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Neotypification of *Dothistroma septosporum* and epitypification of *D. pini*, causal agents of Dothistroma needle blight of pine

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Summary

Dothistroma needle blight (DNB) is one of the most devastating needle diseases on *Pinus* spp. world-wide. Ever since the description of the causal agent of the disease in Europe in 1911, as *Cytosporina septospora*, and independently in the USA in 1941 as *Dothistroma pini*, there has been considerable taxonomic discordance regarding the name of the pathogen used in literature. This was compounded both by the proposal of different varieties of the pathogen based on differences in spore size and the application of dual nomenclature where three names, *Scirrhia pini*, *Eruptio pini* and *Mycosphaerella pini*, were used to describe the sexual morph of the fungus. More recent studies using sequence-based methods revealed that DNB can be caused by either one of two distinct species i.e. *D. septosporum* and *D. pini*. These important species have not been adequately typified and this perpetuates lack of stability for their names. In this study, these names are fixed to reference sequences linked to living cultures representing type specimens. To achieve this goal, we designate an epitype for *D. pini* and a neotype for *D. septosporum*. The known polymorphism in the ITS region, the barcoding gene for these fungi, is characterised and a complete taxonomic history is provided for the genus *Dothistroma*.

1 Introduction

Dothistroma needle blight (DNB), also commonly known as ‘red-band disease’, ‘red spot’ or ‘red band needle blight’, is one of the most important foliage diseases of *Pinus* spp. world-wide (Gibson 1972; Bradshaw 2004; Drenkhan et al. 2016). Symptoms of the disease include reddish spots or bands surrounding black erumpent conidiomata (acervuli) on necrotic needles. Recent reviews of the disease distribution have shown that it occurs in 76 countries spanning a wide array of geographic and climatic conditions (Drenkhan et al. 2016; Woods et al. 2016). The disease occurs in almost all areas where susceptible pines are found and has been documented on 95 *Pinus* species or their sub-species. Rare and sporadic occurrences of the disease have also been recorded on five non-*Pinus* genera of the Pinaceae including *Abies*, *Cedrus*, *Larix*, *Picea*, and *Pseudotsuga*. But in all these cases, heavily diseased *Pinus* spp. have been in close proximity to those conifers (Drenkhan et al. 2016).

DNB can be caused by either one of two different fungal species, i.e. *D. septosporum* (Dorogin) M. Morelet and *D. pini* Hulbary (Barnes et al. 2004). These two species can be clearly distinguished based on DNA sequence data (Barnes et al. 2004; Ioos et al. 2010). However, before 2004, they were considered as one species and the names were commonly used interchangeably. This confusion in their taxonomy stems from two independent roots of the species name, one in Europe and the other in the USA (see Table 1). In the USA, the asexual state of the pathogen was described by Robert L. Hulbary in 1941 as *Dothistroma pini* (Hulbary 1941). The pathogen had also previously been described as *Actinothyrium marginatum* (Saccardo 1920), *Cryptosporium acicola* (Dearness 1928), *Septoria acicola* (Hedgcock 1929) and it was confused with *Lecanosticta acicola* (Sydow and Petrak 1924), a closely related but distinctly different pathogen that causes brown spot needle blight (Evans 1984).

In Europe, the pathogen causing DNB was first described as *Cytosporina septospora* (Doroguine 1911). As was true in the USA, various names were incorrectly applied to the pathogen including *Brunchorstia pinea* (Doroguine 1926) and *Septoriella septosporum* (Trotter 1931). A complete account of the taxonomic history of these fungi is provided in Table 1.

In the late 1960s, Michel Morelet reduced to synonymy all the names applied in the USA and Europe to the causal agent of DNB and referred to the asexual morph of the pathogen as *Dothistroma septosporum* (as ‘*septospora*’) (Morelet 1968a, 1969). Yet, for more than four decades, both the names *D. septosporum* and *D. pini* were interchangeably used with *D. pini* being preferentially applied in the USA, New Zealand and Africa and *D. septosporum* typically used in Europe (Bradshaw 2004). It was not until Barnes et al. (2004), who applied DNA sequence data to a global collection of isolates showed that DNB is caused by two distinct species and both names, *D. septosporum* and *D. pini*, were retained. An on-going initiative, strongly promoted by the objectives of the DIAROD EU COST Action FP1102 (Determining Invasiveness And Risk Of Dothistroma, http://www.cost.eu/COST_Actions/fps/Actions/FP1102?parties) is now in place to continually use molecular methods to correctly identify the species of *Dothistroma* reported in old literature and to establish the current global distribution of both pathogens (Drenkhan et al. 2016).

