruce (*Picea abies* (L.) Karst.) in Europe (Postner 1974, Christiansen & Bakke 1988). In Bhutan, the Eastern Himalayan spruce bark beetle, *I. schmutzenhoferi* Holzschuh is a serious pest in conifer forests at elevations between 2500 and 3800 m a.s.l. (Holzschuh 1988, Schmutzenhofer 1988). This scolytid attacks mainly living trees or infests freshly felled logs of Eastern Himalayan spruce, Himalayan blue pine and occasionally Himalayan larch (*Larix griffithiana* (Lindl. & Gord.) Carrière) (Schmutzenhofer 1988, Tshering & Chhetri 2000). During the 1980s, *I. schmutzenhoferi* caused a destructive outbreak in Western and Central Bhutan, during which more than 2060 ha of forest were affected (Chhetri 1991) and losses of approximately 2 million m^3 of timber occurred (Schmutzenhofer 1988).

Conifer-infesting bark beetles are well known vectors of blue-stain fungi belonging to the ascomycete genera *Ceratocystis* Ellis & Halst. and *Ophiostoma* H. Syd. & P. Syd. and related anamorph genera (Francke-Grosmann 1967, Upadhyay 1981, Whitney 1982, Wingfield *et al.* 1993, Jacobs & Wingfield 2001, Kirisits 2004). These fungi cause blue, grey or black discoloration in the sapwood of living trees, logs and lumber, mostly on conifers. This damage results from the presence of pigmented fungal hyphae in the ray parenchyma cells and tracheids of the

sapwood (Münch 1907, Liese & Schmid 1961, Seifert 1993).

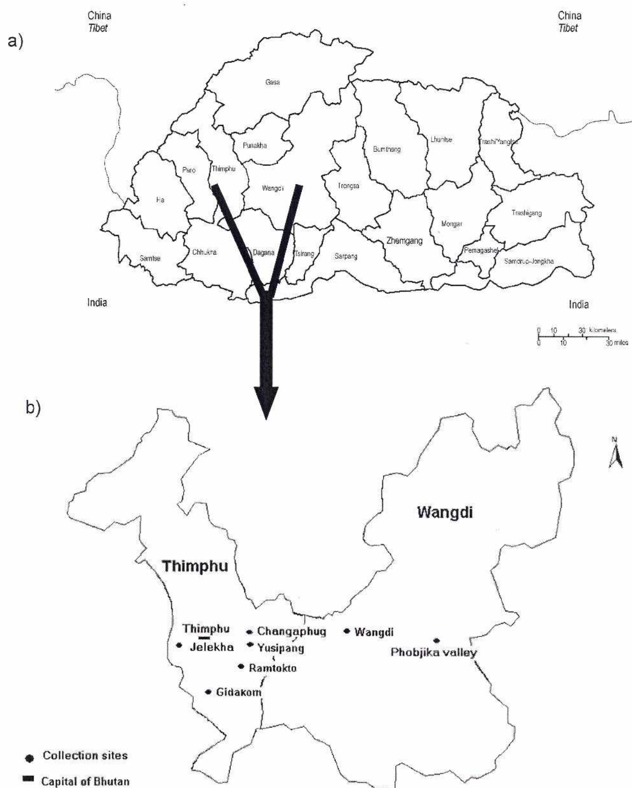

Fig. 1. A. A map of Bhutan showing the administrative districts (dzongkhags) of the country and Bhutan's capital Thimphu. B. A map of the dzongkhags Thimphu and Wangdi (Wangdue Phodrang) showing the localities where samples for fungal isolation were collected from *Picea spinulosa* and *Pinus wallichiana*. *Ceratocystis bhutanensis* was isolated only from individuals of *Ips schmutzenhoferi* obtained from Jelekha.

Damage due to sapstain is cosmetic rather than structural, and results in substantial financial loss, because markets prefer non-stained wood (Münch 1907, Seifert 1993, Uzunovic *et al.* 1999). Some bark beetle-associated blue-stain fungi also cause vascular stain diseases on living conifer trees and are thought to aid their insect vectors in exhausting the defence mechanisms of their host trees (Whitney 1982, Paine *et al*. 1997, Kirisits 2004).

Most fungal associates of bark beetles belong to the genus *Ophiostoma*. In contrast, *Ceratocystis* spp. usually have loose relationships with insects (Kile 1993). However, there are some *Ceratocystis* spp. that are consistently associated with conifer bark beetles (Harrington & Wingfield 1998). *Ceratocystis polonica* (Siemaszko) C. Moreau is associated with *I. typographus* and other species of *Ips* on *Picea abies* in Europe (Solheim 1986, 1992, Krokene & Solheim 1996, Kirisits 2004), and with *I. typographus japonicus* Niijima on *Picea jezoensis* (Sieb. & Zucc.) Carr. in Japan (Yamaoka *et al.* 1997). *Ceratocystis laricicola* Redfern & Minter, is associated with the larch bark beetle *I. cembrae* Heer on *Larix* Miller spp. in Europe and Japan (Redfern *et al*. 1987, Yamaoka *et al.* 1998, Stauffer *et al.* 2001, Kirisits 2004). *Ceratocystis rufipennis* M.J. Wingf., T.C. Harr. & H. Solheim, is associated with the spruce bark beetle *Dendroctonus rufipenniss* Kirby on *Picea engelmannii* Parry and *Picea glauca* (Moench) Voss in Western North America (Solheim 1995, Wingfield *et al.* 1997).

The small number of *Ceratocystis* spp. that are associated with bark beetles, display high levels of pathogenicity, when compared to *Ophiostoma* spp. from the same niches (Solheim 1988, Solheim & Safranyik 1997, Kirisits 1998, Krokene & Solheim 1998). *Ceratocystis polonica* is highly pathogenic to Norway spruce and contributes to tree death following attack by *I. typographus* (Christiansen 1985, Solheim 1988, 1992, Kirisits & Offenthaler 2002). Likewise, *C. laricicola* is considered to play an important role in the death of *Larix* spp. infested by *I. cembrae* (Redfern *et al.* 1987, Yamaoka *et al.* 1998, Kirisits 2001, Harrington *et al*. 2002) and *C. rufipennis* can kill Sitka spruce (*Picea sitchensis* (Bongard) Carrière) in mass inoculation experiments (Solheim & Safranyik 1997).

During a recent survey of ophiostomatoid fungi associated with *I. schmutzenhoferi* in Bhutan, a *Ceratocystis* sp. resembling *C. moniliformis* Hedgcock and *C. moniliformopsis* Z.Q. Yuan & C. Mohammed was isolated. Despite its morphological similarity to these *Ceratocystis* spp., the association of the *Ceratocystis* sp. from Bhutan with a conifer bark beetle aroused suspicion that it might represent an undescribed taxon. This study compares the *Ceratocystis* sp. from Bhutan with *C. moniliformis* and *C. moniliformopsis* and assesses their phylogenetic relationships based on gene sequences of the ITS region of the rDNA operon as well as parts of the β-tubulin and EF1-α regions.

MATERIALS AND METHODS

Fungal cultures and isolations

A survey of ophiostomatoid fungi associated with *I. schmutzenhoferi* in Bhutan was conducted in July 2001. Samples for isolation were collected at several locations in Western and Central Bhutan (administrative districts Thimphu and Wangi) (Fig. 1) where *I. schmutzenhoferi* had infested living trees or freshly felled logs of *Picea spinulosa* and/or *Pinus wallichiana* (Fig. 2). The collection sites included mixed conifer forests at Jelekha (3300 m a.s.l.), Changaphug (3600 m a.s.l.), Phobjikha valley (3100 m a.s.l.), and near the Renewable Natural Resources Research Centre (RNR-RC) in Yusipang (2700 m a.s.l.) as well as wood depots at Gidakom (2200 m a.s.l.) and Ramtokto (2100 m a.s.l.) (Fig. 1).

