Species diversity of *Lecanosticta* and population genetics of *Dothistroma* species: important needle pathogens of *Pinus*

By
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DECLARATION

I, Ms A van der Nest, hereby declare that the thesis which I hereby submit for the degree *Philosophiae Doctor* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

	23/09/2021
Signature	Date

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PREFACE

Dothistroma needle blight (DNB) is an economically important pine needle disease caused by *Dothistroma pini* and *D. septosporum*. DNB is mostly known due to severe outbreaks in clonal *Pinus* plantations in the Southern Hemisphere in the 1960s, but has similarly emerged as an important foliage disease in the Northern Hemisphere in the past twenty years. The disease is caused by *Dothistroma septosporum,* that has a global distribution, and *Dothistroma pini* that is reported only from the Northern Hemisphere. DNB is easily confused with Brown spot needle blight (BSNB) caused by *Lecanosticta* species. This disease is known from the Northern Hemisphere with the most southern record in Colombia. Like DNB, it has become an important disease of *Pinus* in the last twenty years.

This thesis focuses on different research gaps pertaining to the genus, *Lecanosticta,* as a whole, and the two *Dothistroma* species. It is composed of a literature review and three independent research studies written as Chapters. Due to each chapter forming an independent study, duplication of information in the introductory sections and references is unavoidable. The first two chapters are published and are presented in the format required for each journal. The last two chapters are presented in accordance to the author guidelines for *Molecular Plant Pathology* for the sake of conformity in the unpublished chapters.

The first chapter of this thesis is a review of the literature pertaining to *Lecanosticta,* published in the journal *Molecular Plant Pathology* 20(10):1327-1364 in 2019. This was in lieu of the fact that there has not been a review of *Lecanosticta* literature since 1944, and a synthesis of the literature on this genus of emerging importance was needed. The chapter is written as a pathogen profile on *Lecanosticta acicola* and other species in the genus. The review provides a summary on the research of symptoms of BSNB as well as disease management of the disease, and covers topics such as the life cycle, toxin production, biology, host range, host susceptibility and geographic distribution and molecular diagnostics used to identify *Lecanosticta* species.

Research Chapter One was published in 2019 in *IMA Fungus* 10, Article number: 2. *Lecanosticta acicola* was long hypothesized to have a Central American centre of origin. This was due to morphological variation observed between isolates collected from pristine forests in the region, in the 1980s, as well as the morphological description of another species, *L. gloeospora,* in Mexico. In the first molecular study that included isolates from Mesoamerica, two additional species of *Lecanosticta* were described based on sequence phylogenies,

however *L. acicola* was not found. In this chapter a large collection of *Lecanosticta* isolates from infected pine needles from Guatemala, Honduras and Nicaragua are identified based on DNA sequence comparisons with the aim to confirm whether *L. acicola* is present in Central America and to see if those with morphological variations represent new species.

The focus of Research Chapter Two centres on another pine needle pathogen, *Dothistroma pini.* Currently, *D. pini* only occurs in the Northern Hemisphere and through international collaborations, a large collection of *D. pini* has been collected in Europe over a 12-year period. Since the development of species specific microsatellite markers in 2016, only two small scale population studies have been conducted, and very little is known regarding the population structure and diversity as well as modes of spread, of this pathogen. This research chapter comprehensively considers the population structure and diversity of *Dothistroma pini* in Europe as well as the likely modes of reproduction and means of spread on the continent.

In the past decade, severe outbreaks of needle blight diseases have been reported more frequently in the Northern Hemisphere. In Spain severe outbreaks were observed in the Basque country and surveys revealed that both *D. pini* and *D. septosporum* as well as *Lecanosticta* is present in the region. In Cantabria, the province west of the Basque country, severe outbreaks of Dothistroma needle blight was observed in 2015. Research Chapter Three aims to determine what pathogen is involved in the outbreaks and to determine the population structure and genetic diversity of the pathogen involved.

Five additional research outputs were indirectly produced during the completion of my PhD studies and are listed on page IX, ordered by author line. The work of Adamčíková *et al.* (2021) is a population genetic study of *D. pini* in Slovakia. This dataset, in which I significantly contributed towards generating, is also included in the study conducted in Research Chapter Two. In Aglietti *et al.* (2021), LAMP assays for the detection of *D. pini, D. septosporum* and *L. acicola* are developed. In the work of Barnes *et al.* (2016), the history of the *Dothistroma* taxonomy is summarized and additionally, *D. septosporum* is neotypified, and *D. pini* epitypified. In Bradshaw *et al.* (2019), eighteen *Dothistroma septosporum* genomes are compared and genomic traits involved in dothistromin production is investigated. Last but not least, Mesanza *et al.* (2020), reports on new hosts infected with pine needle pathogens in Arboreta in the Basque country in Spain.

The research conducted during the course of the PhD for which this thesis is presented, was performed by myself, Ms Ariska van der Nest, at the Forestry and Agricultural Biotechnology

Institute (FABI) at the University of Pretoria (UP). This research was conducted under the supervision of Professor Irene Barnes and Professor Michael J. Wingfield. Cultures were either obtained by isolating from needles collected by Professor Irene Barnes and a multitude of collaborators, or cultures and DNA were provided by our collaborators, or previously isolated by Professor Barnes. Photos of plantations and trees infected with needle diseases were provided by Professor Barnes. Financial support for the research conducted was provided by Thuthka Grants (No 80670, and No 95875*)* provided by the National Research Foundation as well as the DST/NRF Centre of Excellence in Plant Health Biotechnology (CPHB) and the Tree Protection Co-operative Programme (TPCP). Infrastructure was provided by the University of Pretoria (UP) and FABI. I was supported by a Scarce Skills Doctoral Scholarship (Grant 89086) provided by the National Research Foundation.

SUMMARY

Dothistroma needle blight (DNB), caused by *Dothistroma pini* and *D. septosporum*, and Brown spot needle blight (BSNB), caused by *Lecanosticta* species, are two pine needle diseases of *Pinus* species that have become diseases of emerging importance in the Northern Hemisphere in the past twenty years. This thesis aimed to address three research gaps pertaining to the species diversity of the genus, *Lecanosticta,* as a whole, as well as the population structure and genetic diversity of *D. pini* in Europe, and *D. septosporum* in a recent outbreak of DNB in Spain.

Research Chapter 1 aimed to identify a large collection of *Lecanosticta* isolates from infected pine needles from Guatemala, Honduras and Nicaragua based on sequence comparisons of five gene regions and to determine if *L. acicola* is present in the region. Six species of *Lecanosticta,* of which four are novel taxa, were identified. *Lecanosticta jani, L. pharomachri, L. tecunumanii* and *L. variabilis* were described and new hosts and geographical records were reported for previously described *L. brevispora* and *L. guatemalensis. Lecanosticta acicola* was not found in any of the samples considered and this species was redefined as a Northern Hemisphere taxon with three distinct lineages based on the translation elongation 1-α gene region. It was concluded that due to the high species diversity of *Lecanosticta* in Mesoamerica, that this region is considered a centre of diversity for the genus.

The second research chapter aimed to investigate the population diversity, structure and mode of reproduction and likely means of spread for a large collection of *D. pini* obtained from twelve countries in Europe over a 12-year period. Based on population structure analyses, the *D. pini* populations considered grouped in four main geographic clusters and variable population diversity was observed between countries. Both mating types were detected in most countries but evidence for sexual recombination was only supported in the population from Spain. The observed population structure as well as several shared multilocus haplotypes between non-bordering countries provides evidence that the occurrence and spread of *D. pini* on the continent has been strongly influenced by human mediated activities in Europe.

The last research chapter of the thesis was to address the unprecedented DNB outbreaks observed in Cantabria in Spain, in 2015, by determining the causal agent, and to determine the population structure, genetic diversity and mode of reproduction of the pathogen involved, from three infected sites of planted *Pinus nigra* subspecies. *Dothistroma septosporum* was confirmed as the causal agent. Structure analyses revealed two genetic clusters in the

populations examined. Both mating types were detected amongst the isolates, however, sexual recombination was not statistically supported for any of the sites. The high genetic diversity observed in isolates obtained from Cantabria suggested that the pathogen was not recently introduced into the region.

In conclusion, the findings of this thesis emphasize the importance of quarantine and the need for caution when pine germ plasm and other pine material is moved between continents, countries and different regions. Of the nine species of *Lecanosticta*, for which four novel taxa were described, eight species occur only in Mesoamerica. *Lecanosticta acicola*, that has become an increasingly important pathogen of *Pinus* species, is the only species that occurs outside of this region. The other *Lecanosticta* species are of unknown importance but could pose a threat to *Pinus* species if introduced into new environments in the future especially considering that Mesoamerican pines are being used for plantation development in the Southern Hemisphere. Furthermore, through the first large scale population study of *Dothistroma pini,* it was shown that this pathogen is not new to the European continent, however, human activities have contributed to the spread of this pathogen. The further spread of genetically diverse individuals could lead to future unprecedented outbreaks of *D. pini* as well as the possible introduction of this pathogen into the Southern Hemisphere where it is currently still not present. The unprecedented outbreaks of *D. septosporum* in Cantabria was not due to a recent introduction into the region. It was shown that genetically diverse individuals were present in the infected sites and it is likely that these outbreaks were due to increased precipitation that prompted the high infection levels observed.

SCIENTIFIC OUTPUTS DIRECTLY OR INDIRECTLY EMERGING FROM THIS THESIS

1. JOURNAL PUBLICATIONS

Adamčíková, K., Jánošíková, Z., van der Nest, A., Adamčík, S., Ondrušková, E. and Barnes, I. (2021) Population structure and genetic diversity suggest recent introductions of *Dothistroma pini* in Slovakia. *Plant Pathol.,* **70,** 1883– 1896.

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Barnes, I., van der Nest, A., Mullett, M. S., Crous, P. W., Drenkhan, R., Musolin, D. L. and Wingfield, M. J. (2016) Neotypification of *Dothistroma septosporum* and epitypification of *D. pini*, causal agents of Dothistroma needle blight of pine. *Forest Pathol.,* **46,** 388-407.

Bradshaw, R. E., Sim, A. D., Chettri, P., Dupont, P.-Y., Guo, Y., Hunziker, L., McDougal, R. L., van der Nest, A., Fourie, A., Wheeler, D., Cox, M. P. and Barnes, I. (2019) Global population genomics of the forest pathogen *Dothistroma septosporum* reveal chromosome duplications in high dothistromin-producing strains. *Mol. Plant. Pathol.,* **20,** 784-799.

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van der Nest, A., Wingfield, M. J., Janoušek, J. and Barnes, I. (2019) *Lecanosticta acicola*: A growing threat to expanding global pine forests and plantations. *Mol. Plant. Pathol.,* **20,** 1327-1364.

van der Nest, A., Wingfield, M. J., Ortiz, P. C. and Barnes, I. (2019) Biodiversity of *Lecanosticta* pine-needle blight pathogens suggests a Mesoamerican Centre of origin. *IMA Fungus,* **10,** Article 2 (2019).

2. CONFERENCE PARTICIPATION

van der Nest, A., Wingfield, M.J., Sadiković, D., Mullett, M., Queloz, V., Adamčíková, K., Davydenko K., Barnes, I. 2020. Population structure and diversity of *Dothistroma pini* in Europe suggests human mediated movement of the needle blight pathogen. MSA 2020 'Mycology from the Clouds' virtual meeting. Graphical abstract.