Recent advances in fungal taxonomy have led to the abandonment of the dual nomenclature system for pleomorphic fungi (Hawksworth et al. 2011; Hawksworth 2015). This has led to a situation where the application of names for both sexual and asexual morphs is no longer appropriate. As a consequence, the ‘One Fungus = One Name’ (1F1N) concept is in the process of being implemented (Taylor 2011; Wingfield et al. 2012) and where entire genera are being reclassified with single names being fixed to type species (Crous et al. 2014; Wijayawardene et al. 2014; Rossman et al. 2015). The names for the sexual morphs of the DNB pathogens, described as *Scirrha pini* (Funk and Parker 1966), *Mycosphaerella pini* (Munk 1957) and *Eruptio pini* (Barr 1996) are no longer appropriate and the asexual genus name *Dothistroma* has been retained for both the DNB pathogens (Quaedvlieg et al. 2012).

DNA barcoding has been established to advance and streamline the molecular identification of fungal species and the discovery of potentially new species (Quaedvlieg et al. 2012; Schoch et al. 2012; Stielow et al. 2015). The barcoding region considered most appropriate for fungi is the Internal Transcribed Spacer (ITS) region due to its robust amplification success and the extensive databases that are currently available for this region (Schoch et al. 2012, 2014; Stielow et al. 2015).

The two pathogens causing DNB have very similar morphology and they give rise to the same disease symptoms (Barnes et al. 2011). Consequently, the ITS region is being used to effectively distinguish between them (Piškur et al. 2013; Tsopelas et al. 2013; Queloz et al. 2014). Not surprisingly, diagnostic methods using ITS-RFLP have also been developed to rapidly distinguish between *D. pini* and *D. septosporum* (Barnes et al. 2004; Pehl et al. 2004). However, point mutations in the ITS region of both *D. septosporum* (Mullett and Fraser 2015) and *D. pini* have recently been reported (Barnes et al. 2014). It is consequently not yet known whether the restriction sites used in diagnostic protocols have been affected in these new haplotypes.

Descriptions of the majority of new fungal species being described are supported with DNA sequence data, commonly for more than one gene region. It has consequently become imperative to have DNA sequence data linked to type specimens in order to validate already described species. In this regard, an important challenge is that cultures linked to appropriate type material are often not available; or DNA cannot be extracted from inordinately old fungarium material. This problem can be circumvented by neo-typification, or in the case of living cultures not being available, epi-typification (Ariyawansa et al. 2014).

The original fungarium material of *Cytosporina septospora* collected by Georges Doroguine in 1910 from *P. mugo* Turra subsp. *mugo* (syn. *P. montana* Mill.) in Saint Petersburg, Russia (Doroguine 1911) has been lost. According to the curators of these herbaria, it is neither maintained at the Komarov Botanical Institute of the Russian Academy of Sciences, Saint Petersburg (formerly, Leningrad, LE) nor at the All-Russian Research Institute of Plant Protection (LEP). This implies that the name-bearing type material for *D. septosporum* is no longer available. Although the type material of *D. pini*, collected by James C. Carter in Illinois in 1938 is available, repeated attempts to amplify the ITS region from conidiomata on this specimen have not been successful (Barnes et al. 2004). A serious situation thus exists where there is no appropriate type material or cultures to allow for robust DNA-based classification of *Dothistroma* species, or indeed other members of this genus.

The purpose of this study was to provide a neotype for *D. septosporum* and to designate an epitype for *D. pini* for which cultures and sequence data are available. These strains can then be used as the authentic material for all future morphological and DNA-based comparative studies on *Dothistroma*. Since the ITS region is used as the barcoding gene for the genus, our aim was to characterise the different haplotypes found in the ITS region of both species and provide an ITS map for easy annotation. Lastly, we have provided a complete taxonomic history for *Dothistroma* and the DNB pathogens.

2 Material and methods

2.1 Isolates

In Russia, four sampling areas were chosen for the collection of possible neotype material for *D. septosporum*. All needle samples were collected by Rein Drenkhan and Dmitry L. Musolin in November 2013. The first site (59.991°N, 30.344°E), was the park of Saint Petersburg Forestry Institute, in Lesnoj (now St. Petersburg State Forest Technical University), where Georges Doroguine collected symptomatic needles in 1910 and then described *Cytosporina septospora* (Doroguine 1911, 1912; Fig.1). Although typical DNB symptoms were not found, several needles were collected from various conifer species and the species-specific conventional PCR

(Ioos et al. 2010) was used to directly screen the plant material for the presence of the pathogens.

A second sampling site in St. Petersburg was ca. 3 km from the first site, in Park Sosnovka (60.02278°N, 30.35167°E). This 360 ha area was a natural pine forest used as a recreational area before it was established as a city park in 1960. Needle samples were collected from nine symptomatic local, but planted, *P. sylvestris* and two *P. mugo* trees, ca. 10–15 years old.

The third and fourth sampling sites were from two natural pine stands 30 km from St. Petersburg. At the third location (60.28500°N, 29.81200°E), samples were collected from 19 symptomatic *P. sylvestris* trees 15–20 years old. At the fourth sampling location (60.20060°N, 29.96010°E), 13 symptomatic *P. sylvestris* trees 10–15 years old were sampled. At all sampling sites, 2–3 year-old needles were collected from the lower parts of the tree canopies and needle samples from different trees were placed in separate sterile plastic bags.