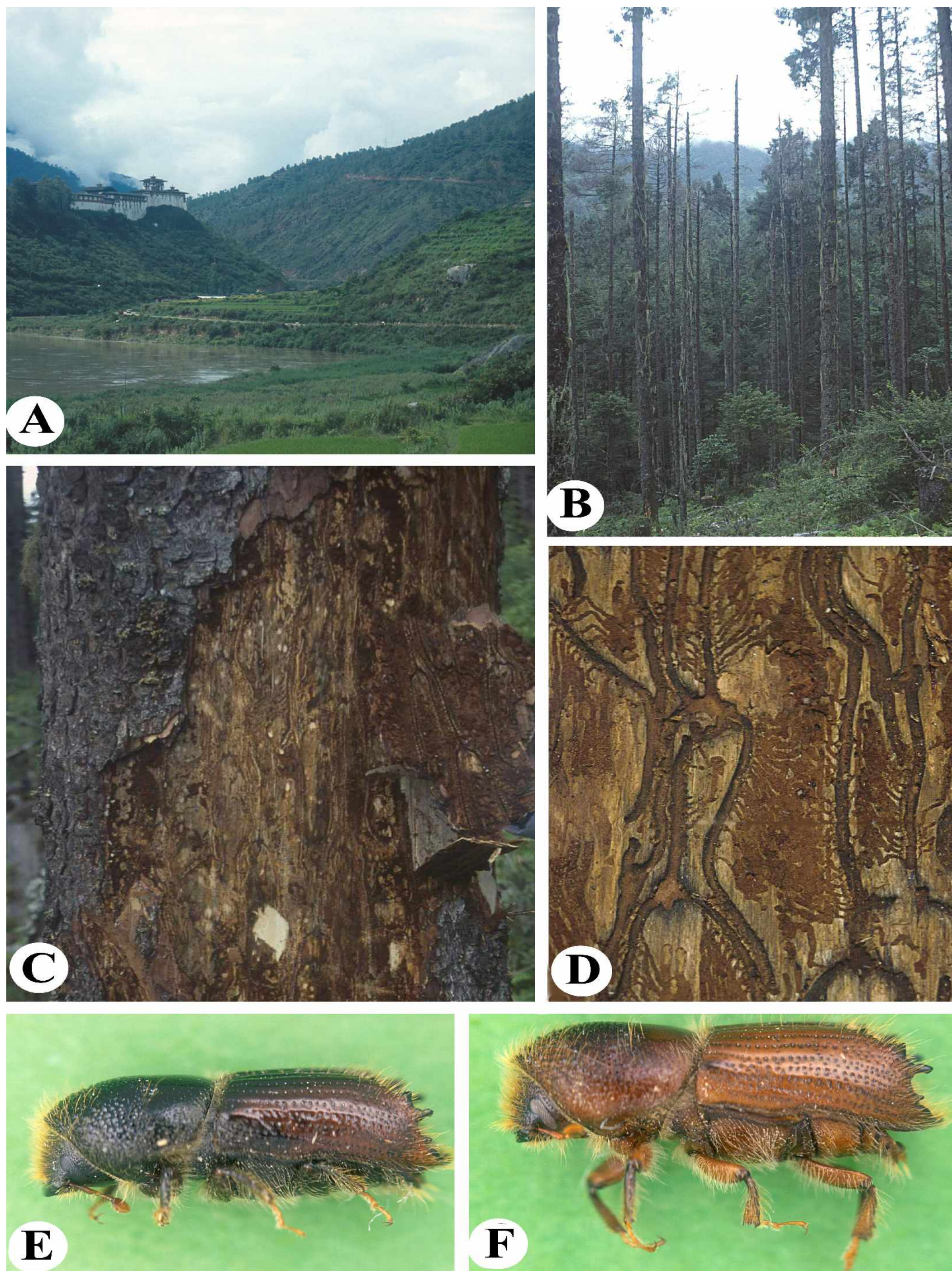

Fig. 2. Eastern Himalayan spruce (*Picea spinulosa*), blue stain and *Ips schmutzenhoferi* in Bhutan. A. A landscape view of the valleys and mountains in Western Bhutan with Wangdi dzong on the left. B. Eastern Himalayan spruce (*P. spinulosa*) dying due to infestation by *I. schmutzenhoferi*. C, D. Blue stain resulting from colonization by ophiostomatoid fungi seen around nuptial chambers and female galleries of *I. schmutzenhoferi* on *P. spinulosa*. E. A female *I. schmutzenhoferi*. F. A male *I. schmutzenhoferi*. The large elongated third spine at the elytral declivity, which bends downwards at the apex, is a distinct characteristic of this bark beetle species.

Logs and standing trees, infested by *I. schmutzenhoferi* were examined for suitable material to conduct fungal isolations (Fig. 2)

Galleries of the insects occurring in the bark or on the surface of the sapwood on logs and standing pine and spruce trees were inspected on site, with the aid of a 10 × magnification hand lens, for the occurrence of sexual and asexual stages of ophiostomatoid fungi. At the research station in Yusipang, adult beetles of *I. schmutzenhoferi* (2nd generation) were collected from a pheromone trap installed specifically for the purpose of insect specimen collection.

Adult and juvenile beetles, breeding galleries, stem discs and stem sections from beetle-infested *P. spinulosa* and *P. wallichiana* trees and logs were collected for further investigation (Fig. 2). All samples were stored in plastic bags and transported to the laboratory at RNR-RC in Yusipang.

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

Species	Isolate no.[d]	Alternative numbers[e]	GenBank accession no.	Year of isolation	Host	Geographical origin	Associated insect	Collector(s)
C. moniliformis	CMW 8240[a]	–	AY528989[f] AY529000[g] AY529010[h]	2001	*Cassia fistula*	Punakha, Bhutan	–	MJW, TK & DBC
C. moniliformis	CMW 8238[c]	CBS 115771	–	2001	*C. fistula*	Wangdi, Bhutan	–	MJW, TK & DBC
C. moniliformis	CMW 9590[a,c]	CBS 116452	AY528985[f] AY528996[g] AY529006[h]	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	–	JR
C. moniliformis	CMW 4114[a]		AY528986[f] AY528997[g] AY529007[h]	1997	*Schizolobium parahybum*	Ecuador, South America	–	MJW
C. moniliformis	CMW 10134[c]		–	2002	*Eucalyptus grandis*	Mpumalanga, South Africa	–	MvW
C. moniliformopsis	CMW 9986[a,c]	CBS 109441	AY528987[f] AY528998[g] AY529008[h]	1999	*Eucalyptus obliqua*	Tazmania, Australia	–	ZQY
C. moniliformopsis	CMW 10214[a,c]	CBS 115792, ORB 33	AY528988[f] AY528999[g] AY529009[h]	1989	*Eucalyptus sieberi*	Victoria, Australia	–	MJ D
C. moniliformopsis	CMW 10215[c]	115793, ORB 346	–	1990	*E. sieberi*	Victoria, Australia	–	MJD
C. bhutanensis	CMW 8215[a]	CBS 114290, PREM 57805	AY528953[f] AY528958[g] AY528963[h]	2001	*Picea spinu-losa*	Jelekha, Bhutan	*Ips schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8242[a,b]	CBS 112907 PREM 57809	AY528951[f] AY528956[g] AY528961[h]	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8217[a,b,c]	CBS 114289 PREM 57807	AY528952[f] AY528957[g] AY528962[h]	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8241[a,b,c]	CBS 115773, PREM 57808	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8396[a]	CBS 114286 BH 8/5, PREM 57812	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8399[a]	CBS 115772, BH 8/8	AY528954[f] AY528959[g] AY528964[h]	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC

C. bhutanensis	CMW 8243[a,b]	CBS 112908, PREM 57810	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8108[a,b]	CBS 112905	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8244[a,b,c]	CBS 114287, PREM 57811	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. bhutanensis	CMW 8241[a,b,c]	CBS 115773, PREM 57808	–	2001	*P. spinulosa*	Jelekha, Bhutan	*I. schmutzenhoferi*	TK & DBC
C. virescens	CMW 3276[a]	–	AY528984[f] AY528990[g] AY528991[h]	1963	*Quercus* sp.	Warrenber, U.S.A.	–	TH

[a]Isolates sequenced. [b]Isolates used for morphological descriptions. [c]Isolates used in growth studies. [d]C.M.W. refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. [e]CBS refers to the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, BH to the culture collection of the 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, Austria and PREM to the National Fungal Herbarium (PREM), Pretoria, South Africa. [f]GenBank accession numbers representing the ITS sequences. [g]GenBank accession numbers representing the β-tubulin sequences. [h]GenBank accession numbers representing the elongation factor sequences. M.J.W. = M.J. Wingfield, T.K. = T. Kirisits, D.B.C. = D.B. Chhetri, T.H. = T. Hinds, M.J.D. = M.J. Dudzinski, Z.Q.Y. = Z.Q. Yuan, M.v.W. = M. Van Wyk, J.R. = J. Roux.

Dry bark samples were sprayed with distilled water and the bags sealed for a few days to create a moist environment, conducive for sporulation of fungi within the beetle galleries. Reference specimens of *I. schmutzenhoferi* were stored in ethanol and are maintained at the 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, Austria. In addition to material obtained from pine and spruce infested by *I. schmutzenhoferi*, wood samples were collected from broken *Cassia fistula* L. trees near Punakha (*ca.* 1300 m a.s.l.) and Wangdi (Wangdue Phodrang) (*ca.* 1100 m a.s.l.). The purpose of these collections was to search for *Ceratocystis moniliformis* on this subtropical hardwood species.