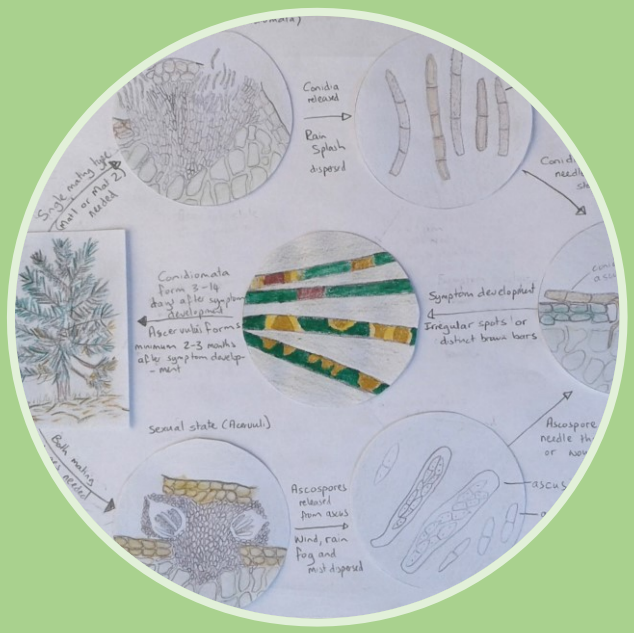
van der Nest, A., Wingfield, M.J., Barnes, I. 2018. The rising threat of novel pine needle pathogens from Central America under expanding global cultivation. 44th Conference of the South African Association of Botanists, 9-12 January 2018, Pretoria, South Africa. p91.

Barnes, I., van der Nest, A., Ortiz, P., Wingfield, M. 2017. New cryptic species and a putative center of diversity for the pine pathogens *Dothistroma* and *Lecanosticta.* IUFRO 125th Anniversary Congress, 18-22 September 2017, Freiburg, Germany. p221.

van der Nest, A., Wingfield, M.J., Barnes, I. 2017. Diversity of *Lecanosticta* species in Central America suggests a centre of diversity for an important group of pine needle pathogens. 50th Anniversary Congress of the Southern African Society for Plant Pathology, 15-18 January 2017, Champagne Sports Resort, Drakensberg. Poster 26.

van der Nest, A., Wingfield, M.J., Barnes, I. 2016. The phylogenetic diversity of *Lecanosticta* in Central America suggests a centre of diversity for this genus. 4th Joint congress of the SA Society for Bioinformatics and the SA Genetics Society, 20-23 September 2016, Durban, South Africa. p35

LITERATURE REVIEW



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Pathogen profile

Lecanosticta acicola: A growing threat to expanding global pine forests and plantations

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SUMMARY

Lecanosticta acicola causes brown spot needle blight (BSNB) of *Pinus* species. The pathogen occurs mostly in the Northern Hemisphere but has also been reported in Central America and Colombia. BSNB can lead to stunted growth and tree mortality, and has resulted in severe damage to pine plantations in the past. There have been increasingly frequent new reports of this pathogen in Europe and in North America during the course of the past 10 years. This is despite the fact that quarantine practices and eradication protocols are in place to prevent its spread.

Taxonomy: Kingdom Fungi; Phylum Ascomycota; Subphylum Pezizomycotina; Class Dothideomycetes; Subclass Dothideomycetidae; Order Capniodales; Family Mycosphaerellaceae; Genus *Lecanosticta*.

Host range and distribution: *Lecanosticta* spp. occur on various *Pinus* species and are found in North America, Central America, South America (Colombia), Europe as well as Asia.

Disease symptoms: Small yellow irregular spots appear on the infected pine needles that become brown over time. They can be surrounded by a yellow halo. These characteristic brown spots develop to form narrow brown bands that result in needle death from the tips down to the point of infection. Needles are prematurely shed, leaving bare branches with tufts of new needles at the branch tips. Infection is usually most severe in the lower parts of the trees and progresses upwards into the canopies.

Useful websites: The EPPO global database providing information on *L. acicola* (https://gd.eppo.int/taxon/SCIRAC)
Reference genome of *L. acicola* available on GenBank (https://www.ncbi.nlm.nih.gov/genome/?term=Lecanosticta+acicola)
JGI Gold Genome database information sheet of *L. acicola* sequenced genome (https://gold.jgi.doe.gov/organism?xml:id=Go0047147)

Keywords: brown spot needle blight, *Lecanosticta acicola*, *Lecanosticta* species, *Mycosphaerella dearnessii*, pine pathogen, *Pinus* spp.

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INTRODUCTION

Lecanosticta acicola is an ascomycete fungus that causes a disease of *Pinus* spp. known as brown spot needle blight (BSNB). The pathogen was first described by de Thümen (1878) and it owes its notoriety to a disease problem that arose in the south-eastern USA on *Pinus palustris*, better known as long leaf pine in that area (Siggers, 1932). This tree species, which is highly susceptible to infection, is peculiar in having a so-called 'grass' stage during the first five years of its growth. This mass of young needles provides a favourable environment for infection to occur.

The BSNB pathogen completes its life cycle (Fig. 1) on pine needles that are shed prematurely. This leads to reduced or stunted growth that can result in significant yield losses (Wakeley, 1970) or tree death. In some cases, pine plantations have been sufficiently damaged that they have needed to be cleared (Huang *et al.*, 1995; Lévy, 1996; Markovskaja *et al.*, 2011).

Lecanosticta acicola has been recorded on 53 different *Pinus* species and hybrids in native and non-native pine stands in the USA, Canada, several European countries and Asia as well as in Central America and Colombia (Table 1). Due to the severity of the disease, the pathogen has been afforded an A1 quarantine status in Africa, Argentina, Chile, Uruguay, Bahrain, Kazakhstan, Ukraine and Russia, and A2 quarantine status in Europe (https://gd.eppo.int/taxon/SCIRAC/categorization). However, reports of new outbreaks of the disease in various European countries have increased significantly since 2008 (Adamson *et al.*, 2015, 2018; Anonymous, 2012; Cleary *et al.*, 2019; Hintsteiner *et al.*, 2012; Jankovský *et al.*, 2009a; Markovskaja *et al.*, 2011; Mullett *et al.*, 2018; Ortíz de Urbina *et al.*, 2017).

Quarantine measures rely on accurately identifying the presence of pathogens on symptomatic tissues. This is complicated in the case of *L. acicola* where the symptoms of BSNB closely resemble those of Dothistroma needle blight (DNB). DNB is caused by two species: *Dothistroma septosporum* and *D. pini* (Barnes *et al.*, 2016). Due to their similar symptoms,

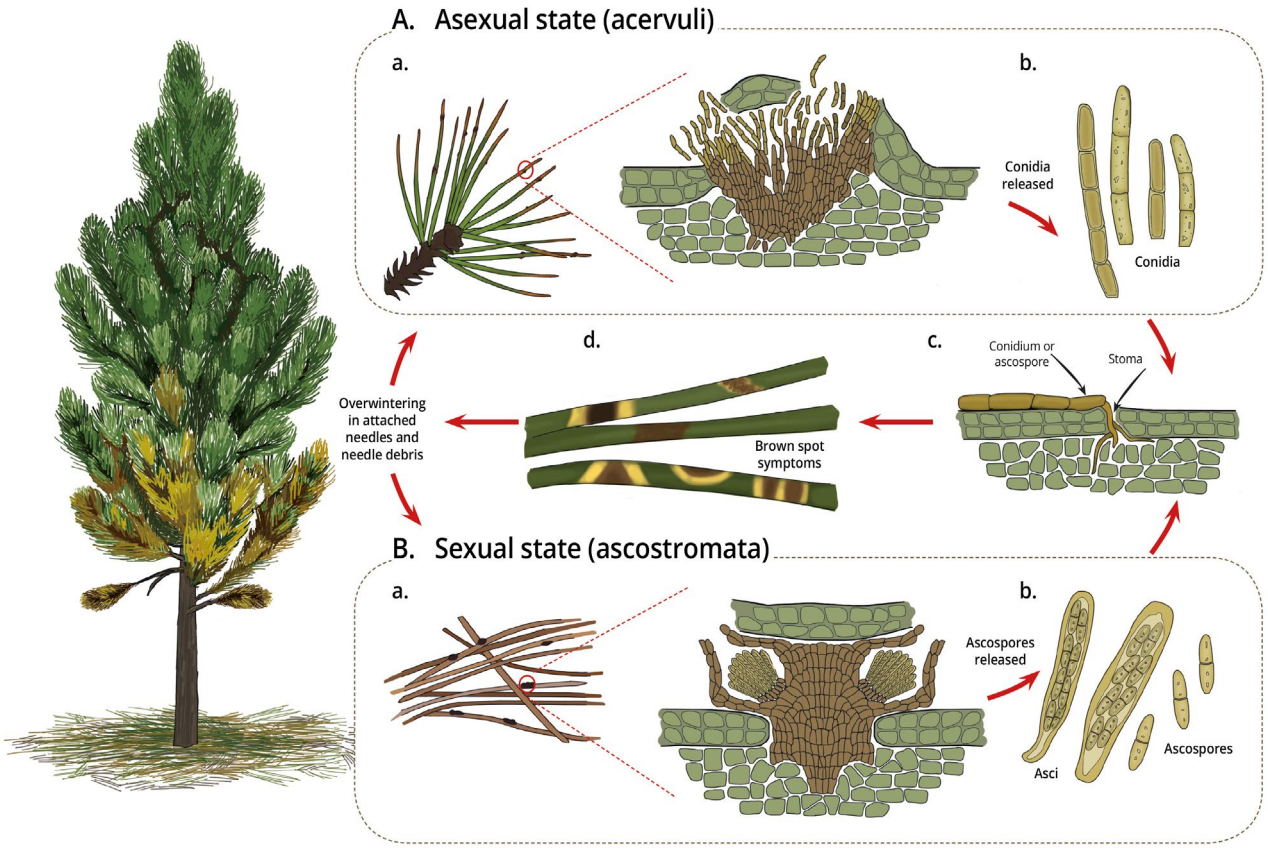

Fig. 1 Life cycle of *Lecanosticta acicola* on *Pinus* spp. (A) Asexual state: acervuli (a) develop on attached needles and needle debris and release conidia (b). Infection occurs through the stomata of new season needles (c), resulting in brown spot symptoms (d). (B) Sexual state: ascostromata develop on dead needles associated with previous season infections (a) and release ascospores in spring (b). Infection occurs through the stomata of new season needles (c), resulting in brown spot symptoms (d).

field diagnoses of the causal agent based on symptoms and/or on morphology alone have commonly been incorrect (Shishkina and Tsanava, 1967; Siggers, 1944; Thyr and Shaw, 1964). Consequently, past reports of *L. acicola* based only on morphological descriptions and symptoms must be treated with caution and verified using molecular identification techniques (van der Nest *et al.*, 2019).

Lecanosticta acicola has been well-known in the southeastern USA since the early 1900s, but is rapidly spreading in northern parts of the USA, Canada and in some parts of Europe (Broders *et al.*, 2015). Its complete host range is not known but appears to be expanding (Mullett *et al.*, 2018). A recent taxonomic re-evaluation of isolates previously identified as *L. acicola*, applying phylogenetic analyses based on DNA sequences, has led to various isolates being recognized as distinct species (Quaedvlieg *et al.*, 2012; van der Nest *et al.*, 2019). This and a number of recent publications (Adamson *et al.*, 2018; Cleary *et al.*, 2019; Mullett *et al.*, 2018; Ondrušková *et al.*, 2018; Ortíz de Urbina *et al.*, 2017; Sadiković *et al.*, 2019; Schneider *et al.*, 2019; Wyka *et al.*, 2017) justifies the need for a review of current knowledge regarding BSNB and the *Lecanosticta* species that cause this disease. This is the first review of the topic to be presented in 75 years subsequent to that of Siggers (1944).

LECANOSTICTA SPECIES

The genus *Lecanosticta,* which includes nine species with the type species being *L. acicola* (previously known as *Mycosphaerella dearnessii,* Table 2), is characterized by stromata and septate, pigmented conidia. The genus was erected by Sydow and Petrak in 1922 (Sydow and Petrak, 1922). The taxonomic history and nomenclature of *Lecanosticta acicola* has been succinctly presented previously (Evans, 1984; Siggers, 1944) and is summarized and updated in Table 2.