In seeking an epitype for *D. pini*, it was not possible for the authors to sample in De Kalb County, northern Illinois where James C. Carter collected the material on *P. nigra* subsp. (var.) *austriaca* in 1938 and from which Robert L. Hulbary made his original descriptions of the genus *Dothistroma* and the species, *D. pini* (Hulbary 1941). However, needle samples collected in 2001 by Gerry Adams from *P. nigra* in Stanton, Montcalm County, Michigan, and cultures generated from this material (Barnes et al. 2004) were used for this purpose.

Most of the remaining isolates used in this study were made from symptomatic needles of different pine species collected from various countries during the course of the last few years (Table 2). Single conidial isolations from mature conidiomata were made from all needle collections following the methods described by Mullett and Barnes (2012), on 2% *Dothistroma* Sporulating Media (DSM: 20 g malt extract, 5 g yeast extract and 15 g agar) supplemented with 100 mg/L streptomycin (Sigma-Aldrich, St. Louis, USA). Additional cultures were also obtained from international culture collections (Table 2). All cultures for DNA isolation were grown on DSM at 21°C at natural day/night light intervals for 3–4 weeks.

2.2 DNA isolation, amplification and analyses

For DNA extractions, mycelium obtained from cultures growing on DSM plates was

freeze-dried overnight and ground into a powder using the Retsch GmbH MM301 homogenizer (Haan, Germany). Total DNA was extracted from (30 mg) ground mycelia using the Zymo Research Fungal DNA MiniPrep kit (Irvine California, USA) and eluted in a volume of 50 μ L. DNA concentrations were measured using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer (Wilmington, DE, USA) and diluted to a working stock of 20 ng/ μ L.

The ITS barcoding region was amplified using the primers ITS1 and ITS4 (White et al. 1990). Each PCR reaction mix included 2.5 μ L of 10 $\times$ FastStart PCR buffer, 2.5 μ L MgCl₂ (25 mM), 0.2 μ L of FastStart Taq polymerase (5 U/ μ L) (Roche Diagnostics, Indianapolis, USA), 2 μ L dNTP mix (10 mM), 0.5 μ L of each forward and reverse primer (10 mM), 1 μ L of DNA and dsH₂O to a total volume of 25 μ L. PCR reactions were run on a Bio-Rad iCycler thermocycler (BIO-RAD, Hercules, CA, USA) with the following thermal cycling conditions: 1 cycle at 95°C for 4 min, 10 cycles of 95°C for 20 s, 56°C for 45 s, 72°C for 45 s, 25 cycles of 95°C for 20 s, 56°C for 45 s (with an increase of 5 seconds after each cycle), 72°C for 45 s, followed by a final extension cycle at 72°C for 10 min. For each sample, 5 μ L of PCR amplicon was electrophoresed on 2% agarose gel (Merck, Darmstadt, Germany) with 2 μ L GelRed™ (Biotium, California) and visualised under UV light using the GelDoc™ EZ Imager (BioRad, Johannesburg, South Africa). Amplicons were purified using G-50 sephadex (SIGMA-Aldrich, Steinheim, Germany) in Centri-sep Spin Columns (Princeton separations Inc., Adelphia, USA).

ITS PCR amplicons were sequenced in both directions using the ABI PRISM™ Big Dye ready reaction kit (Applied BioSystems, Foster City, CA, USA). Sequencing reactions consisted of 0.5 μ L Big Dye reaction mix, 2.1 μ L 5x Big Dye sequencing buffer, 0.5 μ L primer, 60–100 ng amplified PCR product and dsH₂O to a total volume of 12 μ L. Cycling conditions included 1 cycle of 96°C for 10 s and 35 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Sequencing reactions were run on an ABI PRISM™ 3500xl capillary autosequencer (Applied BioSystems).

Forward and reverse sequences were assembled in CLC MAIN WORKBENCH V. 6.6.2 (CLC Bio, www.clcbio.com). To validate the correct orientation and annotation of the ITS sequences for this, and future studies (Nilsson et al. 2014), and to identify the different haplotypes, an ITS map for *Dothistroma* was constructed in CLC MAIN WORKBENCH V. 6.6.2. The full length of the 18S, ITS1, 5.8, ITS2 and 28S sequence of the *D. septosporum* strain NE1 was obtained from Scaffold 18, downloaded from

the Joint Genome Institute (JGI) website (De Wit et al. 2012; <http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html>), and used as the reference strain. The different ITS haplotypes of *D. pini* and *D. septosporum* were identified and annotated using CLC MAIN WORKBENCH V. 6.6.2.