Fungi were isolated on 2 % malt extract agar (MEA) (20 % w/v) (Biolab, Midrand, South Africa) or on 2 % malt agar (MA) (20 % w/v) (DiaMalt, Hefe Schweiz AG, Stettfurt, Switzerland), both supplemented with 100 mg/L streptomycin sulphate (SIGMA or VWR International). In order to obtain a comprehensive view of the fungi associated with *I. schmutzenhoferi,* various isolation methods were applied. Fungi were isolated directly from adult beetles (2nd generation) collected from two spruce logs at Jelekha, from young beetles (1st generation) obtained from a pine log at Ramtokto, and from swarming beetles (2nd generation) collected from a pheromone trap at Yusipang. To obtain isolates directly from the insects, their body parts were dissected and spread onto 2 % MA.

Fungi were also isolated from the sapwood of a spruce tree from Jelekha. Six stem discs (*ca.* 10–15 cm thick, 18–21 cm diam), were cut from the upper part of this tree. These were split vertically and isolations from the sapwood were done along radii beneath *I. schmutzenhoferi* female galleries, following the procedure described by Solheim (1992). Three radii per disc were sampled, resulting in a total of 18 radii. Small pieces of sapwood were transferred onto 2 % MA plates. From each radius, samples were taken 2, 5 and 10 mm apart from the cambium into the sapwood.

Most isolations were made from ascospores and conidia taken directly from sexual and asexual fungal structures occurring in and around female and larval galleries and pupal chambers of the insects. Bark and sapwood samples from spruce and pine collected at Jelekha, Gidakom, Ramtokto, Changaphug (administrative district Thimphu) and Phobjikha valley (administrative district Wangdi) (Fig. 1) were examined with a dissecting microscope at magnifications ranging from 10 × to 40 ×. With a fine needle, ascospores and conidia accumulating at the apices of perithecia and on conidiophores, respectively, were carefully removed and transferred to 2 % MA or 2 % MEA plates. Isolation of *C. moniliformis* from *C. fistula* collected at Punakha and Wangdi was done in a similar manner, from ascospores obtained from perithecia occurring on the wood surface.

A selective method for the isolation of *Ceratocystis* spp. was also used (Moller & DeVay 1968). Fresh carrots were washed and lightly sprayed with 70 % ethanol. Carrot discs (5–10 mm thick) were cut and four to eight discs were placed in plastic Petri dishes (90 mm). Beetles, larvae and pupae of *I. schmutzenhoferi* were dissected and spread over the surface of the carrot discs. Larval frass, collected from the insect galleries was also put onto the carrots. The discs were examined for the incidence of perithecia after 5–10 d incubation at *ca.* 20 °C.

Pure cultures were obtained by transferring ascospore or conidial masses as well as small pieces of mycelium from the primary isolation plates onto fresh 2 % MA or 2 % MEA plates. Fungal cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, the 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, Austria and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Holotype material of the new *Ceratocystis* sp. from Bhutan, consisting of a dried culture of isolate CMW 8217 on 2 % MEA has been lodged at the National Fungal Herbarium (PREM), Pretoria, South Africa (Table 1).

Culture characteristics and morphology

The growth of isolates CMW 8217, CMW 8241 and CMW 8244 representing the *Ceratocystis* sp. obtained from *I. schmutzenhoferi*, was determined on 2 % MEA (Table 3). Three isolates of *C. moniliformis* (CMW 9590, CMW 8238, CMW 10134) and *C. moniliformopsis* (CMW 9986, CMW 10214, CMW 10215) were used for comparisons in the growth studies (Table 3). Prior to the growth assays, the isolates were grown for 2 wk at 25 ºC on 2 % MEA (Fig. 3). Mycelial plugs were taken from actively growing cultures using a 5 mm cork borer and a single mycelial plug was transferred to the centre of a 90 mm Petri dish containing 2 % MEA. For each isolate, five plates were incubated at 4, 10, 15, 20, 25, 30 and 35 ºC, respectively. Colony diameter for each culture was assessed by taking two daily measurements at right angles to each other, for 4 d or until the plates were almost completely covered by mycelium. Averages were computed separately for each isolate and each specified temperature. The entire experiment was repeated once.

Micro-morphological characteristics were described from 10-d-old cultures, on 2 % MEA supplemented with streptomycin sulphate (0.001 g/L) (SIGMA, Steinheim, Germany) and Thiamine (0.001 g/L) (SIGMA, Steinheim, Germany). Fungal struc-

tures were mounted in lactophenol containing cotton blue. Fifty measurements for each taxonomically relevant structure were made from isolate CMW 8217, and 10 further measurements were made for each of seven other isolates of the *Ceratocystis* sp. from Bhutan (Tables 1, 2). Ranges, averages, and standard deviations of the corresponding measurements were calculated. Measurements are given in the format: (minimum–) mean minus standard deviation – mean plus standard deviation (–maximum). The microscopic observations were made using a Carl Zeiss microscope and the photographic images were made with a Zeiss Axio Vision camera system. Colour descriptions were determined using the colour charts of Rayner (1970). The measurements and morphological characteristics of the *Ceratocystis* sp. from Bhutan were compared with those of the descriptions of *C. moniliformis* (Hedgcock 1906, Bakshi 1951, Upadhyay 1981) and *C. moniliformopsis* (Yuan & Mohammed 2002) (Table 2).

PCR, sequencing and analysis

Representative isolates of the *Ceratocystis* sp. from *I. schmutzenhoferi* in Bhutan as well as isolates of *C. moniliformis*, *C. moniliformopsis* and *C. virescens* (R.W. Davidson) C. Moreau (Table 1) were selected for DNA extraction and sequencing. An ascospore mass was transferred from each actively growing and sporulating culture, to 50 mL 3 % ME broth, in Erlenmeyer flasks, and incubated at 25 °C. After 2 wk, the thick mycelial mats were filtered from the broth and lyophilised for two d. The freeze-dried mycelium was placed in liquid nitrogen and ground to a powder using a glass rod, and DNA was extracted using the method described by Barnes *et al.* (2001).

The two ITS regions (ITS1 and ITS2) and the 5.8S gene of the rDNA operon were amplified using primers ITS1 and ITS4 (White *et al.* 1990) at an annealing temperature of 55 °C. The β-tubulin gene was partially amplified using primers βt1a and βt1b at an annealing temperature of 55 °C (Glass & Donaldson 1995) and the EF1-α gene of the rDNA operon was amplified using primers EF1-728F and EF1-986R at an annealing temperature of 56 °C (Carbone & Kohn 1999).

Polymerase chain reaction (PCR) mixtures consisted of 200 nM of the forward and reverse primers, 200 µM of each dNTP, Expand High Fidelity PCR System enzyme mix (1.75 U) (Roche Diagnostics, Mannheim, Germany), 1 × Expand HF Buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 2–10 ng DNA. Reaction volumes were adjusted to 25 µL with sterile water. The PCR programme was set at 96 ºC for 2 min, followed by 10 cycles at 94 ºC for 20 s, x ºC (x = the annealing temperature specified for each set of primers) for 40 s and 72 ºC for 45 s. A further 30 cycles were included with the annealing time altered to 40 s and a 5 s extension after each cycle. A final step of 10 min at 72 ºC completed the programme. Amplification of the respective genes was confirmed on a 2 % agarose (Roche diagnostics, Mannheim, Germany) gel supplemented with ethidium bromide. PCR amplicons were purified using the Magic PCR Preps, Purification System (Promega, Madison, U.S.A.).

PCR amplicons were sequenced in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California). The same primers as those in the PCR reactions were used for sequencing of the respective gene areas. Sequence reactions were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California) and sequences were analysed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

The sequences obtained for the *Ceratocystis* sp. from *I. schmutzenhoferi* were compared with those for morphologically similar *Ceratocystis* spp. (Table 1). Sequences were aligned manually and analysed using PAUP v4.0b10 (Phylogenetic Analysis Using Parsimony) (Swofford 2002). Gaps were treated as “newstate” and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. The heuristic search based on parsimony with tree bisection reconstruction was used to obtain the phylogram. Confidence intervals using 1000 bootstrap replicates were calculated. The out-group taxon, *C. virescens*, was rooted as a midpoint with respect to the in-group taxa. All sequences derived from this study have been deposited in GenBank (Table 1). A partition homogeneity test (Swofford 2002) was used to determine whether the sequence data sets for the three different genome regions could be combined.

The Markov Chain Monte Carlo (MCMC) method (Larget & Simon 1999), with a Bayesian framework was used to estimate the posterior probability of nodes in the phylogenetic tree. One hundred thousand random trees were generated using the MCMC procedure, sampling every 100th tree and printing every 10th tree. To avoid including trees that might have been sampled before convergence of the Markov chain, the chain was assessed for the number of trees that were formed before the stabilization and these trees (8600) were discarded. For the combined analysis of the three gene sequences, gamma rate heterogeneity was set, and no codon-specific sites were included for the ITS gene. For β-tubulin and EF1-α sequences, codon specific sites were specified with a site-specific substitution rate and the site partition was treated as a bycodon.