Lecanosticta acicola is the oldest known species in the genus and owes its notoriety to the disease of long leaf pine, which it was first associated with, in the southeastern USA (Chapman, 1926; Hedgcock, 1929). Although the pathogen was identified in

Table 1 Host and geographical range of *Lecanosticta* species.

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Lecanosticta acicola						
Austria, Lower Austria, Valley of the river Ybbs	1996–2000	*P. mugo, P. sylvestris*	Morphological identifications of the pathogen were performed.		Infected trees were eradicated after which the disease was no longer detected (2001–2002).	Brandstetter and Cech (2003)
Austria, Lower Austria, Hollenstein/Ybbs	2008–2009	*P. sylvestris*	The pathogen was recognized during a forest survey.			Cech and Krehan (2008), Kessler (2009)
Austria, Lower Austria, Hollenstein/Ybbs	2009–2010	*P. mugo* subsp. *mugo, P. mugo* subsp. *uncinata*	Symptoms were observed in a survey.			Kessler and Krehan (2011)
Austria, Lower Austria	1996	*P. mugo*	Fruiting bodies were observed on pine needles.		Isolated occurrence in a garden.	Cech (1997)
Austria, Lower Austria, Hollenstein/Ybbs	1998	*Pinus* sp.	Symptoms were observed in the field.			Brandstetter and Cech (1999)
Austria, Lower Austria	2004	*P. mugo, P. sylvestris*	*TEF 1* sequencing used for identification, both mating types were detected.	*		Janoušek *et al.* (2016)
Austria, Lower Austria	2010	*P. mugo*	*TEF 1* sequencing used for identification, both mating types were detected.	*		Janoušek *et al.* (2016)
Austria, Upper Austria	2010	*P. mugo*	*TEF 1* sequencing used for identification. Mating type 1 was detected.	*		Janoušek *et al.* (2016)
Austria, Upper Austria, Bregenz (Vorarlberg)	2011	*P. mugo* subsp. *mugo*	Symptoms were observed in a survey.			Kessler and Krehan (2011)
Austria, Upper Austria, Gmunden	2011	*P. nigra* var. *nigra, P. mugo* subsp. *mugo*	ITS sequencing used for identification.	*		Hintsteiner *et al.* (2012)
Austria, Upper Austria, Tyrol	2011	*P. mugo* subsp. *uncinata*	Symptoms were observed in a survey.			Kessler and Krehan (2011)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Austria, Upper Austria	2012	*P. nigra*	*TEF 1* sequencing used for identification. Mating type 2 was detected.	*		Janoušek *et al.* (2016)
Austria, Upper Austria, Tyrol	2015	*P. mugo* subsp. *mugo, P. mugo* subsp. *uncinata, P. sylvestris*	The pathogen was detected during a forest survey and confirmed with laboratory tests (method not specified).	*	Detected in area covering more than 60 ha of forest.	EPPO (2015)
Austria, Graz	2016	*P. mugo*	Infected needles were collected by I. Barnes. Isolations were made by I. Barnes and A. van der Nest, and identified by ITS sequencing. Mating type 2 was detected.	*	Trees heavily infected (see Fig. 2A,B).	I. Barnes, FABI, Pretoria, South Africa, personal communication
Austria, Lower Austria	2016	*P. mugo*	Infected needles were collected by T. Cech. Isolations were made by I. Barnes and A. van der Nest, and identified by ITS sequencing. Mating type 2 was detected.	*		I. Barnes, FABI, Pretoria, South Africa, personal communication
Austria, Salzburg	2016	*P. uncinata*	Infected needles were collected by T. Cech. Isolations were made by I. Barnes and A. van der Nest, and identified by ITS sequencing. Mating type 2 was detected.	*		I. Barnes, FABI, Pretoria, South Africa, personal communication
Austria, Upper Austria	2016	*P. mugo*	Infected needles were collected by T. Cech. Isolations were made by I. Barnes and A. van der Nest, and identified by ITS sequencing. Mating type 1 was detected.	*		I. Barnes, FABI, Pretoria, South Africa, personal communication
Belize	1981	*P. caribaea, P. oocarpa*	Morphological identifications were made. Confirmation is needed as molecular identification did not reveal *L. acicola* in Central America (van der Nest *et al.,* 2019).			Evans (1984)
Bulgaria, near Sofia	1938	*P. nigra*	The pathogen was identified based on morphological characteristics. However, the conidial descriptions are not typical of *L. acicola* and therefore this record is doubtful and should be verified.			Kovaćevski (1938)
Canada, Manitoba	1965	*P. banksiana, P. contorta* var. *latifolia*	Symptoms were observed in the field and the presence of the pathogen was confirmed with morphological identifications.		50–90% of *P. contorta* var. *latifolia* was infected, 20% of *P. banksiana* was infected.	Laut *et al.* (1966)
Canada, New Brunswick, Quebec and Ontario	2009	*P. strobus*	*L. acicola* was reported to occur with *Canavergella banfieldii* on all trees sampled and confirmed based on morphological characteristics.			Laflamme *et al.* (2010)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Canada, Quebec	2011	*P. strobus, P. mugo*	*TEF 1* sequencing used for identification, both mating types detected.	*		Janoušek *et al.* (2016)
China, Jiangsu	1958	*P. thunbergii*	Identification method not specified.		Insignificant damage was reported.	Ye and Wu (2011)
China, Fujian province	1982–1985	*P. elliottii*	Morphological identifications of the pathogen.			Li *et al.* (1987)
China, Anhui, Fujian, Guangdong, Guangxi, Jiangsu, Jiangxi and Zhejiang provinces	1986	*P. caribaea, P. clausa, P. echinata, P. elliottii, P. palustris, P. taeda, P. thunbergii*	Morphological characteristics were used to identify the pathogen.		*P. elliottii, P. taeda* and *P. thunbergii* were severely damaged. *P. caribaea, P. clausa, P. echinata* and *P. palustris* were reported as susceptible.	Li *et al.* (1986), Ye and Wu (2011)
China, Fujie	1988	*P. elliottii*	Morphological characteristics and RAPD analysis were used to identify the pathogen. *TEF 1* sequencing was further used for identification and mating type 2 was detected by Janoušek *et al.* (2016).	*		Huang *et al.* (1995), Janoušek *et al.* (2016)
China, Zhejiang	1991	*P. thunbergii*	Morphological characteristics and RAPD analysis were used to identify the pathogen.	*		Huang *et al.* (1995)
China, Jiangxi	1992	*P. elliottii, P. thunbergii*	Morphological characteristics and RAPD analysis were used to identify the pathogen.	*		Huang *et al.* (1995)
China, Guanxi	1992	*P. caribaea, P. elliottii*	Morphological characteristics and RAPD analysis were used to identify the pathogen.	*		Huang *et al.* (1995)
Colombia, Piedras Blancas and Pereira	1978	*P. radiata, P. elliottii, P. patula*	Identification method not specified.		*P. radiata* severely defoliated but on *P. elliottii* and *P. patula* the pathogen was isolated from cast needles found underneath healthy trees.	Gibson (1980)
Colombia, Albán	1981	*P. radiata*	Morphological identification, sexual and asexual state were identified.		Plantations were severely defoliated.	Evans (1984)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Colombia, Refocosta	2011	*P. caribaea*	Infected needles were collected by C.A. Rodas. Isolations were made by I. Barnes. *TEF 1* sequencing was used for identification and mating type 2 was detected by Janoušek *et al.* (2016).	*		Janoušek *et al.* (2016)
Costa Rica, Alajuela	1980	*P. oocarpa*	Morphological identification of pathogen.			Evans (1984)
Croatia, Dalmatia	1975	*P. halepensis*	Morphological identification of *L. acicola.*		The pathogen is not as aggressive as in the USA on this host and it seems to only be aggressive where dense canopies are present with high air humidity. Copper fungicides were applied.	Milatović (1976)
Croatia, Zadar	Not specified	*P. halepensis*	Forest surveys were conducted. It is not specified in the English abstract whether morphological identifications were performed.		500 ha of *P. halepensis* was heavily infected with the pathogen. Highly infected trees and lower infected branches were cut down and it is reported that the trees recovered.	Glavaš and Margaletić (2001)
Croatia, Zadar	2009	*P. halepensis*	*TEF 1* sequencing was used for identification. Mating type 2 was detected.	*		Janoušek *et al.* (2016)
Croatia, Kožino	2015	*P. halepensis*	*TEF 1* sequencing was used for identification. Mating type 2 was detected.	*		Sadiković *et al.* (2019)
Cuba, Baracoa, Guantánamo, Plateau of Mayarí and Master Saw	1980–1998	*P. caribaea, P. cubensis, P. maestrensis*	Symptom identification and morphological confirmation of the fungus.		Mostly seedlings in nurseries were infected.	Lopéz Castilla *et al.* (2002)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Czech Republic, Southern Bohemia, Červené Blato Nature Reserve	2007	*P. uncinata* subsp. *uliginosa*	Morphological identifications were conducted as well as sequencing of the ITS region. The identity of the pathogen was again confirmed with *TEF 1* sequencing by Janoušek *et al.* (2016). Both mating types were detected.	*	Heavy defoliation was reported in 2007. No control measures were taken as the incidence was reported in a natural nature reserve.	Jankovský *et al.* (2009b), Janoušek *et al.* (2016)
Czech Republic, Southern Bohemia, Soběslav, Borkovická Blata National Nature Reserve	2008	*P. uncinata* subsp. *uliginosa*	Morphological identifications were conducted as well as sequencing of the ITS region. The identity of the pathogen was again confirmed with *TEF 1* sequencing by Janoušek *et al.* (2016). Both mating types were detected.	*	No action was taken as the outbreak was in a natural reserve.	Jankovský *et al.* (2009a), Janoušek *et al.* (2016)
Estonia, Hiiumaa Island and Käravere	2014–2015	*P. mugo*	Symptom identification was confirmed with conventional PCR directly from pine needles. *Lecanosticta acicola* was isolated from the needles and confirmed with ITS sequencing. Both mating types were detected.	*		Adamson *et al.* (2015)
Estonia, Tallinn Botanical Garden	2006–2008	*P. ponderosa*	Material of *Dothistroma* was collected and isolated but in culture it was determined to be *L. acicola* based on culture morphology. The *TEF 1* sequences were later determined for representative isolates and mating type 2 was detected (Janoušek *et al.*, 2016).	*		Drenkhan and Hanso (2009), Janoušek *et al.* (2016)
Estonia, Tallinn Botanical Garden	2010–2013	*P. mugo, P. mugo* var. *pumilio, P. ponderosa, P. uncinata*	Symptom identification was confirmed with conventional PCR directly from pine needles. *Lecanosticta acicola* was isolated from the needles and confirmed with ITS sequencing. Mating type 1 was detected.	*		Adamson *et al.* (2015)
Estonia, Tartu county	2016	*P. sylvestris, P. mugo, Pinus* × *rhaetica*	Visual symptom identification was confirmed with conventional PCR and selected isolates were identified using an ITS sequencing PCR. Both mating types were detected.	*		Adamson *et al.* (2018)
Estonia, Tori and Vasula	2012, 2013	*P. mugo*	Symptom identification was confirmed with conventional PCR directly from pine needles. *Lecanosticta acicola* was isolated from the needles and confirmed with ITS sequencing.	*		Adamson *et al.* (2015)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
France, South-West, Aquitaine and western Pyrénées	1993	*P. attenuata* × *P. radiata*	In field observations were made.		Severe tree mortality was observed. French authorities implemented eradication measures and destroyed 127 ha of trees.	Lévy (1996)
France, Gironde	1995	*P. muricata*	*TEF 1* and *BT 2* sequencing used for identification, mating type 1 detected.	*		Ioos *et al.* (2010), Janoušek *et al.* (2016)
France, Landes	1995	*P. attenuata* × *P. radiata*	*TEF 1* and *BT 2* sequencing used for identification, mating type 2 detected.	*		Ioos *et al.* (2010), Janoušek *et al.* (2016)
France, Pyrénées-Atlantiques	1995	*P. radiata*	*TEF 1* and *BT 2* sequencing used for identification.	*		Ioos *et al.* (2010)
France, Ariège	2009	*P. sylvestris*	Forest surveys were conducted.		More than 50% of the trees were affected.	Alvère *et al.* (2010)
France, Tarn-et-Garonne	2009	*P. nigra* var. *laricio*	Forest surveys were conducted.		The trees were moderately affected.	Alvère *et al.* (2010)
France, Pyrénées-Atlantiques	2012	*P. radiata*	*TEF 1* sequencing used for identification, both mating types were detected.	*		Janoušek *et al.* (2016)
Germany, Bavaria	1994	*P. mugo*	The pathogen was identified based on morphological characteristics.			Pehl (1995)
Germany, Bavaria	1994, 2000, 2010, 2011	*P. mugo*	*TEF 1* sequencing used for identification, both mating types were detected.	*		Janoušek *et al.* (2016)
Germany, Bavaria, Munich Botanical gardens	2018	*P. mugo*	Collected by I. Barnes. The identity was confirmed by ITS sequencing. *Dothistroma septosporum* was also present.	*		I. Barnes, FABI, Pretoria, South Africa, personal communication
Guatemala, El Progreso	1983	*P. oocarpa*	Morphological identification methods were used. As *L. acicola* was not identified in Central America using molecular identification techniques (van der Nest *et al.*, 2019), this report will need to be verified.			Evans (1984)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Honduras	1980–1983	*P. caribaea, P. maximinoi, P. oocarpa, P. tecunumanii,*	Morphological identification methods were used. As *L. acicola* was not identified in Central America using molecular identification techniques (van der Nest *et al.,* 2019), this report will need to be verified.			Evans (1984)
Ireland, Wexford county	2016	*P. mugo, P. sylvestris*	ITS sequencing was used for identification purposes. Mating type 1 was detected.	*		Mullett *et al.* (2018)
Italy, Brescia	1997	*P. mugo*	Symptoms were noted in the botanical garden and the presence of the pathogen was confirmed with morphological identifications.		Extensive necrosis and crown defoliation were observed in all 12 of the *P. mugo* trees present in the botanical garden.	La Porta and Capretti (2000)
Italy, Brescia	2008	*P. mugo*	*TEF 1* sequencing used for identification and mating type 1 detected.	*		Janoušek *et al.* (2016)
Japan, Shimane Prefecture (Honshu)	1996	*P. thunbergii, P. densiflora* (tested in controlled environment)	The pathogen was morphologically identified.		*P. thunbergii* was severely infected. Inoculation trials on this host as well as *P. densiflora* also revealed that *P. densiflora* is susceptible although it was not reported in the host's natural environment.	Suto and Ougi (1998)
Japan, Shimane	2010	*P. thunbergii*	*TEF 1* sequencing used for identification, mating type 2 was detected.	*		Janoušek *et al.* (2016)
Latvia, Salaspils	2012	*P. pumila*	Morphological identification. Later it was confirmed with PCR-based methods.	*	Eradication measures were taken.	EPPO (2012a)
Latvia, Salaspils	2016	*P. mugo*	Identification was done by ITS sequencing. Mating type 1 was detected.	*		Mullett *et al.* (2018)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Lithuania, Curonian Spit, Smiltynė Forest District	2009	*P. mugo*	Morphological characteristics as well as ITS sequencing and ITS-RFLP was used to identify the pathogen. This material was again examined by Janoušek *et al.* (2016) and the identity confirmed with *TEF 1*. Mating type 1 was detected.	*	A monitoring programme was initiated and infected trees felled and burned.	Markovskaja *et al.* (2011), Janoušek *et al.* (2016)
Lithuania, Curonian Spit, Smiltynė Forest District and Juodkrantė Forest District	2010	*P. mugo*	Morphological characteristics as well as ITS sequencing and ITS-RFLP was used to identify the pathogen.	*	A monitoring programme was initiated and infected trees felled and burned.	Markovskaja *et al.* (2011)
Lithuania, Curonian Spit, near Juodkrante	2012	*P. mugo, P. sylvestris*	Morphological identifications and PCR-based methods.	*	Phytosanitary methods were implemented.	EPPO (2012b)
Lithuania, Curonian Spit, Smiltyne Smiltynė Forest District and Juodkrantė Forest District	2014	*P. mugo*	Infected needles were collected by S. Markovskaja. Isolations were made by A. van der Nest. A multigene phylogenetic approach was used to determine the identity of the isolates.	*		van der Nest *et al.* (2019)
Mexico, Puebla	1983	*P. patula*	Morphological identification.			Evans (1984)
Mexico	2000	*P. ayacahuite, P. cembroides, P. halepensis*	Morphological characteristics were examined.		High disease severity was reported on *P. halepensis.*	Marmolejo (2000)
Mexico, Nuevo León	2010, 2011	*P. halepensis*	*TEF 1* sequencing used for identification, both mating types detected. KJ938447–KJ938449 were later identified as *L. variabilis* (van der Nest *et al.,* 2019) and the remaining isolates are part of *L. acicola* lineage 3.	*		Janoušek *et al.* (2016)
Nicaragua	1981–1983	*P. caribaea, P. maximinoi, P. oocarpa, P. tecunumanii*	Morphological identification methods were used. As *L. acicola* was not identified in Central America using molecular identification techniques (van der Nest *et al.,* 2019), this report will need to be verified. Both the sexual and asexual state was observed.			Evans (1984)
Portugal, Minho	2016	*P. radiata*	Identification was done by ITS sequencing. Mating type 1 was detected.	*		Mullett *et al.* (2018)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Romania, Vrancea	2017	*Pinus* sp.	The pathogen was detected during a forest survey in a 30-year-old plantation.		Eradication reported to be under way in the 19-hectare forest.	EPPO (2018)