For the phylogenetic analyses, all sequences were aligned using the online version of MAFFT VERSION 7 (Kato and Standley 2013; <http://mafft.cbrc.jp/alignment/server/>) with default settings. Alignments were manually checked and adjusted in MEGA (Tamura et al. 2013). Sequences for the outgroup taxa were downloaded from the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) (Table 2). *Stromatoseptoria castaneicola* and *Amycosphaerella africana* (syn.: *Mycosphaerella ellipsoidea* and *M. africana* respectively) were included in the alignments as they are phylogenetically the most closely related taxa to *Dothistroma* (Quaedvlieg et al. 2012, 2013). *Sphaerulina rhabdoclinis* was included as it was previously considered a member of *Dothistroma* as *D. rhabdoclinis* (Butin et al. 2000; Quaedvlieg et al. 2013). All known *Lecanosticta* species were also included in the analyses as a consequence of their commonly being confused with *Dothistroma* spp. due to similar disease symptoms and morphological characters (Table 1).

Phylogenetic analyses included Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). The MP analysis was conducted using the software package PAUP* VERSION 4.0b10 (Swofford 2003). Gaps were treated as a fifth character state and 1000 random stepwise addition heuristic searches were performed with tree-bisection-reconnection (TBR) selected as the branch-swapping algorithm. The Consistency Index (CI), Homoplasy Index (HI), Rescaled Consistency index (RC), Retention Index (RI) and Tree Length (TL) were recorded for the resulting trees. The branch node confidence levels were estimated by performing 1000 bootstrap replicates.

For both likelihood methods (ML and BI), the best fit substitution models for the data set were determined using JMODELTEST VERSION 0.1.1 (Posada 2008). Maximum likelihood analysis was performed with the program PHYML VERSION 3.0 (Guindon and Gascuel 2003) and the confidence levels for nodes were estimated with 1000 bootstrap replicates.

The BI analysis was conducted in MRBAYES VERSION 3.1.2 (Ronquist et al. 2012) by applying the Markov Chain Monte Carlo (MCMC) method. Four

independent MCMC chains were randomly initiated and run for six million generations, applying the best substitution model determined with JMODELTEST VERSION 0.1.1. Trees were sampled every 100 generations. TRACER VERSION 1.6 (Rambaut et al. 2014) was used to determine burn-in values by comparing the log likelihoods and trees sampled in the burn-in phase (10%) were discarded. The remaining trees were used to construct majority rule consensus trees and to determine posterior probabilities for the tree topology.

2.3 Morphology

For morphological observations, cultures were sub-cultured onto 2% DSM and Spezieller Nährstoffarmer agar (SNA), and incubated at 18°C for two weeks under ultraviolet light to induce sporulation. Slide preparations were made by mounting fungal material in clear, 80% lactic acid and morphological structures were observed using a Zeiss Axioskop microscope (Carl Zeiss, Germany). Images were captured electronically using a Zeiss Axio Vision (Carl Zeiss) camera system and software. Type specimens were deposited in MycoBank (www.MycoBank.org).

3 Results

3.1 Isolates

All attempts to isolate *Dothistroma* from the pine needles collected in the Saint Petersburg State Forest Technical University Park, where the original description of *Dothistroma* was made, were unsuccessful. However, screening conifer needles from 12 different species with species-specific primers as described in Ioos et al. (2010), confirmed the presence of *D. septosporum* in *Pinus sibirica*, *P. ponderosa* and *Pseudotsuga menziesii*.

Successful isolations were made from symptomatic pine material collected at Park Sosnovka and from the two locations near St. Petersburg, but these were only from *P. sylvestris* trees. Pine needles from a single *P. sylvestris* tree, collected at Park Sosnovka, and the associated culture obtained from these needles provided the material to neotypify *D. septosporum* (see Taxonomy section below, Table 2).

All cultures generated in this study are maintained in the culture collection

(CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative reference strains and pine needle specimens were deposited in international culture collections and fungaria including the CBS-KNAW Fungal Biodiversity Centre in Utrecht, The Netherlands, and the Mycological Herbarium of the Estonian University of Life Sciences (TAAM; <http://nataarc.ut.ee/seenekogud.php>) (Table 2).

3.2 DNA isolation, amplification and analyses

Amplification of the ITS region (part of the nuclear rDNA region) in *Dothistroma* generated PCR products in the range of 535–536 bp (Fig. 2). The complete sequenced fragments included 30 bp of the 3' end of the 18S nrRNA gene (SSU), 146–147 bp of the internal transcribed spacer 1 (ITS1), 158 bp of the 5.8S nrRNA gene, 144 bp of the internal transcribed spacer 2 (ITS2) and 58 bp of the 5' end of the 28S nrRNA gene (LSU) (Fig. 2).