Table 2. Comparison of *Ceratocystis bhutanensis* with the morphologically similar species, *C. moniliformis* and *C. moniliformopsis.*

Character	*C. bhutanensis*	*C. moniliformis*	*C. moniliformopsis*

		(Hedgcock 1906)	(Yuan & Mohammed 2002)
ASCOMATA			
Base			
Colour	Dark brown to black	Brown to black	Dark brown to black
Diameter	138–178 µm	90–180 µm	200–300 µm
Ornamentation	Conical spines and hyphal hairs	Conical spines (sparse)	Hyphal hairs & conical spines
Form	Globose	Globose	Ovoid
Neck			
Colour	Dark brown to black becoming pale brown towards apex	Pale brown becoming transparent at the apex[a]	Dark brown to black
Disc-form at base	Yes	Yes[a]	Yes
Length	453–519 µm	730–896 µm[a]	470–780 µm
Width (Tip)	12–14 µm	14 µm [a]	18–22 µm
Width (Base)	34–42 µm	39.2–51.8 µm[a]	40–50 µm
Ostiolar hyphae			
Shape	Divergent	Divergent	Convergent
Measurement	18–26 µm	12–18 × 2 µm	25–45 × 1.5–2 µm
Ascus	Not seen	Fugacious	Not seen
Ascospores			
Colour	Hyaline	Hyaline	Hyaline
Shape (Side view)	Hat-shaped	Oval, one side flat	Hat-shaped
Measurements	4–6 × 2–5 µm	4–5 × 3–4 µm	4–5 × 2–2.5 µm
Aggregation	Mucilaginous	Slimy grey mass	Gelatinous sheath
CONIDIOPHORES			
Measurements	23–39 × 4–6 µm	(1) 3–13.7 × 3.5–8.9 µm[a] (2) 7.3–13.7 × 4.5 µm[a]	5–32.5 × 4–5.3 µm
Shape	Phialides	Phialides (2 types)	Phialides (2 types)
Conidia			
Shape	(1) Cylindrical (2) Barrel-shaped	(1) Oval or cylindrical[a] (2) Cylindrical[a]	(1) Cylindrical (2) Oblong or ellipsoidal
Measurements	(1) 7–9 × 1–3 µm (2) 3–5 × 2–3 µm	(1) 7.3–13.7 × 3.5–8.9 µm[a] (2) 4.3–15.5 × 1–2.5 µm [a]	(1) 13–21 × 2–3 µm (2) 12–17.5 × 5–7.5 µm
CULTURES			
Growth rate	20 mm per day at 25 ºC in the dark	60 mm in 10 days at 22 ºC in the dark[a]	6.3–7.5 mm per day at 22 ºC in the dark
Colour	Cream-buff to dark olive to black	Hyaline to grey to black	Colourless to white grey, centre becoming greenish brown
Odour	Fermenting odour	Pear drops[a]	None
Mycelia	Smooth and granulated	Coarsely granular	Smooth

[a]Description by Bakshi (1951).

RESULTS

Fungal cultures and isolations

Conspicuous blue-stain was observed on the surface of the sapwood and in the bark around nuptial chambers as well as female and larval galleries of *I. schmutzenhoferi* on spruce and pine (Fig. 2). However, intensive blue-stain, deeply penetrating into the sapwood was not seen on any of the wood samples. On stem discs of the spruce from which isolations were made, a narrow zone of desiccation, extending 5 to 8 mm deep into the sapwood and recognizable by its white to yellowish colour, occurred.

The *Ceratocystis* sp. was isolated directly from second-generation beetles collected from galleries on *P. spinulosa* at Jelekha. In a sample of 20 beetles from this site 16 (80 %) yielded the *Ceratocystis* sp. and this fungus was thus among the dominant species recovered from this niche. Fourteen isolates of the *Ceratocystis* sp. representing isolations from separate beetles were initially maintained and 10 of these strains were used for characterization of this fungus (Table 1).

The *Ceratocystis* sp. was neither isolated from beetles obtained from Ramtokto and Yusipang nor from desiccated sapwood of the spruce tree collected at Jelekha. Perithecia and conidiophores were never located in galleries of *I. schmutzenhoferi* on spruce or pine. Attempts to isolate this fungus from adult and juvenile beetles, larvae, and pupae or from larval frass using carrot discs were unsuccessful. Ascomata resembling those of *C. moniliformis* were common on the surface of the wood of broken *C. fistula* trees near Punakha and Wangdi (Wangdue Phodrang). This fungus was easily isolated by transferring ascospores from the perithecial tips to MA and MEA plates. Two

isolates of this fungus from Bhutan were included in the comparisons with the *Ceratocystis* sp. from *I. schmutzenhoferi* (Table 1).

PCR, sequencing and analysis

Amplification of the ITS regions and the 5.8S gene of the rDNA resulted in amplification products of ~500 bp. Amplification products for β-tubulin were ~500 bp, while those for the EF1-α gene were ~300 bp.

In the partition homogeneity tests, all DNA sequence data sets gave P-values greater than the minimum required value of $P = 0.05$ and they could thus be combined. The combined sequences of the three gene areas resulted in a data set that was 1491 bp long, had a single most parsimous tree, with a consistency index (CI) of 0.95, a homoplasy index (HI) of 0.05, a retention index (RI) of 0.93 and a rescaled consistency index (RC) of 0.88. The posterior probability of the branch nodes of the combined tree, generated with the Bayesian inference programme supported the bootstrap values. The posterior probability for the branch nodes for the three clades representing *C. moniliformis*, *C. moniliformopsis* and the *Ceratocystis* sp. isolated from *I. schmutzenhoferi* was 100 %.

A heuristic search resulted in a single well-resolved tree with species of *Ceratocystis* residing in three distinct sub-clades (Fig. 5). One of these sub-clades included the *Ceratocystis* sp. isolated from *I. schmutzenhoferi* in Bhutan, supported by a bootstrap value of 100 %. The other sub-clades included isolates of *C. moniliformis* and *C. moniliformopsis*, respectively (Fig. 5).

Taxonomy

Comparison of DNA sequence data confirmed morphological observations that the *Ceratocystis* sp. from *I. schmutzenhoferi* in Bhutan is related to *C. moniliformis* and *C. moniliformopsis*. The data also provided robust support for the view that this fungus represents a new and previously undescribed species of *Ceratocystis*. The fungus is, therefore, described as follows.

Ceratocystis bhutanensis M. Van Wyk, M.J. Wingf. & T. Kirisits, **sp. nov.** MycoBank MB500092.

Anamorph: *Thielaviopsis* sp.

Etymology: *bhutanensis* refers to Bhutan, the country of origin.

Coloniae juvenes cremeo-fulvidae, infra mellinae, seniores griseo-mustelinae, infra umbrinae, dein atro-olivaceae, infra nigrae. Mycelium plerumque in medio immersum; mycelium album aerium adest. Crescit optime ad 20–25 ºC, nullo incremento supra 35 ºC. Hyphae leves vel granulatae, ad septa non constrictae, 1–3.5 µm latae. Bases ascomatum atrobrunneae vel nigrae, globosae, spinis hyphisque ornatae, spinis atrobrunneis vel nigris, (4.5–)8–19(–27) µm longis, bases (112–)138–178(–206) µm diametro. Colla ascomatum basin versus atrobrunnea vel nigra, apicem versus pallidiora, (450–)453–519 µm longa, ad basim, 34–42(–44) µm lata, basi discoidea dilata. Hyphae ostiolares divergentes, hyalinae, (13–)18–26(–34) µm longae. Asci non visi. Ascosporae e latere visae galeiformes, aseptatae, hyalinae, in vagina investitae, vagina inclusa 4–6 × 2–5 µm, vagina exclusa 2–5 × 2–5 µm. Ascosporae in massis mucilagineis fulvo-luteis in apicibus collorum ascomatum cumulantes. Anamorphe Thielaviopsis: conidiophora in mycelio singula, hyalina, basi tumida, apicem versus angustata, (15–)23–39(–51) µm longa, basi (3–)4–6(–9) µm lata, apice 1–3 µm lata. Conidiogenesis phialidica incremento parietis annulari; conidia in catenis biformibus facta: conidia primaria hyalina, aseptata, cylindrica, (6–)7–9(–10) × 1–3 µm, conidia secundaria hyalina, aseptata, doliiformia, 3–5 × (1.5–)2–3(–3.5) µm.

