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Sirococcus shoot blight on *Picea spinulosa* in Bhutan

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Summary

During a recent survey of forest tree diseases in Western and Central Bhutan, Sirococcus shoot blight and an associated *Sirococcus* sp. were found on saplings and mature trees of Eastern Himalayan spruce (*Picea spinulosa*). Based on morphological characteristics and DNA sequence comparisons of the ITS region of the rDNA operon, representative isolates from Bhutan were unequivocally identified as *Sirococcus conigenus*. The DNA sequence data also showed that these isolates belong to the P group of *S. conigenus*. To our best knowledge, this is the first report of Sirococcus shoot blight from the Himalayas or any other part of Asia. *Sirococcus conigenus* does not appear to cause dramatic damage at the moment, but this fungus has the potential to cause severe disease problems on *P. spinulosa* in Bhutan.

1 Introduction

In contrast to many other countries in Southern Asia, the Kingdom of Bhutan has maintained the majority of its natural forests. Forests cover 64.2% of the area of Bhutan, and another 8.1% is covered by scrub forests (FAO 1999, 2001). Temperate mixed conifer forests constitute the natural vegetation at elevations between about 2600 and 3300 m a.s.l. (Grierson and Long 1983; Rosset 1999). In mixed conifer stands on dry sites, Eastern Himalayan spruce (*Picea spinulosa* [Griffith] A. Henry) is the dominant conifer species (Rosset 1999). This tree has a relatively limited distribution range located in Sikkim as well as in Western and Central Bhutan (Schmidt-Vogt 1977; Rosset 1999). *Picea spinulosa* is amongst the most economically important tree species for forestry in Bhutan.

Sirococcus shoot blight (SSB) is caused by the anamorphic fungus *Sirococcus conigenus* (DC.) P. Cannon and Minter (syn. *S. strobilinus* G. Preuss). The disease was first described on Norway spruce (*P. abies* [L.] Karst.) in Central Europe (Hartig 1890) and has subsequently been reported on a wide range of conifer hosts (mainly *Picea* spp., *Pinus* spp., *Larix* spp., *Tsuga* spp. and *Pseudotsuga menziesii* [Mirb.] Franco) in Europe and North America (Peace 1962; Sutherland 1987; Butin 1995; Smith et al. 2003; Sinclair and Lyon 2005). There is also one report of SSB from *Pinus halepensis* Mill. in Morocco, North Africa (Morelet 1972). Although *S. conigenus* is

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probably best known as pathogen of seedlings in nurseries (Sutherland et al. 1981; Sutherland 1987; Butin 1995), the disease also occurs in young plantations (Hartig 1890; Halmschlager et al. 2000) and even in mature stands. This is for example on Norway spruce in Central Europe (Hartig 1893; Rudolph 1898; Neumüller 1994; Anglberger 1998; Anglberger and Halmschlager 2003) and on red pine (*Pinus resinosa* Aiton) in the north-eastern USA (Ostry et al. 1990).

Based on Inter Simple Sequence Repeats Polymerase Chain Reaction (ISSR-PCR) markers, rDNA sequences, conidium morphology, growth in culture and cultural characteristics, isolates of *S. conigenus* have recently been separated into two host-related groups, which might represent two different species (Smith et al. 2003). One of these entities is referred to as the T group, which consists of isolates mainly originating from hemlock (*Tsuga* spp.) in Western North America. The other group of isolates has been designated as P group, and it mainly occurs on *Pinus* spp., *Picea* spp. and *Larix* spp. in North America and Europe. Smith et al. (2003) also noted that there are isolates that have previously been identified as *S. conigenus*, but reside in neither the P nor T group. These results suggest that what has been referred to as *S. conigenus* consists of a complex of morphologically similar, yet genetically distinct species.

During a recent survey of forest tree diseases in Western and Central Bhutan (Kirisits et al. 2002; Van Wyk et al. 2004; Coetzee et al. 2005) a shoot disease was commonly encountered on *P. spinulosa*. Only current-year shoots were affected and the symptoms and signs of the disease resembled those described for SSB. The purpose of this study was to confirm the identity of the causal agent of shoot blight on *P. spinulosa*. This was achieved based on morphology and comparisons of DNA sequence data.