Russia, Krasnodar region, Sochi	2016	*P. mugo* subsp. *mugo, P. thunbergii*	Identification was done by ITS sequencing. Mating type 2 was detected.	*		Mullett *et al.* (2018)
Slovenia, Bled	2008–2009	*P. mugo, P. sylvestris*	Morphological identifications. The identity of isolates on *P. mugo* were confirmed with *TEF 1* sequencing and mating type 2 was detected (Janoušek *et al.*, 2016; Sadiković *et al.*, 2019).	*	All affected trees were eradicated.	Jurc and Jurc (2010), Janoušek *et al.* (2016), Sadiković *et al.* (2019)
Slovenia, Čatež	2015	*P. mugo*	*TEF 1* sequencing was used for identification. Mating type 1 was detected.	*		Sadiković *et al.* (2019)
Slovenia, Ljubljana	2008–2009	*P. mugo, P. sylvestris*	Morphological identifications. The identity of isolates from *P. mugo* were confirmed with *TEF 1* sequencing by Sadiković *et al.* (2019).	*	All affected trees were eradicated.	Jurc and Jurc (2010), Sadiković *et al.* (2019)
Slovenia, Ljubljana	2013	*P. mugo*	*TEF 1* sequencing was used for identification. Mating type 1 was detected.	*		Sadiković *et al.* (2019)
Slovenia, Tolmin	2016	*P. nigra*	*TEF 1* sequencing was used for identification. Mating type 1 was detected.	*		Sadiković *et al.* (2019)
Slovenia, Trenta	2014–2015	*P. mugo*	*TEF 1* sequencing was used for identification. Mating type 2 was detected.	*		Sadiković *et al.* (2019)
South Korea, Naju	2010–2011	*P. thunbergii*	*L. acicola* symptoms were observed and confirmed with ITS sequencing. *TEF 1* sequencing was used for identification by Janoušek *et al.* (2016) and mating type 2 was detected.	*	Low incidence, less than 1%.	Janoušek *et al.* (2016), Seo *et al.* (2012)
Spain	1942	*P. radiata*	Probably oldest official report of *L. acicola* in Europe based on morphological identification.			Martínez (1942)
Spain, Cantabria	2012	*P. radiata*	*TEF 1* sequencing used for identification, mating type 2 was detected.	*		Janoušek *et al.* (2016)
Spain, Spanish Atlantic climate region	2015	*P. nigra, P. radiata*	Sequenced directly from needles using conventional PCR (Ioos *et al.*, 2010). Both mating types were detected.	*	*Lecanosticta acicola* was detected on 44.7% of trees that were surveyed.	Ortíz de Urbina *et al.* (2017)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Sweden	2017	*P. mugo* 'Hesse'	Morphological identification and ITS sequencing.	*	Single tree in arboretum that was severely affected.	Cleary *et al.* (2019)
Switzerland, Zollikon	1995	*P. mugo, P. uncinata*	Morphological identification of the pathogen.		Control measures were initiated in accordance with the phytosanitary policy of the EPPO.	Holdenrieder and Sieber (1995)
Switzerland, Canton St Gallen	1999	*P. mugo*	*TEF 1* sequencing used for identification.	*		Janoušek *et al.* (2016)
Switzerland, Canton Zug	2009	*P. mugo*	Symptoms were observed in the field. Later, *TEF 1* sequencing was used to confirm identification (Janoušek *et al.*, 2016). Mating type 1 was detected.	*		Angst (2011), Janoušek *et al.* (2016)
Switzerland, Zürich	2009	*P. mugo*	Symptoms were observed in the field. Later, *TEF 1* sequencing was used to confirm identification (Janoušek *et al.*, 2016). Mating type 1 was detected.	*		Angst (2011), Janoušek *et al.* (2016)
Switzerland, Bern and Zürich	2017	*P. mugo*	Detection with qPCR and a conventional PCR directly from pine needles.	*		Schneider *et al.* (2019)
Switzerland, Schwyz	2017	*P. sylvestris*	Detection with qPCR and a conventional PCR directly from pine needles.	*		Schneider *et al.* (2019)
USA, Alabama	1929	*P. palustris*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Alabama	1944	*P. echinata, P. palustris, P. taeda*	Siggers reported *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Alabama	1948–1967	*P. palustris*	Symptoms were observed annually on seedlings and the proportion of seedlings affected were recorded.		In a 4-year study, 78% or more seedlings were infected yearly with *L. acicola.*	Boyer (1972)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Arkansas	1929	*P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Arkansas	1944	*P. taeda*	Siggers reported *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Arkansas	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)
USA, Florida	1929	*P. caribaea, P. glabra, P. palustris, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Florida	1944	*P. attenuata, P. caribaea, P. coulteri, P. jeffreyi, P. glabra, P. halepensis, P. latifolia, P. muricata, P. palustris, P. pinaster, P. pinea, P. ponderosa* var. *scopulorum, P. radiata, P. thunbergii*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Georgia	1929	*P. palustris, P. taeda, P. virginiana*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Georgia	1944	*P. caribaea, P. palustris, P. taeda, P. virginiana*	Siggers reported *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Idaho	1929	*P. ponderosa*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture. According to Siggers (1944) the identification was based on characteristics that do not fit *Lecanosticta* and therefore this record should be verified.			Hedgcock (1929)
USA, Iowa	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)
USA, Kansas	1929	*P. nigra* var. *austriaca*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture. According to Siggers (1944) the identification was based on characteristics that do not fit *Lecanosticta* and therefore this record should be verified.			Hedgcock (1929)
USA, Kansas	1951	*P. nigra, P. ponderosa*	Reports in the field and mycological identification.			Rogerson (1953)
USA, Kansas	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)
USA, Kentucky	1929	*P. nigra* var. *austriaca*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture. According to Siggers (1944) the identification was based on characteristics that do not fit *Lecanosticta* and therefore this record should be verified.			Hedgcock (1929)
USA, Kentucky	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Louisiana	1929	*P. palustris, Pinus* × *sondereggeri, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Louisiana	1929–1930, 1960	*P. palustris*	Symptoms were observed and the proportion of seedlings affected were recorded at 4–5 years of age and again at 30 years.		Most of the trees were affected.	Wakeley (1970)
USA, Louisiana	1944	*P. attenuata, P. caribaea, P. contorta* var. *latifolia, P. echinata, P. nigra* var. *laricio, P. palustris, P. pinaster, P. ponderosa* var. *scopulorum, P. radiata, P. rigida, P. serotina, P. sabiniana, Pinus* × *sondereggeri, P. taeda*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Maine	2011	*P. strobus*	Isolates were collected and morphologically identified in a survey. These isolates were later identified with *TEF 1* sequencing and both mating types were detected.	*		Munck *et al.* (2012), Janoušek *et al.* (2016)
USA, Maine	2011–2012	*P. strobus*	*Lecanosticta acicola* was identified as part of a complex of pathogens that cause white pine needle damage (WPND). Morphological identifications and selected ITS PCR sequencing was performed to confirm the presence of *L. acicola*.	*	It was observed that affected trees were defoliated annually.	Broders *et al.* (2015)
USA, Michigan	2016	*P. sylvestris*	*TEF 1* sequencing used for identification, mating type 2 was detected.	*		Janoušek *et al.* (2016)
USA, Minnesota	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Minnesota and Wisconsin	1970–1972	*P. banksiana, P. glauca, P. nigra, P. palustris, P. resinosa, P. strobus, P. sylvestris, Picea glauca*	Symptoms were observed in the field and the proportion of needles affected were noted.		These species were tested for susceptibility in a field trial by planting the hosts underneath heavily infected *P. sylvestris.* Four varieties of *P. sylvestris,* as well as *P. nigra* and *P. resinosa,* were the most susceptible. *P. strobus* was moderately resistant. *P. banksiana* was the most resistant. Less than 1% of *Picea glauca* was infected.	Skilling and Nicholls (1974)
USA, Mississippi	1929	*P. caribaea, P. palustris, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Mississippi	1944	*P. caribaea, P. palustris, P. pinaster, P. taeda, P. thunbergii*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Mississippi	1952–1953	*P. palustris*	Microscopic identification. Both the sexual and asexual states were observed.			Henry (1954)
USA, Mississippi	1966–1967	*P. palustris*	Morphological identifications. Both the sexual state and asexual state were observed throughout the year on infected *P. palustris.*			Kais (1971)
USA, Mississippi	2012	*P. palustris, P. taeda*	*TEF 1* sequencing used for identification, both mating types detected.	*		Janoušek *et al.* (2016)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Missouri	1929	*P. nigra* var. *austriaca*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture. According to Siggers (1944) the identification was based on characteristics that do not fit *Lecanosticta* and therefore this record should be verified.			Hedgcock (1929)
USA, Missouri	1947–1949	*P. ponderosa*	Symptoms were observed in the field and morphological identifications were made. Both the sexual state and asexual state were observed.		All trees were affected. Excessive needle defoliation and in some cases tree mortality was observed.	Luttrell (1949)
USA, Missouri	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)
USA, New England	2016	*P. strobus*	Severe needle browning was observed and *L. acicola* was identified as part of a complex of species causing premature defoliation. This is possibly WPND although it was not defined as such.			Brazee (2016)
USA, New Hampshire	2011	*P. strobus*	Isolates were collected and morphologically identified in a survey. These isolates were later identified with *TEF 1* sequencing and mating type 1 was detected.	*		Munck *et al.* (2012), Janoušek *et al.* (2016)
USA, New Hampshire	2011–2012	*P. strobus*	*Lecanosticta acicola* was identified as part of a complex of pathogens that cause WPND. Morphological identifications and selected ITS PCR sequencing confirmed the presence of *L. acicola*.	*	It was observed that affected trees were defoliated annually.	Broders *et al.* (2015)
USA, New York	1976	*P. mugo*	*Lecanosticta acicola* was identified with morphological methods and brown spot needle blight symptoms confirmed on trees. Specimens are in the Cornell University Plant Pathology Herbarium.			Sinclair and Hudler (1980)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, North Carolina	1929	*P. echinata, P. palustris, P. rigida, P. taeda, P. virginiana*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, North Carolina	1944	*P. palustris, P. rigida, P. strobus, P. taeda, P. virginiana*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, North Carolina	1957, 1958	*P. strobus*	Morphological identifications of *L. acicola.*			Boyce (1959)
USA, Ohio	1944	*P. contorta* var. *latifolia, P. coulteri, P. jeffreyi*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Oregon	1929	*P. attenuata*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Oregon	1944	*P. attenuata*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Pennsylvania	1929	*P. rigida*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Pennsylvania	1987–1989	*P. strobus*	Morphological identifications were done.			Stanosz (1990)
USA, South Carolina	1876	*P. echinata* (*P. variabilis*)	Morphological description of *Cryptosporium acicolum.*			de Thümen (1878)
USA, South Carolina	1929	*P. caribaea, P. echinata, P. palustris, P. serotina, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, South Carolina	1944	*P. caribaea, P. palustris, P. taeda*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Tennessee	1929	*P. rigida, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Tennessee	1944	*P. palustris, P. ponderosa* var. *scopulorum, P. rigida, P. taeda*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Texas	1929	*P. palustris, P. taeda*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)
USA, Texas	1929	*P. palustris, P. taeda*	Symptoms were observed on trees inside and surrounding the nurseries.		Low severity recorded. Nursery beds were sprayed with Bordeaux 4-4-50 with good results.	Webster (1930)
USA, Texas	1944	*P. caribaea, P. palustris, P. pinaster, P. taeda*	Siggers reported on *Lecanosticta* isolates that are in the collections in the Division of Forest Pathology in Louisiana and Maryland, USA. These reports should be verified.			Siggers (1944)
USA, Vermont	2008	*P. mugo, P. resinosa, P. sylvestris, P. strobus*	Forest surveys were conducted and the pathogen identified based on symptomology.			Gibbs and Sinclair (2008)
USA, Vermont	2011	*P. strobus*	Isolates were collected and morphologically identified in a survey. These isolates were later identified with *TEF 1* sequencing and both mating types were detected.	*		Munck *et al.* (2012), Janoušek *et al.* (2016)
USA, Vermont	2011–2012	*P. strobus*	*Lecanosticta acicola* was identified as part of a complex of pathogens that cause WPND. Morphological identifications and selected ITS PCR sequencing confirmed the presence of *L. acicola.*	*	It was observed that affected trees were defoliated annually.	Broders *et al.* (2015)
USA, Virginia	1929	*P. rigida*	Hedgcock reported on collections of the pathogen at the office of Forest Pathology at Washington, D.C. and the Mycological collections of the US Department of Agriculture.			Hedgcock (1929)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
USA, Wisconsin	1966–1970	*P. sylvestris*	A forest survey was conducted and symptoms of *L. acicola* was observed.		Approximately 3000 acres in 55 plantations were severely infected. Short leaf French and Spanish *P. sylvestris* were severely affected. Long leaf *P. sylvestris* varieties were reported as resistant.	Prey and Morse (1971)
USA, Wisconsin	1967–1971	*P. sylvestris*	Symptoms were observed in the field and the proportion of needles affected were noted. In some cases, microscopic examinations of conidia were used for identification.			Skilling and Nicholls (1974)
USA, Wisconsin	1970	*P. resinosa*	Symptoms were observed in the field and morphological identifications were made.		After the pathogen was observed in pine stands, an inoculation trial revealed that *P. resinosa* is highly susceptible to *L. acicola*.	Nicholls and Hudler (1972)
USA, Wisconsin	2010	*P. sylvestris*	*TEF 1* sequencing used for identification, mating type 2 was detected.	*		Janoušek *et al.* (2016)
Lecanosticta brevispora						
Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*		van der Nest *et al.* (2019)
Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	2010	*P. pseudostrobus*	Multigene phylogenetic analysis.	*		van der Nest *et al.* (2019)
Guatemala, Lugar, La Soledad, Jalapa site II	2012	*P. oocarpa*	Multigene phylogenetic analysis.	*		van der Nest *et al.* (2019)
Honduras	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*		van der Nest *et al.* (2019)
Mexico	2000	*Pinus* sp.	Multigene phylogenetic analysis.	*		Quaedvlieg *et al.* (2012)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Lecanosticta gloeospora						
Mexico, Nuevo León, Iturbide-Galeana	1983	*P. pseudostrobus*	Morphological identification. The type was later sequenced using multiple genes (van der Nest *et al.*, 2019).	*		Evans (1984), Marmolejo (2000), van der Nest *et al.* (2019)
Lecanosticta guatemalensis						
Guatemala, Baja Verapaz	1983	*P. oocarpa*	The type culture was previously identified as *L. acicola* based on morphological characteristics (Evans, 1984). Multigene phylogenetic analysis revealed it as a new species, *L. guatemalensis.*	*		Quaedvlieg *et al.* (2012), van der Nest *et al.* (2019)
Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Chiquimula	2011	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Jalapa, Finca Forestal Soledad	2012	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Coban, San Juan Chamelco	2012	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Nicaragua	1982	*P. tecunumanii*	This isolate was previously identified as *L. acicola* based on morphological characteristics (Evans, 1984). Multigene phylogenetic analysis revealed it to be *L. guatemalensis.*	*		van der Nest *et al.* (2019)
Nicaragua, Matagalpa	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Honduras, Yoro	1981	*P. caribaea, P. oocarpa*	These isolates were previously identified as *L. acicola* based on morphological characteristics (Evans, 1984). Multigene phylogenetic analysis revealed it to be *L. guatemalensis.*	*		van der Nest *et al.* (2019)
Lecanosticta jani						
Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Chiquimula	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Jalapa, Finca Forestal Soledad	2012	*P. maximinoi*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	2012	*P. tecunumanii*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)