Colonies of young cultures with submerged mycelium honey-coloured (19''b), aerial mycelium cream-buff (19''d). Cultures that were ± 14-d-old, had umber (15 m) submerged mycelium, aerial mycelium ecru-drab (13'''''d) (Fig. 3). Cultures that were ± 28-d-old, had black (7'''''k) submerged mycelium, aerial mycelium abundant, dark olive (21''m). *Mycelium* submerged in medium, abundant white aerial mycelium present. *Optimal temperature* range 20–25 ºC, no growth at 35 ºC. Isolates growing up to 20 mm per day at 20 ºC. *Hyphae* smooth or granulated, not constricted at septa, 1–3.5 µm wide. *Ascomatal bases* dark brown to black, globose, ornamented with spines and hyphae, spines dark brown to black, (4.5–)8–19(–27) µm long, bases (112–)138–178(–206) µm diam. *Ascomatal necks* dark brown to black at base, becoming light brown towards the apex, (450–)453–519 µm long, 34–42(–44) µm wide at the base, (11–)12–14(–17) µm wide at the apex, with a disc-like (disciform) base. *Ostiolar hyphae* divergent, hyaline, (13–)18–26(–34) µm long. *Asci* not observed. *Ascospores* cucullate in side view, aseptate, hyaline, invested in a sheath, 4–6 × 2–5 µm with sheath, 2–5 × 2–5 µm without sheath. Ascospores accumulating in white and later buff-yellow (19d) mucilaginous masses on the apices of ascomatal necks.

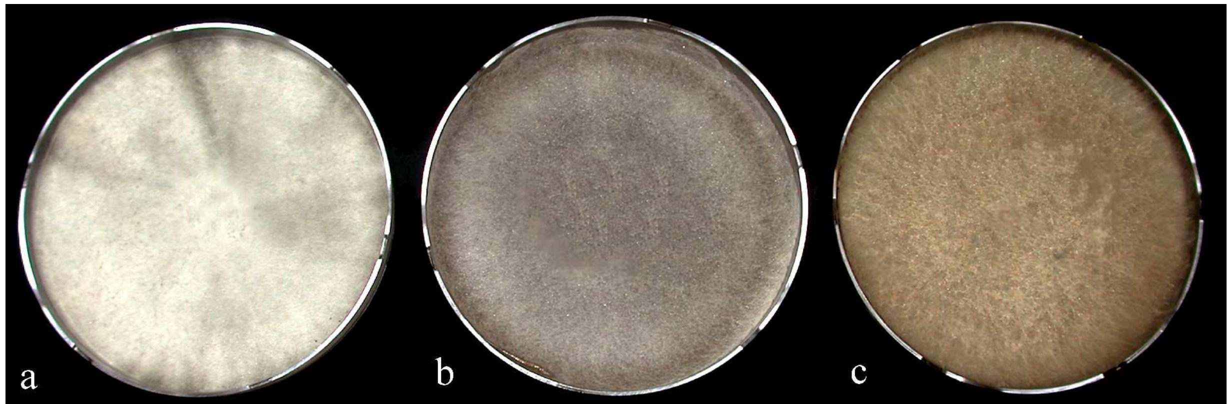

Fig. 3. Characteristics of the cultures of *Ceratocystis bhutanensis* and the two *Ceratocystis* spp. most closely related to it. A. White to pale mouse grey (15”’”’d) colony of *C. moniliformis* (CMW 9590). B. Grey to black (7”’”’k) colony of *C. bhutanensis* (CMW 8217). C. Buffy brown (17”’i) culture of *C. moniliformopsis* (CMW 9986). All cultures were grown on 2 % MEA at 20 ºC in the dark for approximately 10 d.

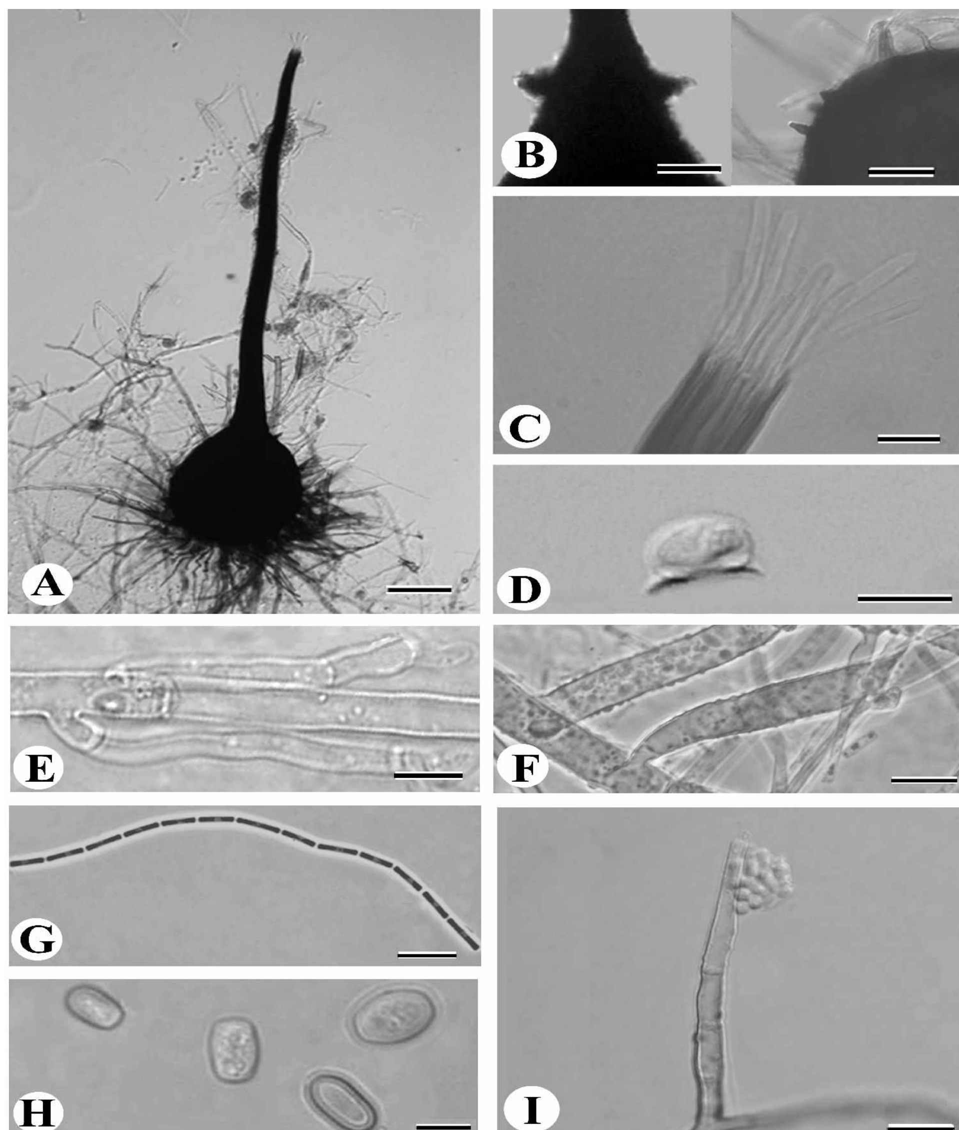

Fig. 4. Morphological characteristics of *Ceratocystis bhutanensis* (PREM 57807, CMW 8217, CBS 114289). A. Globose ascoma with long neck. B. Neck base with disc and ascoma base with short, conical spines. C. Divergent ostiolar hyphae on the top of the ascomatal neck. D. Hat-shaped ascospore in side view. E. Hyphae with smooth walls. F. Hyphae with rough walls. G. Cylindrical conidia forming a chain. H. Barrel-shaped conidia. I. Phialidic conidiophore with emerging cylindrical conidia. Scale Bars: A, B = 40, C = 10, D–F, I = 5 µm, G = I = 30 µm.

Thielaviopsis anamorph: conidiophores occurring singly on mycelium, hyaline, swollen at the base, tapering towards the apex, (15–)23–39(–51) µm long, (3–)4–6(–9) µm wide at the base, 1–3 µm wide at the apices. Phialidic *conidium* development through ring wall building, *conidia* of two types formed in chains: primary conidia hyaline, aseptate, cylindrical, (6–)7–9(–10) × 1–3 µm, secondary conidia hyaline, aseptate, barrel-shaped, 3–5 × (1.5–)2–3(–3.5) µm.