Four different ITS haplotypes were identified in *D. septosporum* (Ds_HAP.1, Ds_HAP.2, Ds_HAP.3, Ds_HAP.4), and five in *D. pini* (Dp_HAP.1, Dp_HAP.2, Dp_HAP.3, Dp_HAP.4, Dp_HAP.5) based on either point mutations or single nucleotide insertions (Table 2; Fig. 3). To construct the ITS map for *Dothistroma*, the sequence of *D. pini* haplotype 4 (Dp_HAP.4) was used (Table 2). This isolate from France contained an extra A in position 75 thus making it the longest ITS fragment. Polymorphisms were observed at 11 sites in the ITS fragment (Fig. 3), eight of which were found in the ITS1 region and three in the ITS2 region. Of these polymorphisms, four were fixed and distinct between *D. septosporum* and *D. pini* (see sites at bp 99, 146, 349, and 472) and were used to define the species. The polymorphism at site 349, a G in *D. septosporum* and an A in *D. pini*, gave rise to the *AluI* restriction site in *D. pini*, which can also be used to distinguish between the two species using an ITS-RFLP method (Barnes et al. 2004). This restriction site was maintained regardless of the mutations observed in the different haplotypes within the two species.

For the phylogenetic analyses, the final dataset consisted of 59 taxa with 547 aligned nucleotides, including gaps. In the MP analysis, 367 characters were constant, 72 characters were parsimony-uninformative and 108 characters were parsimony-informative. The CI, HI, RC, RI, and TL were 0.936, 0.064, 0.906, 0.968 and 233 respectively. After the heuristic search, two trees were retained of which one was

chosen for presentation (Fig. 4). The best fit substitution model for ML and BI was selected by Akaike Information Criterion (AIC) and was TIM2 (Posada 2008) with rate variations among sites (+G). Because the MP, ML and BI analyses all resulted in similar tree topologies, significant bootstrap support (for MP and ML) and posterior probabilities (for BI), are all indicated on the branches of the MP tree (Fig. 4).

Ds_HAP.1 was the most frequent haplotype (88%) found in *D. septosporum* (Fig. 4). The other three haplotypes were represented by only 1–2 individuals each. Ds_HAP.2 is represented by an isolate of *D. septosporum* that was obtained from *Cedrus atlantica* subsp. *glauca* and has a 1 bp difference from Ds_HAP.1 at site 159 (Fig. 3). Isolates from the USA had the most variable number of *D. septosporum* haplotypes (three of the four). This was also true for the *D. pini* haplotypes where three out of the five haplotypes of *D. pini* occurred in the USA isolates. The sequences of haplotypes represented by only one individual were double-checked to ensure they had not been generated as a result of a sequencing error. All ITS sequences generated in this study were deposited in GenBank (Table 2).

3.3 Taxonomy

The morphological and culture characteristics of the isolates from Russia, St. Petersburg, Park Sosnovka (CMW 44656 and CMW 44657; Fig. 6) were the same as those described for *D. septosporum* in Barnes et al. (2004). In addition, the regions sequenced confirmed the identity of these isolate as *D. septosporum* (Fig. 4). The morphological characteristics and DNA sequence data of isolate *D. pini* isolate CMW 10951 were previously presented in Barnes et al. (2004) and were available for this study (Fig. 5). These results allowed us to designate a neotype for *D. septosporum* and an epitype for *D. pini*. These typifications are described below.

Classification: *Dothistroma*, Mycosphaerellaceae, Capnodiales, Dothideomycetidae, Dothideomycetes, Pezizomycotina, Ascomycota, Fungi

Dothistroma pini Hulbary, Bull. Ill. St. Nat. Hist. Surv. 21: 235. 1941. **Figs 3-5**, (type of the genus).

366

367 See Barnes et al. (2004) for a full description of *D. pini* based on isolate CBS 116487.

368 **Holotype:** USA, northern Illinois, De Kalb County from *P. nigra* subsp. (var.)

369 *austriaca*, 29 November 1938, J. Cedric Carter, MBT128093, herb. ILLS 27093,

370 herb. CBS H-12211 (= isotype).

371

372 **Epitype designated here:** USA, Michigan, Montcalm County, Stanton, Evergreen

373 Township, from *Pinus nigra*, 2001, G. Adams, MBT62987, herb. CBS H-12211,

374 culture ex-epitype CMW 10951 = CBS 116487.

375

376 **Notes:** No ex-type cultures are available from the holotype material and DNA could

377 not be recovered from the herbarium material. The epitype designated here represents

378 *Dothistroma pini* ITS haplotype 1 (DP_HAP.1) (Figs. 3, 4, Table 2). Sequences

379 available on GenBank: Genome (PRJNA212510), ITS (AY808302), BT1

380 (AY808197), BT2 (AY808232), TEF1 α (AY808267).

381

382 *Dothistroma septosporum* (Dorogin) M. Morelet (as '*septospora*'), Bull. Soc. Sci.

383 Nat. Archéol. Toulon Var. 177: 9. 1968. **Figs. 3, 4, 6.**

384 *Basionym.* *Cytosporina septospora* Dorogin, Bull. Trimestriel Soc. Mycol. France 27:

385 106. 1911.

386 = *Septoriella septospora* (Dorogin) Sacc. apud Trotter, Syll. Fung. 25: 480. 1931.