2 Material and methods

2.1 Study sites and collection of isolates

Observations on the shoot disease of *P. spinulosa* were made during July 2001, in mixed conifer forests along the road leading from Yusipang (27°28′13″N, 89°42′32″E) up to Dochula (27°29′40″N, 89°45′08″E) (Thimphu dzongkhag, 2700–3200 m a.s.l.), at Jelekha (Thimphu dzongkhag, 27°26′09″N, 89°31′15″E, 3300 m a.s.l.) and at Phobjikha valley (Wangdue Phodrang dzongkhag, 27°30′30″N, 89°47′32″E, 3400 m a.s.l.) (Fig. 1). Isolations of fungi were done by removing conidial masses oozing from pycnidia occurring on diseased shoots and needles with a sterile needle and transferring them to malt extract agar (MEA; 20 g DiaMalt malt extract, Hefe Schweiz AG, Stettfurt, Switzerland; 16 g 'Becoagar' agar, W. Behrens & Co, Hamburg, Germany; 1000 ml tap water), supplemented after autoclaving with 100 mg streptomycin sulphate to suppress bacterial growth. Isolations were also made by placing needles and parts of shoots onto MEA plates after surface sterilization of symptomatic shoots for 1 min in 96% ethanol and subsequently rinsing them once in sterile distilled water. Isolation plates were incubated at about 20°C under artificial light. Pure cultures were obtained by transferring mycelium or conidia to fresh MEA plates. Representative isolates of the shoot blight pathogen on *P. spinulosa* in Bhutan are maintained in the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Austria, and they have also been deposited at the Centralbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). Three isolates from Bhutan (BH-S 1, BH-S 3 and BH-S 8, Table 1) were selected for morphological characterization and DNA sequencing to identify the causal agent of shoot blight on *P. spinulosa*.