Specimens examined: **Bhutan**, Thimphu dzongkhag, Jelekha, isolated from *Ips schmutzenhoferi* collected from galleries on *Picea spinulosa*, Jul. 2001, T. Kirisits and D.B. Chhetri, **holotype** Herb. PREM 57804; culture ex-type CMW 8217 = CBS 114289; Thimphu dzongkhag, Jelekha, isolated from *Ips schmutzenhoferi* collected from galleries on *Picea spinulosa*, Jul. 2001, T. Kirisits and D.B. Chhetri, **paratype** PREM 57808; culture ex-paratype CMW 8241 = CBS 115773; same collection data, **paratype** PREM 57809; culture ex-paratype CMW 8242 = CBS 112907; same collection data, culture ex-paratype CMW 8108 = CBS 112904; **paratype** PREM 57811; culture ex-paratype CMW 8244 = CBS 114287; **paratype** PREM 57810, culture ex-type CMW 8243 = CBS 112908.

Cultural characteristics and morphology

All three *Ceratocystis* spp. compared for growth characteristics grew very rapidly in culture, at least at their optimum growth temperatures. *Ceratocystis bhutanensis*, *C. moniliformis* and *C. moniliformopsis* differed considerably in their growth profiles at different temperatures (Table 3). The optimum temperature range for growth of *C. bhutanensis* was 20–25 °C, whereas *C. moniliformis* and *C. moniliformopsis* had optimum growth ranges at 25–30 ºC and 15–20 ºC, respectively (Table 3). *Ceratocystis bhutanensis* was able to grow at 4 ºC, whereas *C. moniliformis* and *C. moniliformopsis* showed no growth at this temperature. *Ceratocystis moniliformopsis* did not grow at temperatures above 25 ºC, but *C. moniliformis* grew rapidly at 25 ºC and 30 ºC and ceased to grow at 35 ºC. *Ceratocystis bhutanensis* displayed rapid growth at 25 ºC, diminished growth at 30 ºC for two of the isolates (CMW 8244 and CMW 8217), and no growth for one isolate (CMW 8241), and also did not grow at 35 ºC (Table 3). In addition to differences in their ability to grow at different temperatures, *C. moniliformis*, *C. moniliformopsis* and *C. bhutanensis* differ in their rates of growth at various temperatures (Table 3).

On MEA, cultures of *C. bhutanensis* were pale in colour when young, but turned grey and finally black with age. In young cultures, the submerged mycelium had a honey (19"'b) colour and the aerial mycelium was cream-buff (19"d). In older cultures (± 14 d), the submerged mycelium was umber (15 m) with the aerial mycelium ecru-drab (13'""'d) (Fig. 3). In even older cultures (± 28 d), the submerged mycelium tended to be black (7'""'k) and the abundant aerial mycelium dark olive (21"m). This is very different from *C. moniliformis* that produced white to cream-buff (19"d) mycelium (Fig. 3) and the *C. moniliformopsis* isolates, described as having a colourless to white-grey appearance with the centre becoming greenish brown due to sporulation of the ascomata (Yuan & Mohammed 2002). Our observations showed that cultures of *C. moniliformopsis* had a buff-brown (17"'i) colour (Fig. 3).

Isolates of *C. bhutanensis* produced an unpleasant rotten odour. This was in contrast to the pleasant banana-oil odour typically produced by cultures of *C. moniliformis* (Davidson 1935) and little to no odour produced by *C. moniliformopsis* (Yuan & Mohammed 2002). *Ceratocystis bhutanensis* produced ascomata within a few (3–4) d, which were then overgrown by dense aerial mycelium making the detection of ascomata difficult. *Ceratocystis moniliformis* and *C. moniliformopsis* also produced perithecia within a few days, and these could be clearly seen in older cultures, that tended to have sparse aerial mycelium. When older cultures were examined using a dissecting microscope, pigmented, thick-walled cells were obvious and initially thought to represent chlamydospores. They were later identified as accumulations of aged conidia produced by the *Thielaviopsis* anamorph.

Table 3. Comparision of growth of *Ceratocystis moniliformopsis*, *C. moniliformis* and *C. bhutanensis* in culture[a].

		Temperature (ºC)						
Species	**Isolates**	**4**	**10**	**15**	**20**	**25**	**30**	**35**
C. moniliformopsis	CMW 9986	0.0 (0.0)	17.9 (0.2)	35.3 (2.1)	36.3 (1.3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	CMW 10214	0.0 (0.0)	19.3 (4.7)	38.7 (0.9)	50.4 (2.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	CMW 10215	0.0 (0.0)	12.3 (1.0)	24.3 (2.0)	32.8 (5.2)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
C. moniliformis	CMW 9590	0.0 (0.0)	12.1 (3.0)	33.2 (0.8)	61.6 (0.89)	73.1 (1.2)	90.0 (0.0)	0.0 (0.0)
	CMW 8238	0.0 (0.0)	0.0 (0.0)	24.1 (2.6)	53.7 (0.3)	66.5 (1.5)	61.5 (1.0)	0.0 (0.0)
	CMW 10134	0.0 (0.0)	0.0 (0.0)	16.2 (1.9)	21.39 (5.5)	28.6 (15.5)	16.0 (0.6)	0.0 (0.0)
C. bhutanensis	CMW 8217	18.0 (1.6)	14.8 (1.5)	30.4 (1.0)	59.8 (1.2)	62.1 (2.7)	9.4 (0.4)	0.0 (0.0)
	CMW 8244	17.7 (2.1)	16.1 (0.8)	31.6 (2.5)	66.7 (3.4)	50.7 (2.9)	12.0 (0.9)	0.0 (0.0)
	CMW 8241	17.7 (1.2)	14.4 (0.5)	28.3 (2.7)	76.2 (5.9)	45.2 (3.6)	0.0 (0.0)	0.0 (0.0)

[a]Growth assessed as average colony diameter for five cultures per isolate on 2 % MEA after 3 d. Standard deviations are presented in parentheses.

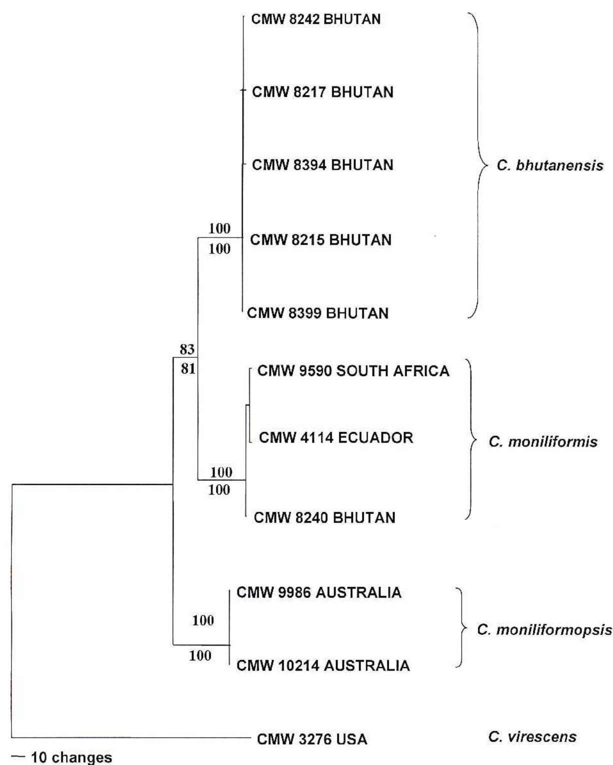

Fig. 5. A phylogenetic tree based on the combined sequence data of three gene regions (ITS, β-tubulin and EF1-α). The phylogram was obtained using the heuristic search option based on parsimony and *Ceratocystis virescens* was treated as the out-group. Bootstrap values are indicated above of the branches while Bayesian values are indicated below the branches.

Cultures of *C. bhutanensis* tended to degenerate on MEA. This phenomenon has also been observed in *C. moniliformis* and in *C. moniliformopsis*. Degenerated cultures became white in colour, displayed reduced growth and ceased to produce ascomata in culture

DISCUSSION

There is very little known regarding the occurrence of ophiostomatoid fungi in the Himalayas or their role as tree pathogens and agents of blue-stain. Prior to the present study, only one ophiostomatoid fungus, *Ophiostoma himal-ulmi* Brasier & M.D. Mehrotra was known from the Western Himalayas, where it occurs on *Ulmus wallichiana* Planchon and is associated with elm bark beetles (Brasier & Mehrotra 1995). The discovery of *C. bhutanensis* and the detection of *C. moniliformis* in Bhutan represent the first reports of species of *Ceratocystis* from this country or the Himalayas in general. *Ceratocystis bhutanensis*, described in this study is also the first fungus to be recorded as an associate of bark beetles, specifically of *I. schmutzenhoferi* in Bhutan. Many other ophiostomatoid fungi, including species of *Ophiostoma*, *Ceratocystiopsis* Upadhyay & W.B. Kendr., *Leptographium* Lagerb. & Melin and *Pesotum* Crane & Schoknecht were found in the survey of these fungi in Bhutan (Kirisits *et al.* 2002) and these are currently being examined and identified.