387 = *Septoria septospora* (Dorogin) Arx, Proc. Kon. Ned. Akad. Wetensch. C 86, 1:

388 33. 1983.

389 = *Actinothyrium marginatum* Sacc., Nuovo Giorn. Bot. Ital. 27: 83. 1920.

390 = *Mycosphaerella pini* Rostr., in Munk, Dansk Bot. Ark. 17(1): 312. 1957.

391 = *Eruptio pini* (Rostr.) M. E. Barr, Mycotaxon 60: 438. 1996.

392 = *Dothistroma pini* var. *lineare* Thyr & C. G. Shaw (as '*linearis*'), Mycologia 56:

393 107. 1964.

394 = *Dothistroma septosporum* (as '*septospora*') var. *lineare* (Thyr & C. G. Shaw) B.

395 Sutton, The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata

396 (Kew): 173. 1980.

397 = *Scirrhia pini* A. Funk & A. K. Parker, Canad. J. Bot. 44: 1171. 1966.

398 = *Mycosphaerella pini* (A. Funk & A. K. Parker) Arx, Proc. Kon. Ned. Akad.
 399 Wetensch., Ser. C 86(1): 33 (1983) (homonym, nom. illegit., Art. 53).

400 = *Dothistroma pini* var. *keniense* M. H. Ivory (as “*keniensis*”), Trans. Brit. Mycol.
 401 Soc. 50: 294. 1967.

402 = *Dothistroma septosporum* (as ‘*septospora*’) var. *keniense* (M. H. Ivory) B. Sutton,
 403 in Sutton, The Coelomycetes. Fungi imperfecti with pycnidia acervuli and stromata
 404 (Kew): 174. 1980.

405
 406 See Barnes et al. (2004) for a full description of *D. septosporum* based on isolate CBS
 407 116488.

408 **Holotype:** *Cytosporina septospora* Dorogin, Lesnoj, near to St. Petersburg, *Pinus*
 409 *montana* Mill., summer 1910, G. Doroguine.

410
 411 **Neotype designated here:** Park Sosnovka, St. Petersburg, Russia, from planted but
 412 native *P. sylvestris*, 14 November 2013, R. Drenkhan and D. L. Musolin,
 413 MBT202423, herb. CBS H-22299, culture ex-neotype CMW 44656 = CBS 140339 =
 414 TAAM 168554A.

415 **Notes:** The herbarium material of the holotype has been lost from the Cryptogamic
 416 herbarium of the Komarov Botanical Institute, St. Petersburg, and no longer exists. It
 417 is also not preserved at LEP. A neotype is designated here and represents *Dothistroma*
 418 *septosporum* ITS haplotype 1 (DS_HAP.1) (Figs. 3, 4, Table 2). Ex-neotype
 419 sequences available on GenBank: ITS (KU948400), BT1 (KX364412), BT2
 420 (KX364411), TEF1 α (KX364410).

421 **Other specimens examined:** **Russia**, Park Sosnovka, St. Petersburg, from planted but
 422 native *P. sylvestris*, 14 November 2013, R. Drenkhan and D. L. Musolin, herb. CBS
 423 H-22300, culture CMW 44657 = CBS = TAAM 168554A; **Russia**, near St.
 424 Petersburg, from natural pine stand of *P. sylvestris*, 13 November 2013, R. Drenkhan
 425 and D. L. Musolin, herb. CBS H-22301, culture CMW 44658 = CBS 140340 =
 426 TAAM 168555; **Russia**, near St. Petersburg, from natural pine stand of *P. sylvestris*,
 427 13 November 2013, R. Drenkhan and D. L. Musolin, herb. CBS H-22302, culture
 428 CMW 44659 = CBS 140684 = TAAM 168552.

4 Discussion

The results of this study have made it possible to provide reliable specimens on which the names *D. septosporum* and *D. pini* can be stabilised in the future. The lack of such material arose for a number of reasons including the long-standing and confused taxonomy of these fungi. Both species were described before molecular genetic tools were available to provide insights into species boundaries and have consequently lacked DNA barcodes (Doroguine 1911; Hulbary 1941; Schoch et al. 2012). The need to neotypify *D. septosporum* has been recognized for many years and dates back to the time when Morelet (1968a) provided a new combination of *D. septosporum*. Because the holotype material has been lost, proposals to establish a neotype based on material linked to Doroguine's original collection were recommended (Morelet 1968a). This material, labeled as *Cytosporina septospora* Dorogin, was collected in Ukraine, Kiev Guberniya, in the town of Smiela, from *P. sylvestris* on the 25 March, 1914, by L. Kaznowski (LE 116244, herb. CBS 11381). DNA could not be obtained from this specimen and it was thus not considered as appropriate material for the present study. Similarly, DNA could not be extracted from the holotype material of *D. pini* (Hulbary 1941; Barnes et al. 2004). In this study, we were able to fix the application of the names by generating DNA barcodes for the neotype designated here for *D. septosporum* and the epitype for *D. pini* after appropriate fresh specimens had been collected. Ex-neotype and ex-epitype cultures have also been secured and these can be used in future comparative studies that should ensure taxonomic stability of two of the world's most important pine pathogens.