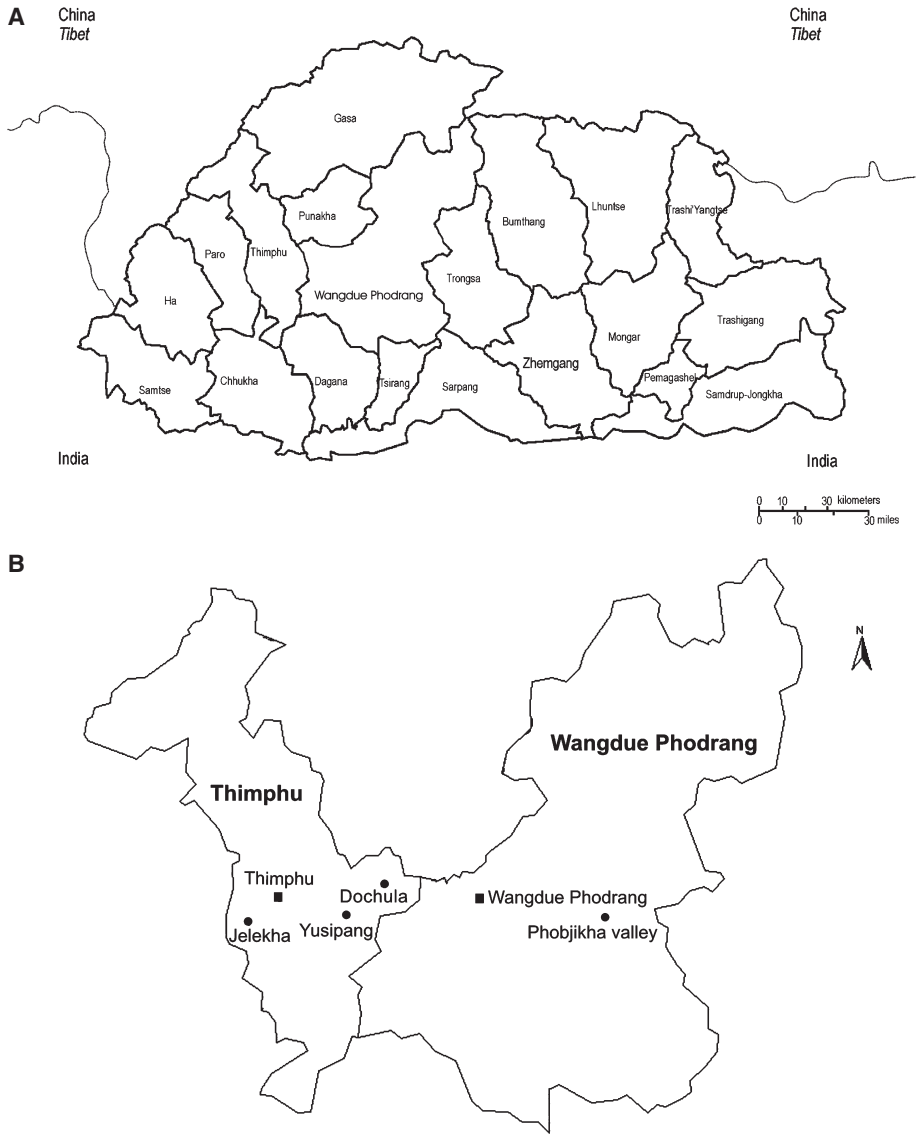

Fig. 1. (A) Map of Bhutan showing the administrative districts (dzongkhags) of the country. (B) Map of the dzongkhags Thimphu and Wangdue Phodrang showing Bhutan's capital Thimphu, the district capital Wangdue Phodrang and the sites (Jelekha, Yusipang to Dochula and Phobjikha valley) where observations on *Sirococcus conigenus* were made

2.2 Conidium morphology

The shape and size of conidia were examined for three isolates associated with shoot blight on *P. spinulosa* in Bhutan, following a similar procedure to that described by Smith et al. (2003). Norway spruce cones, which were free of pycnidia of *S. conigenus* were collected near Vienna, Austria. Cone scales were removed, autoclaved and partly embedded in 1.6%

Table 1. **Isolates of *Sirococcus conigenus* and *Sirococcus clavigignenti-juglandacearum* used for ITS rDNA sequence comparisons**

Sirococcus species and subgroup	Isolate no.	Host	Locality	Year of isolation	Collected/ provided by	Other isolate no.	ITS GenBank accession no.
S. conigenus, P group	BH-S 1	*Picea spinulosa*	Near Dochula, Bhutan	2001	T. Kirisits, D.B. Chhetri	CBS 116475	AY437759
S. conigenus, P group	BH-S 3	*Picea spinulosa*	Near Dochula, Bhutan	2001	T. Kirisits, D.B. Chhetri	CBS 116476	AY437760
S. conigenus, P group	BH-S 8	*Picea spinulosa*	Near Dochula, Bhutan	2001	T. Kirisits, D.B. Chhetri	CBS 116477	AY437764
S. conigenus, P group	621	*Picea abies*	Schöneben, Austria	1998	A. Gabler		AY437757
S. conigenus, P group	632	*Picea abies*	Kobernausserwald, Austria	1995	H. Anglberger		AY437749
S. conigenus, P group	600	*Picea abies*	Bavarian Forest, Germany	1991	A. Wulf	S 289	AY437762
S. conigenus, P group	602	*Picea abies*	Hyytiälä, Finland	1998	A. Uotila		AY437747
S. conigenus, P group	661	*Picea abies*	Uppsala, Sweden	1999	O. Holdenrieder	990622.11	AY437758
S. conigenus, P group	612	*Pinus resinosa*	Nova Scotia, Canada	1973	W. Harrington	FSC-673	AY437756
S. conigenus, P group	618	*Picea glauca*	New Brunswick, Canada	1984	C.M.B. Dobson	FSC-748	AY437745
S. conigenus, T group	01-22[1]	*Tsuga heterophylla*	British Columbia, Canada	Not known	P. Axelrood	9543-4	AY163787
S. conigenus, T group	98-36[1]	*Tsuga heterophylla*	Oregon, USA	Not known	M. Putnam		AY163788
S. clavigignenti-juglandacearum	DB550	*Juglans cinerea*	Berlin, Vermont, USA	1998	D. Bergdahl		AY437754
S. clavigignenti-juglandacearum	DB560	*Juglans cinerea*	Jericho, Vermont, USA	1998	D. Bergdahl		AY437755

[1]These isolates were investigated by SMITH et al. (2003) and their ITS sequences were obtained from GenBank.