Table 1 (Continued)

Country, region, locality	Year collected	Host	Identification method and additional notes	Identification verified using molecular methods (*)	Reported severity of infections and applied eradication methods	Report references
Nicaragua, Matagalpa	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Lecanosticta pharomachri						
Guatemala, Baja Verapaz, San Jerónimo, Salamá	2012	*P. tecunumanii*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	2010–2012	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Honduras	2010	*P. oocarpa*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Lecanosticta tecunumanii						
Guatemala, Baja Verapaz, San Jerónimo, Salamá	2012	*P. tecunumanii*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Lecanosticta variabilis						
Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	2010	*P. oocarpa*	Multigene phylogenetic analysis. Both mating types were present (Janoušek *et al.*, 2016).	*	Very low.	van der Nest *et al.* (2019)
Guatemala, Jalapa, Finca Forestal Soledad	2012	*P. maximinoi*	Multigene phylogenetic analysis.	*	Very low.	van der Nest *et al.* (2019)
Honduras, Santa Barbara, Lago de Yojoa	1984	*P. caribaea*	This isolate was previously identified as *L. acicola* in a morphological study by Evans (1984). A multigene phylogenetic analysis indicated that this is a new species, *L. tecunumanii*.	*	Very low.	Evans (1984), van der Nest *et al.* (2019)
Mexico	2000	*Pinus* sp.	Multigene phylogenetic analysis.	*		van der Nest *et al.* (2019)
Mexico	2010	*P. arizonica* var. *stormiae, P. halepensis*	Multigene phylogenetic analysis. The isolates were previously identified as *L. acicola* (Janoušek *et al.*, 2016) and both mating types were detected.	*		van der Nest *et al.* (2019)

Central America based on morphological characteristics (Evans, 1984), it is now recognized as a Northern Hemisphere pathogen for which phylogenetic analyses of the translation elongation factor 1-α gene (*TEF 1*) sequences have revealed three distinct lineages (van der Nest *et al.*, 2019). One of these lineages includes isolates from Canada, the northern parts of the USA (Maine, Michigan, New Hampshire, Vermont and Wisconsin) and Central and Northern Europe (Austria, Croatia, Czech Republic, Estonia, Germany, Italy, Lithuania, Slovenia, Switzerland) (van der Nest *et al.*, 2019). A second lineage includes isolates from China, Colombia, France, Japan, Spain, South Korea and the southern part of the USA (Mississippi) (van der Nest *et al.*, 2019). A third lineage includes isolates only from Mexico (van der Nest *et al.*, 2019).