Ceratocystis bhutanensis is morphologically similar to *C. moniliformis* and *C. moniliformopsis.* Its occurrence in Bhutan on a conifer tree, in association with a bark beetle provided the first suspicion that it might represent a new species. The unusual colony morphology and distinct odour of this *Ceratocystis* sp., also suggested that it might be new. Morphological comparisons of *C. bhutanensis* with *C. moniliformis* and *C. moniliformopsis* revealed minor differences between these fungi. DNA sequence comparisons for three nuclear gene regions provided unequivocal evidence that isolates of this fungus were unique.

In addition to *C. bhutanensis*, there are six *Ceratocystis* spp. that have hat-shaped ascospores. These include *C. fimbriata* Ellis & Halst. (Upadhyay 1981), *C. moniliformis* (Davidson 1935), *C. albifundus* M.J. Wingf., De Beer & M.J. Morris (Wingfield *et al.* 1996), *C. moniliformopsis* (Yuan & Mohammed 2002), *C. pirilliformis* Barnes & M.J. Wingf. (Barnes *et al.* 2003b) and *C. acericola* H.D. Griffin (Griffin 1968). Of these species, only *C. moniliformis* and *C. moniliformopsis* have spines on their ascomatal bases and both have very characteristic disc-shaped bases at their ascomatal necks. *Ceratocystis bhutanensis* can be distinguished from both of these species based on host, biogeography, association with a conifer bark beetle, and odour in culture, as well as various morphological characteristics (Table 2). *Ceratocystis moniliformopsis* has convergent ostiolar hyphae while these structures are divergent in *C. bhutanensis* (Table 2, Fig. 3). The ascomatal bases in these two species differ in that they are ovoid in *C. moniliformopsis* and globose in *C. bhutanensis* (Table 2). Two types of conidiogenous cells have been described for *C. moniliformopsis*, while *C. bhutanensis* has only one morphological form for these structures (Table 2). *Ceratocystis bhutanensis* has hyphae with walls that are smooth and granulated while *C. moniliformis* has only smooth-walled hyphae (Table 2).

Comparison of DNA sequences for three gene regions provided strong support for the recognition of *C. bhutanensis* as a new species. Sequence data for the ITS regions alone did not provide convincing separation between *C. bhutanensis*, *C. moniliformis* and *C. moniliformopsis*. However, addition of β-tubulin and EF1-α sequences provided clear resolution of the clades, in which these three species reside. Phylogenetically, *C. bhutanensis* grouped within the larger *C. coerulescens* clade (Witthuhn *et al.* 1998) together with *C. moniliformis* and *C. moniliformopsis* as its closest relatives. This clade is separate from the *C. fimbriata* clade, in which the other *Ceratocystis* spp. with hat-shaped ascospores reside (Witthuhn *et al.* 1999, Barnes *et al.* 2003a). This study also pro-

1999, Barnes *et al*. 2003a). This study also provides the first DNA sequence data for *C. moniliformopsis* and supports the view that this is a distinct species, even though it is morphologically very similar to *C. moniliformis* (Yuan & Mohammed 2002).

Besides *C. polonica*, *C. laricicola* and *C. rufipennis*, *C. bhutanensis* is the fourth *Ceratocystis* sp. known to be associated with a conifer-infesting bark beetle. The new *Ceratocystis* sp. from Bhutan is, however, very different to the other three species, morphologically, phenotypically and phylogenetically. *Ceratocystis polonica*, *C. laricicola* and *C. rufipennis* are closely related to each other and form part of the *C. coerulescens* species complex on conifers (Harrington & Wingfield 1998, Witthuhn *et al.* 1998). In contrast, *C. bhutanensis* is more distantly related to species in this complex and groups closely with *C. moniliformis* and *C. moniliformopsis* that typically occur on hardwoods.

The strong aroma produced by *C. bhutanensis* is of special interest, since this is a general characteristic of *Ceratocystis* spp. that are not specifically associated with bark beetles. Species of *Ceratocystis* that produce strong aromas are typically carried by non-specific insects that are attracted to fermenting organic material and that also visit wounds on trees (Kile 1993, Harrington & Wingfield 1998). In contrast to *C. bhutanensis*, cultures of *C. polonica*, *C. laricicola* and *C. rufipennis* lack strong aromas, which is considered as a modification to their consistent association with bark beetles (Yamaoka *et al*. 1997, Harrington & Wingfield 1998).

Ips schmutzenhoferi is an insect that is biologically very similar to *I. typographus* and *I. cembrae* (Postner 1974, Christiansen & Bakke 1988, Schmutzenhofer 1988). Both of the latter insects carry a wide range of *Ophiostoma* spp. and their anamorphs, and they are particularly interesting in that they are also consistently associated with a pathogenic *Ceratocystis* spp. (Solheim 1986, Redfern *et al.* 1987, Solheim 1992, Yamaoka *et al*. 1997, Yamaoka *et al*. 1998, Kirisits 2001, 2004). In this respect, we might have expected to encounter a *Ceratocystis* sp. associated with *I. schmutzenhoferi* in Bhutan. However, the fact that *C. bhutanensis* was isolated only from adult insects at one locality and not from beetles obtained at other sites or from galleries or symptomatic sapwood tissue is intriguing. It also raises the question regarding the intimacy of the relationship between this fungus and *I. schmutzenhoferi*.

Ceratocystis bhutanensis may be a rare associate of *I. schmutzenhoferi* or it may display a restricted geographical distribution. Variation in the assemblages of fungi associated with bark beetles at different study sites has also been well documented. This might explain the isolation results in the present study. For example, *C. polonica* has been reported as a frequent or even as the dominant associate of *I. typographus* in some parts of Europe, while it was not recorded or occurred rarely in studies conducted in other parts of the continent (Solheim 1986, 1992, Kirisits 2001, 2004). It has also been suggested that the population dynamics of *I. typographus* has a strong influence on the incidence and frequency of *C. polonica*, the fungus occurring less frequently during endemic periods, but becoming more frequent during outbreaks of the insect (Solheim 1993, Kirisits 2004). Similar phenomena may also occur in the *I. schmutzenhoferi* – *C. bhutanensis* system.

It is possible that the isolation of *C. bhutanensis* from *I. schmutzenhoferi* was accidental and that this fungus is casually associated with this conifer bark beetle. Its unusual features for a *Ceratocystis* sp. associated with conifer bark beetles, especially its intensive aroma, and its close phylogenetic relationship to two *Ceratocystis* spp. from hardwoods might support this view. At present, the ecology of *C. bhutanensis* remains enigmatic. Further investigations, especially isolations from various niches at the locality where the fungus was first discovered and from the entire distribution range of *I. schmutzenhoferi* would be useful. In addition, pathogenicity tests with the fungus on spruce and pine to consider its role as an associate of *I. schmutzenhoferi* are planned.

ACKNOWLEDGEMENTS

We thank the Conifer Research and Training Partnership (CORET) http://woek.boku.ac.at/coret/, funded by the Austrian Development Co-operation (Austrian Ministry of Foreign Affairs) and the Royal Government of Bhutan for the opportunity and partial funding to undertake this study. G. Gratzer, G. Glatzel, L. Norbu, K. Wangdi, P. Namgyel and P.B. Chhetri helped to organise the research visit of T. Kirisits and M.J. Wingfield to Bhutan in July 2001. We also acknowledge the National Research Foundation (NRF) of the Republic of South Africa, and the Tree Pathology Co-operative Programme (TPCP) for financial support. Thanks are also due to Sylvia Presslauer (IFFF-BOKU) and staff of the Renewable Natural Resources Research Centre (RNR-RC), Yusipang and the Forestry and Agricultural Biotechnology Institute (FABI), for technical assistance in the field and laboratory and to Dr H. Glen for providing the Latin description.

REFERENCES

Bakshi BK (1951). Studies on four species of *Ceratocystis*, with a discussion of fungi causing sap-stain in Britain. *Mycological Papers* **35**: 1–16.

Barnes I, Roux J, Coetzee MPA, Wingfield MJ (2001). Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. *Plant Disease* **85**: 317–321.

Barnes I, Roux J, Wingfield BD, O'Neil M, Wingfield MJ (2003a). *Ceratocystis fimbriata* infecting *Eucalyptus*

grandis in Uruguay. *Australasian Plant Pathology* **32**: 361–366.

Barnes I, Roux J, Wingfield MJ, Old KM, Dudzinski M (2003b). *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* **95**: 865–871.

Brasier CM, Mehrotra MD (1995). *Ophiostoma himal-ulmi* sp. nov., a new species of Dutch elm disease fungus endemic to the Himalayas. *Mycological Research* **99**: 205–215.

Carbone I, Kohn LM (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Chhetri DB (1991). History of bark beetle outbreak in Western Bhutan. *Tsenden* **3**: 68–72.