Table 2. **Dimensions of conidia[1] of three isolates of *Sirococcus conigenus* from Bhutan under controlled conditions**

	Mean (range) of conidial dimensions[2]		
Isolate	Length (μm)	Width (μm)	Length–width ratio
BH-S 1	11.4 a (8.9–13.9)	2.8 b (2.2–4.0)	4.1 a (2.5–5.7)
BH-S 3	14.2 c (10.9–17.8)	2.5 a (2.0–3.0)	5.8 c (3.9–8.5)
BH-S 8	13.2 b (8.9–18.8)	2.5 a (2.0–3.0)	5.4 b (3.0–9.5)

[1]From each isolate 80 conidia from pycnidia produced on *Picea abies* cone scales partly embedded in water agar were measured after 2 months of incubation at 20°C in the dark. Values refer to water as mounting fluid.
[2]Values (within columns) followed by different letters were significantly different according to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$).

water agar in 9-cm plastic Petri dishes. Each cone scale was inoculated with a small block of MEA bearing mycelium of the respective isolate of *Sirococcus* sp. from *P. spinulosa*. Isolates were grown separately in different Petri dishes. Three dishes (with 6–8 cone scales each) were prepared for each isolate. Plates were incubated for 2 months at 20°C in the dark. Thereafter, numerous pycnidia with droplets of conidia at their apices had formed on the spruce cone scales.

Squash mounts of mature pycnidia in water were prepared and conidia were observed using differential interference contrast microscopy (Photomikroskop Axiophot; Carl Zeiss, Oberkochen, Germany). The lengths and widths of conidia were measured at 1000× magnification using an ocular micrometer. Values of conidium length, width and length/width ratio for each isolate are presented as the mean and the range (minimum–maximum) of 80 measurements (Table 2). Differences in the conidium dimensions (length, width and length/width ratio) between isolates were tested by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. The program SPSS for Windows, version 12.0.1 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

2.3 DNA extraction, ITS amplification and sequencing

DNA sequences for part of the ITS region of the ribosomal DNA operon of three isolates of the *Sirococcus* sp. from Bhutan were compared with those of reference strains of *S. conigenus* from Europe and North America (Table 1). For DNA isolation, strains were grown on MEA (composition as before) overlaid with cellophane for 2 weeks. Fungal mycelium was scraped off the cellophane using a sterile spatula and total genomic DNA was isolated from the fresh tissue by chlorophorm-isoamyl extraction, following the protocol of ZOLAN and PUKKILA (1986). The internal transcribed spacer (ITS) region (including ITS1, 5.8S ribosomal DNA and ITS2) was amplified using primers ITS1 and ITS4 (WHITE et al. 1990). PCR conditions were as described by KONRAD et al. (2002), however, the annealing temperature was set at 55°C. Resulting bands were visualized on a 1% agarose gel stained with ethidium bromide.

PCR-products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hildesheim, Germany) and sequenced using primer ITS4. Reactions were performed on an ABI PRISM 377 automated DNA sequencer, with an ABI PRISM Dye terminator cycle sequence kit (Perkin Elmer Applied Biosystems, Inc., Foster City, CA, USA). Sequences were aligned using the software package CLUSTALX (THOMPSON et al. 1997) with the default settings and manual editing of the alignment afterwards. ITS sequences of two isolates representing the T group of *S. conigenus* (Table 1, Genbank accession nos. AY163787 and AY163788) were included in the data analysis.