The eight other species described in *Lecanosticta* during the course of the past 35 years are present only in Mesoamerica (Tables 1 and 2) (Evans, 1984; Marmolejo, 2000; Quaedvlieg *et al.*, 2012; van der Nest *et al.*, 2019). Evans (1984) recognized

Table 2 A summarized history of the taxonomy and nomenclature of the genus *Lecanosticta*.

Year	Species epithet	Reference	Sexual state reported	Country, location	Host	Description	Notes
Lecanosticta acicola							
1878	*Cryptosporium acicolum Thüm*	de Thümen (1878)	Asexual	USA, South Carolina, Aiken	*Pinus echinata* (*P. variabilis*)		*P. variabilis* is a synonym of *P. echinata*. In Wolf and Barbour (1941), it was mentioned that it was in fact on *P. caribaea* and that the host was previously incorrectly identified.
1884	*Septoria acicola* (Thüm) Sacc	Saccardo (1884)	Asexual	USA, Carolina, Aiken	*P. variabilis*	Saccardo moved *C. acicolum* to *Septoria* due to the characteristic septate conidia.	
1922	*Lecanosticta pini*	Sydow and Petrak (1922)	Asexual	USA, Arkansas and Oregon	*P. taeda* and *P. palustris* in Arkansas and *P. attenuata* in Oregon	The genus *Lecanosticta* was erected to accommodate *L. pini,* a fungus with erumpent stromata and pigmented conidia.	
1924	*Lecanosticta acicola*	Sydow and Petrak (1924)	Asexual	USA	–	The authors recognized that *L. pini* was *C. acicolum*. The genus was retained and the name *L. acicola* was proposed as the valid name.	
1926	*Oligostroma acicola*	Dearness (1926, 1928)	Sexual	USA, Florida, Silver Springs	*P. palustris*	The sexual state of *L. acicola* was isolated from old pine needles from which the asexual state was previously isolated. In 1928, it was proposed that the asexual state *Septoria acicola* fits better in the genus *Cryptosporium* and that *Oligostroma acicola* could be *Cryptosporium acicolum*'s sexual state (Dearness, 1928).	
1939	*Schirria acicola*	Siggers (1939)	Sexual	USA, Arkansas, Florida, Georgia, Louisiana, North Carolina, Texas	*P. palustris, P. taeda, P. thunbergii*	Ascospores as well as conidia were plated onto media and morphologically examined to come to the conclusion that the sexual and asexual state are connected. *Oligostroma acicola* was changed to *Schirria acicola* as erumpent acervuli were observed, characteristic of *Schirria*.	

Table 2 (Continued)

Year	Species epithet	Reference	Sexual state reported	Country, location	Host	Description	Notes
1941	*Systremma acicola*	Wolf and Barbour (1941)	Sexual	USA	*Pinus* spp.	It was recognized that the pathogen was better suited in the Dothideaceae and therefore the fungus was moved to the genus *Systremma* and all the above names for the sexual state synonymized with *S. acicola*.	
1967	*Dothistroma acicola*	Shishkina and Tsanava (1967)	Asexual	–	–	The name *D. acicola* was incorrectly assigned to both *D. pini* and *L. acicola*. The name was not used in subsequent literature.	Due to similarities between symptoms caused by *Dothistroma* and *Lecanosticta* the asexual states of *D. pini* (presently *D. septosporum*) and *L. acicola* were synonymized and renamed as *D. acicola* and furthermore associated with the sexual state *Systremma acicola*.
1972	*Mycosphaerella dearnessii*	Barr (1972)	Sexual	USA	–	*Systremma acicola* was synonymized with *Mycosphaerella dearnessii*. *Mycosphaerella dearnessii* was assigned as the type for *Mycosphaerella* subgenus *Mycosphaerella* section *Caterva*.	MycoBank accession number: 318138.
1996	*Eruptio acicola*	Barr (1996)	Sexual	–	–	According to Barr (1996), *Mycosphaerella dearnessii* did not fit the description of *Mycosphaerella* and the new genus *Eruptio* M.E. Barr was erected to accommodate *Eruptio acicola* and *Eruptio pini* (sexual state of *Dothistroma septosporum*).	The validity of the genus *Eruptio* was questioned as *Lecanosticta acicola* phylogenetically clusters with other *Mycosphaerella* anamorphs (Crous *et al.* 2001). *Eruptio* is not widely used in literature.
2012	*Lecanosticta acicola*	Quaedvlieg *et al.* (2012)	Both	Europe and North America	*Pinus* spp.	As it was recognized that the genus *Mycosphaerella* should only be used with the anamorphic genus *Ramularia* (Crous *et al.*, 2007; Crous, 2009), *Lecanosticta acicola* was selected as the valid name and type of the genus under the one fungus = one name rule. An epitype was designated for *L. acicola* in this study (Quaedvlieg *et al.*, 2012).	MycoBank accession number: 255702 Epitype CBS H-21113, Ex-epitype CBS 133791.

Table 2 (Continued)

Year	Species epithet	Reference	Sexual state reported	Country, location	Host	Description	Notes
Other *Lecanosticta* species							
1984	*Lecanosticta cinerea*	Evans (1984); Marmolejo (2000)	Asexual	Honduras	*Pinus* sp.	The species name was proposed as the correct name for *Gloeocoryneum cinereum*.	This name is not validly published (Marmolejo, 2000) as no basionym was established. The new combination *Leptomelanconium pinicola* was proposed because it had previously been established that *Gloeocoryneum cinereum* is a synonym of *Stilbospora pinicola* and that the genera *Gloeocoryneum* and *Leptomelanconium* have the same characteristics (Marmolejo, 2000).
1984	*Lecanosticta gloeospora*	Evans (1984)	Asexual	Mexico, Iturbide-Galeana, Nuevo León	*P. pseudostrobus*	The second valid species in the genus to be described based on morphological characteristics.	MycoBank accession number: 106975 Holotype IMI 283812 Ex-type IMI 283812.
2000	*Lecanosticta longispora*	Marmolejo (2000)	Asexual	Mexico, Nuevo León	*P. culminicola*	The third species described in the genus based on morphological characteristics.	No type was assigned but Quaedvlieg *et al.* (2012) epitypified the species in 2012.
2012	*Lecanosticta longispora*	Quaedvlieg *et al.* (2012)	Asexual	Mexico, Nuevo León	*P. culminicola*	The first phylogenetic study to include this species. An epitype *L. longispora* was designated here.	MycoBank accession number: 466255 Epitype CBS H-21111, Ex-epitype CBS 133602.
2012	*Lecanosticta brevispora*	Quaedvlieg *et al.* (2012)	Asexual	Mexico	*Pinus* sp.	This isolate is the fourth species described in the genus based on phylogenetic inference and morphology.	MycoBank accession number: 801940 Holotype CBS H-21110, Ex-type CBS 133601.
2012	*Lecanosticta guatemalensis*	Quaedvlieg *et al.* (2012)	Asexual	Guatemala	*P. oocarpa*	The ex-type of *L. guatemalensis* (IMI281598) was initially described as *L. acicola* based on morphological characteristics (Evans, 1984). The isolate was phylogenetically delineated as a new species in 2012 and subsequently described (Quaedvlieg *et al.*, 2012).	MycoBank accession number: 801941 Holotype CBS H-21108, Ex-type IMI 281598.
2018	*Lecanosticta jani*	van der Nest *et al.* (2019)	Asexual	Guatemala, Nicaragua	*P. maximinoi, P. oocarpa, P. tecunumanii*	New species described based on phylogenetic and morphological data.	MycoBank accession number: 826875 Holotype PREM 62185, Ex-type CBS 144456.

Table 2 (Continued)

Year	Species epithet	Reference	Sexual state reported	Country, location	Host	Description	Notes
2018	*Lecanosticta pharomachri*	van der Nest *et al.* (2019)	Asexual	Guatemala, Honduras	*P. oocarpa, P. tecunumanii*	New species described based on phylogenetic and morphological data.	MycoBank accession number: 826876 Holotype PREM 62188, Ex-type CBS 144448.
2018	*Lecanosticta tecunumanii*	van der Nest *et al.* (2019)	Asexual	Guatemala	*P. tecunumanii*	New species described based on phylogenetic and morphological data.	MycoBank accession number: 826877 Holotype PREM 62191, Ex-type CBS 144450.
2018	*Lecanosticta variabilis*	van der Nest *et al.* (2019)	Asexual	Guatemala, Honduras	*P. caribaea, P. maximinoi, P. oocarpa*	New species described based on phylogenetic and morphological data. The ex-type was initially described as *L. acicola* by Evans (1984) based on morphological observations.	MycoBank accession number: 826878 Holotype PREM 62195, Ex-type CBS 144453 = IMI 281561.

considerable morphological variation amongst his collections of *L. acicola*. In that study, he described a second species, *L. gloeospora* from *Pinus pseudostrobus* in Mexico, and the fungus remains known only from Mexico on this host (Evans, 1984; Marmolejo, 2000). The novelty of this species was recently validated using DNA sequence data (van der Nest *et al.*, 2019).

Lecanosticta longispora was first described based on morphological features from *P. culminicola* in Nuevo León, Mexico (Marmolejo, 2000). This species was characterized in a phylogenetic study by Quaedvlieg *et al.* (2012), and was distinguished from *L. acicola* based on differences in the *TEF 1* and β-tubulin 2 (*BT 2*) gene sequences. That study was the first to delineate species of *Lecanosticta* based on phylogenetic inference (Quaedvlieg *et al.*, 2012). These authors included several samples from Central America that had previously been identified as *L. acicola,* as well as the collection used by Marmolejo (2000) to typify *L. longispora*. In their phylogenetic analyses (Quaedvlieg *et al.*, 2012), *L. acicola* was not identified from Central America but two new species, *L. brevispora* and *L. guatemalensis*, were described (Tables 1 and 2).

Evans (1984) observed that ecotypes or morphotypes exist amongst isolates of *L. acicola* in Central America, depending on the altitude and hosts from which the isolations were made. He therefore hypothesized that Central America could be the centre of origin of *Lecanosticta*. This was later supported by analysis of *TEF 1* sequence data that revealed high genetic diversity in this geographical region (Janoušek *et al.*, 2016). An extensive collection of isolates from Central America was recently studied using a phylogenetic approach (van der Nest *et al.*, 2019). Interestingly, *L. acicola* was not identified amongst isolates from Guatemala, Nicaragua or Honduras. Furthermore, the isolates considered to be *L. acicola* by Evans (1984) were sequenced and identified as *L. guatemalensis* and a new species, *L. variabilis* (van der Nest *et al.*, 2019, Table 1). *Lecanosticta brevispora* was identified in Guatemala and Honduras on *Pinus oocarpa* and *P. pseudostrobus* (Table 1), expanding the host range and distribution for that species. Likewise, *L. guatemalensis* was also identified in Guatemala, Honduras and Nicaragua on *P. caribaea, P. oocarpa* and *P. tecunumanii* (Table 1). The study of van der Nest *et al.* (2019) introduced four new species, including *Lecanosticta jani* from Guatemala and Nicaragua, *L. pharomachri* from Guatemala and Honduras, *L. tecunumanii* from Guatemala and *L. variabilis* from Mexico, Guatemala and Honduras (van der Nest *et al.*, 2019). Although Central America could not be confirmed as a centre of origin of *L. acicola,* the diversity of species recognized by van der Nest *et al.* (2019) suggests strongly that Mesoamerica is a centre of diversity for *Lecanosticta*.