A search on Index Fungorum (<http://www.indexfungorum.org/Names/Names.asp>) shows that in the 120 years during which the fungi causing the red-band needle disease have been studied, five different species have been linked to the name *Dothistroma*. The taxonomy of the pathogens causing the disease is beset with confusion that has been exacerbated by the lack of easily accessible or available literature, often compounded by problems such as language barriers. This is evident throughout the history of the pathogen as outlined in Table 1.

One of the most vivid examples illustrating the problems relating to the

taxonomy of *Dothistroma* is found in the description of *Dothistroma flichianum*. In 1896, Jean P. Vuillemin produced a detailed description of a fungus that causes red-band symptoms on pine (Vuillemin 1896). He established a new genus, *Hypostomum* to accommodate this fungus and named the pathogen *Hypostomum flichianum* Vuill. The epithet '*flichianum*' honored M. Fliche who collected the material during 1860 from infected *P. austriaca* and *P. mugo* subsp. *mugo* (syn. *P. montana*) in the Champfêtu woods, Theil-sur-Vanne, close to Sens (Yonne), France. This literature remained in complete obscurity for nearly 70 years until Morelet (1980) established the new combination *Dothistroma flichianum* (Vuill.) M. Morelet (as '*flichiana*') (= *Hypostomum flichianum* Vuill), but without any explanation (Morelet 1980).

The genus *Hypostomum* is monotypic and older than *Dothistroma* (Vuillemin 1896; Hulbary 1941). Consistent with the rules of the *International Code of Botanical Nomenclature* (ICBN) at that time, *Dothistroma* should have been reduced to synonymy under *Hypostomum*. It is not known why Morelet retained the name *Dothistroma* instead. The only possible explanation is that there was no type material available for *Hypostomum* for comparison, and the taxonomy of this genus could thus not be confirmed. It does not serve any purpose at this point in time to revert the current name of *Dothistroma* back to *Hypostomum* because it would cause substantial confusion amongst plant pathologists. The decision made here is to retain the name *Dothistroma*, which is now a well-established genus name applied to globally important tree pathogens. Although it is highly likely that *D. flichianum* represents *D. septosporum* (or *D. pini*), it is not possible to validate this fact. In the absence of material linked to the name, the species *Dothistroma flichianum* will remain a taxonomic obscurity. However, in order to ensure long-term stability a formal application to conserve *Dothistroma* and the species name '*septosporum*' linked to it, is currently underway.

The names of the two other *Dothistroma* species listed in Index Fungorum (*Dothistroma acicola* and *Dothistroma rhabdoclinis*) are no longer accepted (Table 1). *Dothistroma acicola* (Thüm) Schischkina & Tsanova was reduced to synonymy with the brown spot fungus as *Lecanosticta acicola* (Thüm.) Syd (Quaedvlieg et al. 2012). *Dothistroma rhabdoclinis*, originally described as a hyperparasite of *Rhabdocline pseudotsugae* on Douglas fir (*Pseudotsuga menziesii*) (Butin et al.

2000), was transferred to *Sphaerulina* based on multigene DNA sequence data (Quaedvlieg et al. 2013). As a consequence of the dual nomenclature for fungi being abandoned (Hawksworth et al. 2011), all names associated with the teleomorph/sexual morph of *Dothistroma*, including *Mycosphaerella pini* (Munk 1957), *Scirrhia pini* (Funk and Parker 1966), *Scirrhia pini* var. *pini* (Morelet 1968b), *Scirrhia pini* var. *galliensis* (Morelet 1968b) and *Eruptio pini* (Barr 1996) (Table 1) are redundant and, therefore, obsolete. The rejection of all varietal names based on morphology by Evans (1984) and phylogenetic inference by Barnes et al. (2004), results in only two valid species in the genus *Dothistroma* with one name each: *D. pini* and *D. septosporum*.

The two pathogens causing DNB have very similar morphology, produce disease symptoms that are indistinguishable, and in some cases they have been found infecting the same needle (Barnes et al. 2008b; Ioos et al. 2010). The only reliable means to differentiate between *D. septosporum* and *D. pini* is by applying DNA-based molecular methods (Barnes et al. 2004; Groenewald et al. 2007; Ioos 2010). These methods include DNA sequence comparisons for the ITS, β -tubulin and Elongation factor (EF1- α) gene regions (Barnes et al. 2004, 2011), amplification using species-specific mating type markers (Groenewald et al. 2007), conventional and real-time PCR markers (Ioos et al. 2010), and ITS-RFLPs (Barnes et al. 2004; Pehl et al. 2004).