Phylogenetic analyses were performed using PAUP version 4.0b10 (SWOFFORD 2001). The optimality criterion was set to parsimony. A total of 545 unordered characters, including gaps, were utilized. Each gap was treated as a fifth character (newstate) in heuristic searches, with tree-bisection-reconnection (TBR) branch swapping and MULTREES (saving of all optimal trees) effective. Ninety-one of the characters were parsimony-informative and 454 were constant. Bootstrap analyses were based on 1000 replications. Two isolates of *Sirococcus clavigignenti-juglandacearum* N. B. Nair, Kostichka & Kuntz from *Juglons cinerea* L. (Table 1) were used as the outgroup in the phylogenetic analyses.

3 Results

3.1 Disease symptoms and signs

Symptoms and signs resembling those of SSB were observed on current-year shoots of *P. spinulosa* trees growing in mixed conifer forests along the road leading from Yusipang to Dochula in Western Bhutan. Symptoms included needle browning at the base or in the middle of current-year shoots, drooping of tips of the shoots as well as needle loss (Fig. 2). Dead needles often remained attached to the tips of dead and otherwise defoliated shoots (Fig. 2). Light brown pycnidia with drops or tendrils of conidial masses were abundant on dying and dead needles and shoots collected in the field. Conidia from these pycnidia resembled those of *S. conigenus* (see SMITH et al. 2003 and references therein).

Both saplings and mature trees of *P. spinulosa* were commonly affected by this shoot blight. Disease incidence appeared to be high. Individual saplings were seriously affected, however, the overall disease severity appeared to be low. On mature trees, shoots showing symptoms were scattered singly or in small groups in an irregular pattern throughout the crown. Besides SSB, needles of current-year shoots were commonly affected by Chrysomyxa needle rust, caused by *Chrysomyxa* sp.

In mixed conifer forests at the two other sites in Western (Jelekha) and Central Bhutan (Phobjikha valley) (Fig. 1) no shoot disease was observed on *P. spinulosa*. However, pycnidia of a fungus resembling *Sirococcus* sp. occurred abundantly on the scales of cones, collected from the forest floor. Sporulation of *Sirococcus* sp. on spruce cones on the ground was also common in forest stands between Yusipang and Dochula.

3.2 Conidium morphology

The shape and size of conidia produced by three isolates (BH-S 1, BH-S 3 and BH-S 8, Table 1) of the shoot blight pathogen on *P. spinulosa* (Table 2; Fig. 3) were consistent with descriptions of *S. conigenus* (e.g. HARTIG 1890; BUTIN 1995; SMITH et al. 2003 and references therein; SINCLAIR and LYON 2005). Conidia were fusiform, usually straight, but occasionally slightly curved, always one-septate and sometimes possessed a slight constriction at the septum (Fig. 3). The three isolates from Bhutan showed statistically significant differences in the length and length/width ratio of conidia (Table 2). Likewise, isolate BH-S 1 had significantly wider conidia than the two other isolates (Table 2).

Mean conidial lengths for two isolates from Bhutan (BH-S 3: 14.2 μm and BH-S 8: 13.2 μm) were larger than those reported by SMITH et al. (2003) for *S. conigenus* P group isolates (range: 11.8–12.5 μm), whereas conidia of the third isolate from Bhutan (BH-S 1: 11.4 μm) were slightly shorter. Isolates from Bhutan had on average longer conidia than isolates belonging to the *S. conigenus* T group (range of mean conidial lengths: 10.4–10.7; SMITH et al. 2003). Mean conidial widths for two isolates from Bhutan (BH-S 3 and BH-S8, Table 2) were within the range of P group isolates of

Fig. 2. Symptoms of Sirococcus shoot blight on *Picea spinulosa* in Bhutan

S. conigenus (2.4–2.7 μm, Smith et al. 2003). However, isolate BH-S 1 had wider conidia (mean: 2.8 μm) than both *S. conigenus* P and T group isolates examined by Smith et al. (2003).