With only one exception, which is probably a taxonomic incongruity, *Lecanosticta* species are all associated with *Pinus* species. Petrak (1954) described *Phragmogloeum gaubae* on *Callistemon sieberi* in Australia (Petrak, 1954). von Arx (1983) attempted to reduce various species with overlapping characteristics to fewer genera and found that *Phragmogloeum* had the same morphological

Fig. 2 Symptoms of *Lecanosticta acicola*. (A) *Pinus mugo* in Austria displaying symptoms of both brown spot needle blight (BSNB) and Dothistroma needle blight (DNB) on the same branches. (B) Both the characteristic brown spots associated with BSNB (black arrow) and the red banding associated with DNB (white arrow) can be observed. (C)–(E) Symptoms of BSNB vary from only brown spots as observed on *P. mugo* (C) to distinct brown bands as observed on *P. radiata* (D) to irregular mosaic spots as observed on *P. palustris* (E). (F) *Lecanosticta acicola* conidiogenous cells giving rise to conidia on malt extract agar. (G) *Lecanosticta acicola* septate conidia with verruculose surfaces and truncate bases.

characteristics as *Lecanosticta*. He proposed the new combination *Lecanosticta gaubae*. After the genus *Eruptio* was erected to accommodate *Lecanosticta acicola* and *Dothistroma septosporum* (Barr, 1996), *Lecanosticta gaubae* was transferred to that new genus (Crous, 1999). The genus *Eruptio* was further evaluated and it was found that *L. acicola* and *D. septosporum* were not congeneric (Crous, 2009). Consequently, *Lecanosticta* was selected as the correct name for *Eruptio acicola* following the one fungus one name convention (Crous *et al*., 2009; Hawksworth *et al*., 2011). Because *Eruptio gaubae* is morphologically similar to *Lecanosticta*, phylogenetic analyses are required to resolve this taxonomic confusion.

Lecanosticta acicola is the only species in the genus known to be a significant pathogen. This is particularly important because it is spreading rapidly in Europe and the northeastern parts of North America. Therefore, all data collected over time regarding *Lecanosticta* pertain to the organism that was assigned the name *L. acicola*, and the remainder of the review will focus on this species. However, it is relevant to recognize that other species of *Lecanosticta* cause symptoms similar to those of *L. acicola* and that they have the potential to emerge as pine pathogens if they were accidentally moved to new environments. They would then be recognized as members of a complex of BSNB pathogens.

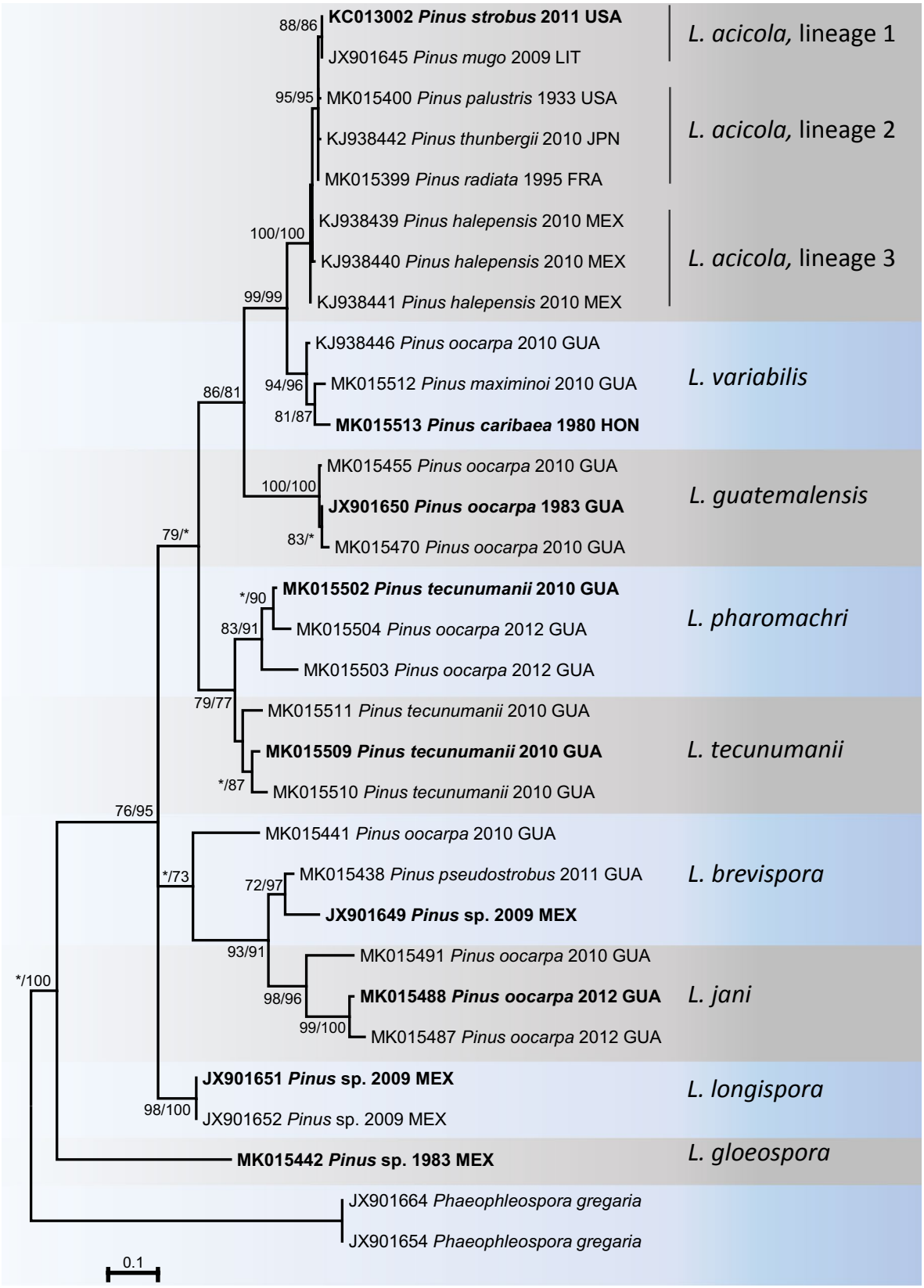
88/86
KC013002 Pinus strobus 2011 USA
JX901645 Pinus mugo 2009 LIT
L. acicola, lineage 1
95/95
MK015400 Pinus palustris 1933 USA
KJ938442 Pinus thunbergii 2010 JPN
MK015399 Pinus radiata 1995 FRA
L. acicola, lineage 2
100/100
KJ938439 Pinus halepensis 2010 MEX
KJ938440 Pinus halepensis 2010 MEX
99/99
KJ938441 Pinus halepensis 2010 MEX
L. acicola, lineage 3
KJ938446 Pinus oocarpa 2010 GUA
94/96
MK015512 Pinus maximinoi 2010 GUA
86/81
81/87
MK015513 Pinus caribaea 1980 HON
L. variabilis
MK015455 Pinus oocarpa 2010 GUA
100/100
JX901650 Pinus oocarpa 1983 GUA
79/*
83/*
MK015470 Pinus oocarpa 2010 GUA
L. guatemalensis
*/90
MK015502 Pinus tecunumanii 2010 GUA
83/91
MK015504 Pinus oocarpa 2012 GUA
MK015503 Pinus oocarpa 2012 GUA
L. pharomachri
79/77
MK015511 Pinus tecunumanii 2010 GUA
MK015509 Pinus tecunumanii 2010 GUA
*/87
MK015510 Pinus tecunumanii 2010 GUA
L. tecunumanii
76/95
MK015441 Pinus oocarpa 2010 GUA
*/73
72/97
MK015438 Pinus pseudostrobus 2011 GUA
JX901649 Pinus sp. 2009 MEX
L. brevispora
93/91
MK015491 Pinus oocarpa 2010 GUA
*/100
98/96
MK015488 Pinus oocarpa 2012 GUA
99/100
MK015487 Pinus oocarpa 2012 GUA
L. jani
JX901651 Pinus sp. 2009 MEX
98/100
JX901652 Pinus sp. 2009 MEX
L. longispora
MK015442 Pinus sp. 1983 MEX
L. gloeospora
JX901664 Phaeophleospora gregaria
JX901654 Phaeophleospora gregaria
0.1

Fig. 3 Maximum likelihood (ML) tree representing the nine known species of *Lecanosticta* as well as the three lineages of *L. acicola* generated from the translation elongation 1-α region. ML bootstrap support (>70%) are indicated first, followed by maximum parsimony (MP) bootstrap support values (ML/MP, * = insignificant value). *Phaeophleospora gregaria* was used as the outgroup taxa. All represented type species are indicated in bold.

SYMPTOMS OF BROWN SPOT NEEDLE BLIGHT

Symptoms of infection can vary depending on the host species affected. Typically, a small and yellow, sometimes light grey-green or reddish brown, irregular circular spot, with defined margins, appears at the point of infection (Hedgcock, 1929) (Fig. 2C–E). These spots soon become brown as the infections mature and they are often surrounded by a yellow halo (Skilling and Nicholls, 1974). In severe cases, infections can occur on several parts of a needle, leading to more rapid necrosis (Fig. 2E). The characteristic brown spots are the first conspicuous symptoms on the pine needles and this has led to the common name 'brown spot needle blight' proposed by Siggers (1932). These brown spots can also appear resin-soaked depending on the host species (Skilling and Nicholls, 1974). In some cases, as has been reported in *P. strobus*, symptoms may only be displayed as chlorosis of the needles without banding (Broders *et al.*, 2015). Infected needles die from the apex to the base (Fig. 2B) and they are eventually shed from the trees (Hedgcock, 1929; Skilling and Nicholls, 1974). Usually only the second- and third-year needles are affected, leaving healthy new growth at the tips of the branches. The new growth tips are then infected in the subsequent season by inoculum on older needles (Skilling and Nicholls, 1974). Generally, infection is more severe in the lower parts of the canopy and then progresses upwards in the trees (Sinclair and Lyon, 2005; Skilling and Nicholls, 1974).

An asymptomatic phase in which *L. acicola* establishes within needles can last several days (Setliff and Patton, 1974) to 3 months (Skilling and Nicholls, 1974). This is dependent on the strain of the pathogen (Kais, 1972) and length of the wet season. This delay in symptom development could lead to the accidental movement of infected plants to new areas.

The symptoms of BSNB (Fig. 2) can easily be confused with those of DNB, which is caused by *Dothistroma septosporum* and *D. pini* (Barnes *et al.*, 2004, 2016). On some host species, symptoms of DNB are similar to those of BSNB (Fig. 2B) but rather than the characteristic brown discoloration and spots, a distinct red band forms around the point of infection in the case of DNB (Pehl and Cech, 2008). However, in some cases the characteristic red banding pattern associated with DNB is not formed or alternatively the red bands are sufficiently dark to give a false impression of brown spots. This can easily lead to incorrect pathogen diagnoses (Barnes *et al.*, 2016; Petrak, 1961).

LIFE CYCLE

Lecanosticta acicola can occur in either its asexual or sexual state (Fig. 1) (Siggers, 1939). The pathogen overwinters in acervuli (asexual) (Fig. 1Aa) or ascostromata (sexual) (Fig. 1Ba) in the dead tissue of either dead or living pine needles. It can also overwinter as vegetative mycelium in the infected needles that remain attached to the host (Siggers, 1944). Conidia are released in gelatinous masses (Fig. 1Ab) or ascospores are released from asci in ascostromata (Fig. 1Bb) on the needles when the light, temperature and humidity are favourable (Kais, 1975; Tainter and Baker, 1996).