The ITS region is currently the most widely used gene region to distinguish between the DNB pathogens (Hanso and Drenkhan 2008; Piškur et al. 2013; Tsopelas et al. 2013; Queloz et al. 2014; Rodas et al. 2016). In the present study, with the annotated ITS map generated, we have identified at least nine different ITS haplotypes in these pathogens. Our investigations of the positions of the point mutations have shown that the phylogenetic species concept remains sound for these species based on at least three fixed polymorphisms (Figs. 3, 4). Although variations exist in eight other positions, mainly in the ITS1, none of the known polymorphisms disrupt the *AluI* restriction site and this can still be used with confidence for ITS-RFLP diagnostic purposes (Barnes et al. 2004).

Nothing is known regarding the variability in pathogenicity of the different *Dothistroma* ITS haplotypes; neither is anything known about the difference in pathogenicity between the two DNB pathogens. Preliminary studies have, however, shown that different strains of *D. septosporum* produce varied levels of dothistromin (Bradshaw et al. 2000), a toxin that has been shown to be a virulence factor during

infection (Kabir et al. 2015). McTaggart et al. (2016), have recently alluded to the fact that name-based taxonomy fails to provide an adequate knowledge base for biosecurity. They emphasise the fact that there is a pressing need to reconsider how quarantine and biosecurity issues are considered and that genotypes rather than species names need to be considered more seriously. This is highlighted by the fact that a unique ITS haplotype of *D. septosporum* was isolated from a non-pine species, *Cedrus atlantica* var. *glauca* (Mullett and Fraser 2015).

Currently, lists of species names are utilized by phytosanitary services to implement biosecurity measures and it is essential that these remain up-to-date with current taxonomic changes. *D. septosporum* has, for example, been on the EU Annex II/A2 list since 1992 and the IAPSC A2 list since 1989 (EPPO 2016), but there is still no quarantine status for *D. pini*. This is despite the fact that this pathogen has been clearly defined since 2004 (Barnes et al. 2004). The results of this study show that plant material having haplotypes (genets), of either *D. septosporum* or *D. pini*, different to those present in a country should be actively excluded by quarantine services. In this case, quarantine measures should especially target countries where many different haplotypes of these pathogens occur. Accidental introductions of new haplotypes could pose a serious risk for local populations of *Pinus* spp. with potentially serious economical outcomes for commercial forestry (Wingfield et al. 2015).

Substantial efforts are currently underway to clarify the different geographic locations and host ranges of the two *Dothistroma* species responsible for DNB (Drenkhan et al. 2016; <http://arctis.mendelu.cz/monitoring/>). This study contributes to this goal in providing DNA data confirming the presence of *D. septosporum* from 11 new geographical locations in six countries (Denmark, England, Russia, Scotland, Spain, and the USA) and *D. pini* in the Czech Republic and Romania. The DNA sequence data linked to type material of *D. septosporum* and *D. pini* emerging from the present study will also contribute substantially to future studies on *Dothistroma* and will provide a sound basis for molecular comparisons between species and genotypes. This is essential where phylogenetic analyses are conducted or where new species descriptions are being considered.

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- 814
 815
- 816 (Legend for figure 4 below that was not captured during PDF building on the online
 817 submission)
- 818
- 819 *Fig. 4.* The most parsimonious tree representing the four different haplotypes of
 820 *Dothistroma septosporum* and five of *D. pini* generated from the ITS region. MP
 821 bootstrap support (>70%) are indicated above branches while ML, below branches.
 822 Bold branches indicate BI values > than 0.95. *Lecanosticta* species were used as the
 823 outgroup taxon. All represented type species are indicated with a T.

Cytosporina septospora nov. spec.

Справа—больная хвоя, въ серединѣ—поперечн. разрѣзъ черезъ плодовместилища гриба и больную хвою (увелич. 450 разъ), слѣва одна камера плодовместилища со спорами (увелич. 600).

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	75	86	99	129	146	159	161	164	349	401	472
Ds_HAP.1	-	T	C	C	T	T	A	G	G	A	-
Ds_HAP.2	-	T	C	C	T	C	A	G	G	A	-
Ds_HAP.3	-	T	C	T	T	T	A	G	G	A	-
Ds_HAP.4	-	T	C	C	T	T	A	G	G	C	-
Dp_HAP.1	-	T	T	C	A	T	A	G	A	A	T
Dp_HAP.2	-	T	T	C	A	T	G	G	A	A	T
Dp_HAP.3	-	C	T	C	A	T	A	G	A	A	T
Dp_HAP.4	A	T	T	C	A	T	A	G	A	A	T
Dp_HAP.5	-	C	T	C	A	T	A	C	A	A	T

efp_12304_f3.png

ITS

Author Manuscript

Ds_HAP.1

Dothistroma septosporum

Ds_HAP.2

Ds_HAP.3

Ds_HAP.4

Dp_HAP.1

Dothistroma pini

Dp_HAP.2

Dp_HAP.3

Dp_HAP.5

Dp_HAP.4

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