3.3 ITS rDNA sequencing

Sequences of a 545-bp portion of the rDNA, spanning the entire ITS1, 5.8S rDNA and ITS2 (= ITS region) were obtained for all isolates by PCR and DNA sequencing. Sequence divergence between isolates from *P. spinulosa* in Bhutan and *S. conigenus* P group isolates

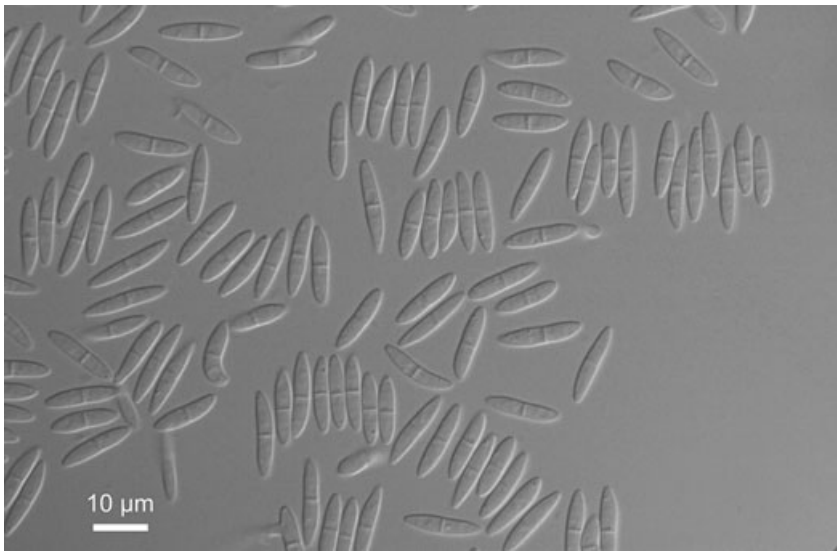

Fig. 3. Fusiform, hyaline, one-septate conidia of *Sirococcus conigenus* isolate BH-S 3 from Bhutan

from Europe and North America was very small (<1%). Phylogenetic analysis placed the isolates from Bhutan within the clade of the P group isolates with strong bootstrap support (Fig. 4). The two isolates from *Tsuga heterophylla* (Raf.) Sarg. in Western North America formed a separate clade, which is representative for the *S. conigenus* T group (Fig. 4).

4 Discussion

Morphological comparisons and phylogenetic analyses of the ITS region of the rDNA operon demonstrated that the causal agent of shoot blight on *P. spinulosa* in Bhutan is *S. conigenus*. Designation of the fungus from *P. spinulosa* to *S. conigenus* was initially indicated based on disease symptoms and signs. Morphological characteristics of the causal agent of the shoot disease on Eastern Himalayan spruce were clearly similar to those described for *S. conigenus*. However, because isolates resembling *S. conigenus* have recently been shown to consist of several groups (Smith et al. 2003), identification based on morphology was confirmed by using DNA-based comparisons.

The DNA sequence comparisons (Fig. 4) showed that the fungus from *P. spinulosa* in Bhutan belongs to the P group of *S. conigenus* and that it is phylogenetically different from isolates forming the T group of this pathogen. Isolates within the P group have formerly been reported to occur mainly on various spruce and pine species in Europe and North America (Smith et al. 2003). The occurrence of isolates of the P group on *P. spinulosa* thus agrees well with the previously known host range of this biological entity of *S. conigenus*.

Measurements of conidia in the present study confirm the finding by Smith et al. (2003) that *S. conigenus* P group and T group isolates can be separated based on conidial size. This is especially true if comparisons are made under controlled conditions and if a large number of conidia per isolate are examined. Conidia of *S. conigenus* P group isolates from Bhutan (Table 2) were longer than those reported by Smith et al. (2003) for T group isolates. Moreover, two of the Bhutanese isolates had longer conidia and the third isolate from Bhutan had slightly shorter conidia (Table 2) than the P group isolates examined by