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, Bakke A (1988). The spruce bark beetles of Eurasia. In: *Dynamics of forest insect populations. Pattern, causes, implications* (Berryman AA, ed.). Plenum Press, New York and London: 479–503.

Davidson RW (1935). Fungi causing stain in logs and lumber in the Southern states, including five new species. *Journal of Agricultural Research* **50**: 789–807.

FAO (1999). *Forest Resources of Bhutan – Country report*. Rome, Italy: Forest Resources Assessment Programme (FRA), Working Paper **14**: 71.

FAO (2001). *Global Forest Resources Assessment 2000 – Main report*. Rome, Italy: *FAO Forestry Paper* **120**: 479.

Francke-Grosmann H (1967). Ectosymbiosis in wood-inhabiting insects. In: *Symbiosis* (Henry SM, ed.). Academic Press, New York and London: 141–205.

Glass NL, Donaldson GC (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.

Griffin HD (1968). The genus *Ceratocystis* in Ontario. *Canadian Journal of Botany* **46**: 689–718.

Harrington TC, Pashenova NV, McNew DL, Steimel J, Konstantinov M (2002). Species delimination and host specialization of *Ceratocystis laricicola* and *C. polonica* to larch and spruce. *Plant Disease* **86**: 418–422.

Harrington TC, Wingfield MJ (1998). The *Ceratocystis* species on conifers. *Canadian Journal of Botany* **76**: 1446–1457.

Hedgcock GG (1906). Studies upon some chromogenic fungi which discolor wood. *Missouri Botanical Garden Annual Report* **17**: 59–111.

Holzschuh C (1988). Eine neue Art der Gattung *Ips* aus Bhutan (Coleoptera, Scolytidae). *Entomologica Brasiliensia* **12**: 481–485.

Jacobs K, Wingfield MJ (2001). *Leptographium species: tree pathogens, insect associates and agents of blue-stain.* APS Press, St. Paul, Minnesota.

Kile GA (1993). Plant diseases caused by species of *Ceratocystis* sensu stricto and *Chalara*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds.). APS Press, St. Paul, Minnesota: 173–183.

Kirisits T (1998). Pathogenicity of three blue-stain fungi associated with the bark beetle *Ips typographus* to Norway spruce in Austria. *Österreichische Zeitschrift für Pilzkunde* **7**: 191–201.

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.* Rerum Naturalium Technicarum Doctoral Thesis, Universität für Bodenkultur Wien (BOKU), Vienna, Austria.

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* (Lieutier F, Day KR, Grégoire JC, Evans H, eds). Kluwer, Dordrecht, The Netherlands: 181–235.

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.

Kirisits T, Wingfield MJ, Chhetri DB (2002). Ophiostomatoid fungi associated with the Eastern Himalayan spruce bark beetle *Ips schmutzenhoferi* and other bark beetles in Bhutan. In: *The 7th International Mycological Congress*, 11–17 August 2002, Oslo, Norway, IMC 7 Book of Abstracts (Ryvarden L, Schumacher T, eds.). 94, Abstract no. 296.

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). Pathogenicity of four blue-stain fungi associated with aggressive and non-aggressive bark beetles. *Phytopathology* **88**: 39–44.

Larget B, Simon DL (1999). Markov Chain Monte Carlo alogorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750–759.

Liese W, Schmid R (1961). Licht- und elektronenmikroskopische Untersuchungen über das Wachstum von Bläuepilzen in Kiefern- und Fichtenholz. *Holz als Roh- und Werkstoff* **19**: 329–337.

Moller W, DeVay J (1968). Insect transmission of *Ceratocystis fimbriata* in deciduous fruit orchards. *Phytopathology* **58**: 1499–1508.

Münch E (1907). Die Blaufäule des Nadelholzes. I-II. *Naturwissenschaftliche Zeitschrift für Land- und Forstwirtschaft* **5**: 531–573.

Paine TD, Raffa KF, Harrington TC (1997). Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology* **42**: 179–206.

Postner M (1974). *Scolytidae* (*Ipidae*), Borkenkäfer. In: *Die Forstschädlinge Europas. Bd. 2.* (Schwencke W, ed.). Paul Parey Verlag, Hamburg, Berlin: 334–482.

Rayner RW (1970). *A Mycological Colour Chart.* Commonwealth Mycological Institute and British Mycological Society, Kew, Surrey.

Redfern DB, Stoakley JT, Steele H, Minter DW (1987). Dieback and death of larch caused by *Ceratocystis laricicola* sp. nov. following attack by *Ips cembrae*. *Plant Pathology* **36**: 467–480.

Schmutzenhofer H (1988). Mass outbreaks of *Ips* bark beetles in Bhutan and the revision of the genus *Ips* de Geer for the Himalayan region. In: *Integrated control of Scolytid bark beetles* (Payne TL, Saarenmaa H, eds.). Proceedings of the IUFRO working party and XVII. International Congress of Entomology Symposium, “Inte-

grated control of Scolytid bark beetles", Vancouver, B. C., Canada: 345–355.

Seifert KA (1993). Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity* (Wingfield MJ, Seifert KA, Webber JF, eds.). APS Press, St. Paul, Minnesota: 141–151.

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

Solheim H (1988). Pathogenicity of some *Ips typographus*-associated blue-stain fungi to Norway spruce. *Meddelelser fra Norsk Institutt for Skogforskning* **40**: 1–11.

Solheim H (1992). Fungal succession in sapwood of Norway spruce infested by the bark beetle *Ips typographus*. *European Journal of Forest Pathology* **22**: 136–148.

Solheim H (1995). A comparison of blue-stain fungi associated with the North American spruce bark beetle *Dendroctonus rufipenniss* and the Eurasian spruce bark beetle *Ips typographus*. In: *Forest pathology research in the Nordic countries 1994. Proceedings from the SNS-meeting in forest pathology at Skogbrukets Kurssenter, Biri, Norway 9–12 August 1994* (Aamlid D, ed.). *Aktuelt fra Skogforsk* 4/95: 61–67.

Solheim H, Safranyik L (1997). Pathogenicity to Sitka spruce of *Ceratocystis rufipennis* and *Leptographium abietinum*, blue-stain fungi associated with the spruce beetle. *Canadian Journal of Forest Research* **27**: 1336–1341.

Stauffer C, Kirisits T, Nussbaumer C, Pavlin R, Wingfield MJ (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.

Swofford DL (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods).* Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts.

Tshering G, Chhetri DB (2000). *Important forest insect pests and diseases of Bhutan with control measures*. Renewable Natural Resources Research Centre, Yusipang and Natural Resources Training Institute, Lobesa. MoA, Field guide 2000/1.

Upadhyay HP (1981). *A monograph of Ceratocystis and Ceratocystiopsis*. University of Georgia Press. Athens, GA.

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

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A sequencing guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds.). Academic Press, San Diego: 315–322.

Whitney HS (1982). Relationships between bark beetles and symbiotic organisms. In: *Bark Beetles in North American Conifers* (Mitton JB, Stugeon KB, eds.). University of Texas Press, U.S.A.: 183–211.

Wingfield MJ, De Beer C, Visser C, Wingfield BD (1996). A new *Ceratocystis* species defined using morphological and ribosomal DNA sequence comparisons. *Systematic and Applied Microbiology* **19**: 191–202.

Wingfield MJ, Harrington TC, Solheim H (1997). Two species in the *Ceratocystis coerulescens* complex from conifers in western North America. *Canadian Journal of Botany* **75**: 827–834.

Wingfield MJ, Seifert KA, Webber JF (eds.) (1993). *Ophiostoma and Ceratocystis*: *Taxonomy, Biology and Pathology*. APS Press, St.Paul, Minnesota.

Witthuhn RC, Wingfield BD, Wingfield MJ, Harrington TC (1999). PCR-based identification and phylogeny of species of *Ceratocystis sensu stricto*. *Mycological Research* **103**: 743–749.

Witthuhn RC, Wingfield BD, Wingfield MJ, Wolfaardt M, Harrington TC (1998). Monophyly of the conifer species in the *Ceratocystis coerulescens* complex based on DNA sequence data. *Mycologia* **90**: 96–101.

Wood SL, Bright DE (1992). A catalogue of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic Index, Volumes A and B. *Great Basin Naturalist Memoirs* **13**: 1–1553.

Yamaoka Y, Wingfield MJ, Ohsawa M, Kuroda Y (1998). Ophiostomatoid fungi associated with *Ips cembrae* in Japan. *Mycoscience* **39**: 367–378.

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

Yuan ZQ, Mohammed C (2002). *Ceratocystis moniliformopsis sp. nov*., an early colonizer of *Eucalyptus obliqua* logs in Tasmania, Australia. *Australian Systematic Botany* **15**: 125–133