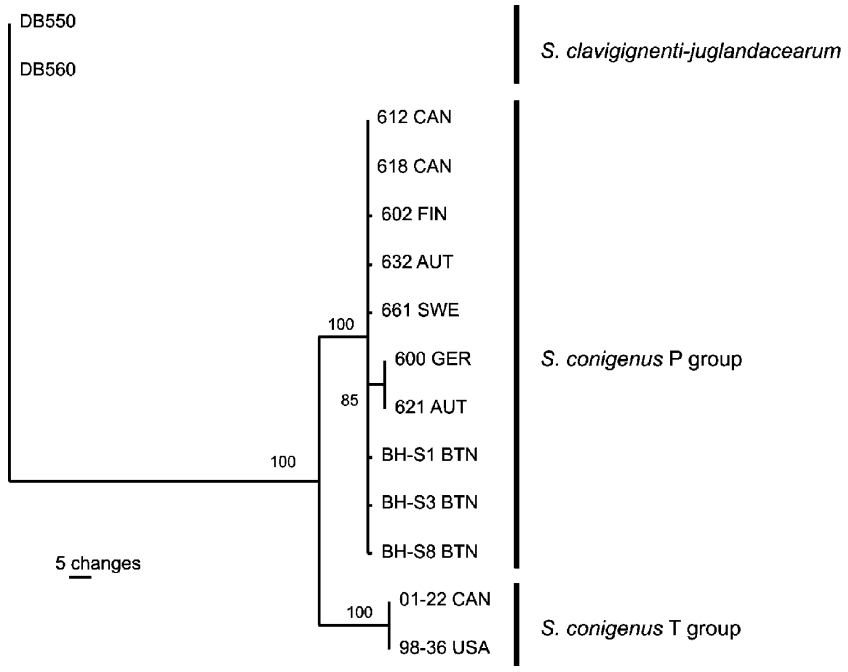

Fig. 4. ITS tree (tree length = 107, consistency index = 0.9346, homoplasy index = 0.0654) derived from maximum parsimony analysis showing two major clades, representing the P group and the T group of *Sirococcus conigenus* according to Smith et al. (2003). Numbers at nodes indicate bootstrap values (only values above 50 are shown). Sequences of T group isolates 01-22 and 98-36 were obtained from GenBank (accession nos. AY163787 and AY163788, respectively)

Smith et al. (2003). This is not surprising, because Smith et al. (2003) included only three *S. conigenus* P group isolates in their morphological studies. Their measurements thus underestimated the breadth of variation in conidial size occurring in this biological entity of *S. conigenus*.

To our best knowledge, this report of SSB and the associated pathogen, *S. conigenus* on *P. spinulosa* in Western and Central Bhutan represents the first record of the disease and the pathogen in the Himalayas or any other part of Asia. The discovery of the pathogen on Eastern Himalayan spruce in Bhutan emphasizes the fact that *S. conigenus* occurs on a wide range of conifers and is widely distributed in conifer forests in the Northern hemisphere (Peace 1962; Morelet 1972; Sutherland 1987; Butin 1995; Smith et al. 2003 and references therein; Sinclair and Lyon 2005). This report of *S. conigenus* on *P. spinulosa* in Bhutan might suggest that the pathogen also occurs on other spruce species and other conifers in the Himalayas and probably also in other parts of Asia. The lack of previous reports of *S. conigenus* from the Himalayas or elsewhere in Asia probably reflects the general low level of knowledge regarding forest tree diseases in these parts of the world. It is also possible that SSB has attracted little attention in the Himalayas, because the disease has not caused notable damage. We expect that reports of SSB from the Himalayas and other regions of Asia may follow this first record of the disease from the continent.

The present observations on SSB are not sufficient to provide a definitive appraisal of the importance of this disease in the conifer forests of Bhutan. Although *S. conigenus* was found in forest stands at three locations, the disease caused by this pathogen was observed

at only one location, in mixed conifer forests along the road from Yusipang to Dochula. Here, SSB occurred at a high level of incidence, but with low disease severity on young and mature trees of *P. spinulosa*. In these mixed conifer forests, foggy and cloudy weather situations are common and we assume that these conditions favour the development of SSB on *P. spinulosa*. Experience in Europe and North America has shown that *S. conigenus* can cause considerable disease problems in nurseries, on natural regeneration, in young plantations and sometimes also in mature stands. This indicates that the pathogen also has the potential to be a problem in Bhutan, particularly in forest nurseries. We, therefore, recommend that the incidence and severity of this disease on *P. spinulosa* and other conifers is monitored in Bhutan. This would provide information facilitating the management of Sirococcus shoot and seedling blight in this Himalayan country.

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