Conidia begin to germinate on the needle surfaces by developing one to four germ tubes, depending on the number of cells in the conidia (Setliff and Patton, 1974). It is uncertain whether the germ tubes are attracted to the stomata, or whether they grow randomly over the needle surface (Patton and Spear, 1978; Setliff and Patton, 1974). Light plays an indirect, but essential role in the infection process as it stimulates the opening of stomata, allowing the germ tube to penetrate the needle (Fig. 1c) (Kais, 1975). Infections can also occur through wounds (Kais, 1978). Once a germ tube enters the stomatal antechamber, it increases in diameter and becomes thick-walled and melanized (Patton and Spear, 1978). Appresoria, such as those found in *Dothistroma* (Gadgil, 1967), have never been seen (Patton and Spear, 1978).

Once the mesophyll tissue has been invaded by *L. acicola* mycelium, conidiomata begin to form. These begin to integrate with the needle tissue and increase in size until they are visible to the naked eye (Wolf and Barbour, 1941). The conidiophores produce conidia towards the leaf exterior (Evans, 1984), which exerts pressure on the needle epidermis. This causes the epidermis to rupture, leaving a flap that partly covers the conidiomata (Wolf and Barbour, 1941). The conidia are released from the conidiomata during wet weather and the disease cycle is repeated.

In the case of the sexual state, asci are formed within the ascostromata on necrotic distal parts of living needles or on dead needles (Henry, 1954; Jewell, 1983). Ascospores are released from asci and dispersed through wind and rain. Asci and ascospores develop more rarely than conidia and have been reported only from Nicaragua, Honduras, Colombia and the southern parts of the USA (Table 1) (Evans, 1984; Henry, 1954; Kais, 1971; Luttrell, 1949; Siggers, 1944). The reports from Nicaragua and Honduras probably represent species other than *L. acicola.*

TOXIN PRODUCTION

Many plant pathogenic fungi have adapted to produce toxic secondary metabolites in their plant hosts and these could influence colonization and sporulation, as has been seen in *D. septosporum* (Kabir *et al.*, 2015). *Lecanosticta acicola* is known to produce the toxic compounds LA-I and LA-II, which are heat-resistant and non-host specific phytotoxins (Yang *et al.*, 2002, 2005). The two

compounds interact with the host independently and do not promote or inhibit the interaction of one another (Yang *et al.*, 2002). Different *Pinus* species have different reactions to LA-I and LA-II. When rooted cuttings of *P. thunbergii* were exposed to the toxin, they showed little sensitivity to it. In contrast, when *P. elliottii* and *P. taeda*, both highly susceptible to BSNB infection, were exposed to the toxin, the results showed high sensitivity to LA-I (Ye and Qi, 1999). It seems likely that these toxins are involved in the destruction of mesophyll tissue of the pine needles at the point of infection (Jewell, 1983).

BIOLOGY AND DISSEMINATION

Conidia and ascospores are released throughout the year at temperatures ranging from –5.5 to 28 °C (Kais, 1971; Siggers, 1944; Wyka *et al.*, 2018). However, warm and wet weather is particularly conducive for the development of BSNB, irrespective of whether infection takes place by sexual or asexual spores. The conidia do not germinate below 5 °C, although most survive this temperature and commence germination once the temperature increases (Siggers, 1944). At the other extreme, tolerance to high temperature was found to vary depending on the strain of *Lecanosticta* involved. It was shown that conidia of isolates from the northern parts of the USA could not germinate at 32 °C, whereas cultures isolated from the southern parts of the USA, as well as China, had a germination success of 80% at the same temperature (Huang *et al.*, 1995). This physiological distinction is reflected in population genetic studies which define two lineages of the pathogen in the USA (Janoušek *et al.*, 2016). The success of the pathogen may therefore be a result of isolates in each lineage adapting to local temperature conditions.

The maximum temperature for the germination of *L. acicola* conidia is 35 °C (Siggers, 1944). It was also found that high humidity pre- and post-infection is required for high levels of infection (Kais, 1975). The optimal temperature for infection to occur is 30 °C during the day and 21 °C at night, and Kais (1975) showed that these temperatures gave positive results in inoculation trials.

Conidia are dispersed predominantly by rain splash to adjacent trees, and they contribute significantly to rapid disease build-up in pine stands (Tainter and Baker, 1996). High levels of conidial dispersal were recorded during the rainy season in the USA, especially between late spring and summer, as well as when there were rain spells after a long period of dryness (Kais, 1971). In other reports, conidial production and dispersal were recorded throughout the year (Siggers, 1944). Dispersal was not influenced by the temperature range but conidial release was connected to rainfall patterns. In Wisconsin, two peaks of conidial release were recorded, with the first peak in early summer when young pine needles are present and the second in late summer (Skilling and Nicholls, 1974), which was similar to that found in the northeastern USA (Wyka *et al.* 2018). In Japan, it was found that conidia were produced by the pathogen from early spring to autumn with peak dispersal in mid-summer. However, for a second year of infection, the dispersal was most abundant from late summer to mid-autumn the following year (Suto, 2002). A study in Fujian province (China) showed that the greatest number of conidia were detected between early spring and mid-summer and again in late summer to late autumn in *Pinus elliottii* plantations (Li *et al.*, 1987). It consequently appears that conidial dispersal varies depending on the rainfall season in any particular geographical region.

Spore traps in several studies failed to capture ascospores (Kais, 1971; Siggers, 1939; Wyka *et al.*, 2018). It was found, however, that conidia could be dispersed to a distance of up to 60 m (Wyka *et al.* 2018). A recent investigation of the dispersal of *Dothistroma*, where the mechanisms of conidial and ascospore dispersal are similar to those in *L. acicola*, showed that conidia could be naturally disseminated over more than 1 km (Mullett *et al.*, 2016). The assumed distance of dispersal in *L. acicola* may, consequently, be similar.

The ascospores of *L. acicola* are forcibly expelled into the air (Wolf and Barbour, 1941) and dispersed by wind currents (Kais, 1971) or rain splash driven by wind (Siggers, 1939). Ascospores can also be released during periods of fog, rain and dew (Tainter and Baker, 1996). Ascospores were recorded in the USA mainly during periods when temperatures were above 15 °C and are found in late summer to autumn. Small numbers of ascospores were detected when temperatures were below 10 °C (Kais 1971).

The main component that facilitates spread of conidia and ascospores is moisture, but other factors may also aid in their dispersal. Insect dissemination was suggested as a mechanism of conidial spread when two Lepidopteran wing scales were found to have conidia attached to them (Skilling and Nicholls, 1974). Given the biology of *L. acicola*, it seems unlikely that insects are involved in its dissemination. It has also been suggested that animals grazing in forests might aid in dissemination of the conidia when spores stick to their coats or hooves (Skilling and Nicholls, 1974; Tainter and Baker, 1996). Again, this mode of dissemination seems unlikely to be particularly important.

Anthropogenic movement of infected plant material has contributed to the dissemination of many tree pathogens (Wingfield *et al.*, 2015). This has been clearly demonstrated for *Dothistroma septosporum* (Barnes *et al.*, 2014), which has a biology very similar to that of *L. acicola*. A study that used microsatellite markers has demonstrated that two separate lineages of *L. acicola* have most likely been introduced into Europe from North America (Janoušek *et al.*, 2016). Long distance dispersal of *L. acicola* is, therefore, likely to be the result of anthropogenic movement of infected plant material. This would not include seed transmission as *L. acicola* conidia cannot survive on a pine seed's surface

longer than 30 to 34 days and it is thus not considered seed-borne (Jianren and Chuandao, 1988).

DISEASE MANAGEMENT

Several measures have been suggested to prevent BSNB during plantation establishment. The most effective is to plant disease-free seedlings of superior quality (Cordell *et al*., 1990; Skilling and Nicholls, 1974). It is also advisable to avoid establishing new plantations alongside old, infected pines that could potentially serve as reservoirs of inoculum (Tainter and Baker, 1996). For natural pine stands, the application of thinning treatments was investigated as a silvicultural practice against pine needle diseases (McIntire *et al*., 2018). This practice, conducted on native stands of *P. strobus* in the USA, showed promise in reducing the fungal load of *L. acicola*, resulting in reduced severity of the disease over time in stands already infected with the pathogen (McIntire *et al*., 2018). This practice is recommended as a preventative measure in stands that are at risk of infection by *L. acicola* and other pine needle pathogens (McIntire *et al*., 2018).

Pruning of infected pines can contribute to the spread of BSNB if it is conducted during rainy or wet periods. This is because conidia are exuded during these conditions and can attach to the pruning shears, providing a means of spread from infected to healthy trees (Skilling and Nicholls, 1974). Cutting blades should be cleaned during pruning and clipped needles and shoots should be removed (Kais, 1978). In the case of infection on *Pinus palustris*, which begins growth as a grass stage, stimulation of growth during the first 3 years of growth reduces the levels of infection (Tainter and Baker, 1996). Because this treatment is economical, effective and environmentally safe, it is widely used in the southeastern USA (Cordell *et al*., 1990), where BSNB occurs on *P. palustris*.

Breeding for resistance to *L. acicola* has been successfully used to reduce the impact of the disease on *P. palustris* in Alabama. The source population of these trees found in south-western Alabama was used in breeding programmes (Snyder and Derr, 1972) where seed was made available to the public (Phelps *et al*., 1978). Since 1982, resistant phenotypes of *P. elliottii* have also been selected for in plantations affected by BSNB in the Fujian province in China. Over time, and using artificial inoculations, resistant clones were selected and resistant seed orchards were established (Ye and Wu, 2011).

Fungicide treatment can protect pine seedlings from infection by *L. acicola.* For example, when *P. palustris* was sprayed with fungicide, the seedlings displayed increased diameter growth in a single growing season, compared to untreated plants (Siggers, 1932). Seedlings, seed orchard trees and Christmas tree plantations have been protected by Bordeaux mixture of copper sulphate and lime, which inhibits conidial germination, by a benomyl root treatment or by ferbam (Fermate®). Chlorothalonil, a broad-spectrum organochlorine pesticide (products include Bravo®, Daconil® and Maneb®), has also been applied to provide efficient control against BSNB. Chlorothalonil is also very effective against *Lophodermium* needle cast, which could be advantageous when both pathogens are present (Cordell *et al*., 1990; Kais *et al*., 1986; Skilling and Nicholls, 1974). Practical details and recommendations concerning fungicide treatment can be found in Skilling and Nicholls (1974). However, the use of chemicals is not considered a desirable solution for disease control due to negative environmental factors and many of these treatments are no longer available.

Controlled burning in pine forests can eliminate competing vegetation and reduce the impact of needle pathogens, especially in *P. palustris* where a grass stage is relevant (Barnett, 1999; Chapman, 1932). This pine species is completely adapted to survive fires as it concentrates all its energy into root development during the first 5 years of growth (Chapman, 1932). Siggers (1934) showed that a single controlled fire can significantly decrease BSNB in *P. palustris* until the next season and that during the initial growth stage, before seedlings begin to increase in height, a winter burn every 3 years is the most beneficial for disease control. The efficacy of controlled burns differs depending on the *Pinus* spp. involved and on the ability to tolerate fire damage.

In countries and regions where *L. acicola* is a quarantine organism, it is suggested that complete eradication of diseased trees or pine stands should be performed once the disease is detected (Pehl and Cech, 2008). This is achieved by felling and burning of infected trees and litter found under infected trees (Sosnowski *et al*., 2009). In Lithuania, for instance, after positive identification of the pathogen in the Curonian Spit in 2009 and 2010, effective eradication measures were implemented (Markovskaja *et al*., 2011). Due to this rapid action, the disease has remained under control in that country and is under constant monitoring by the state plant service of the Ministry of Agriculture of Lithuania (https://gd.eppo.int/taxon/SCIRAC/distribution/LT). Eradication efforts are, however, not always effective, and the best preventative method is to limit the movement of plant material across borders and between regions. As new knowledge is emerging regarding different genetic entities of the pathogen, including strains of different mating types (Sadiković *et al*., 2019), the importance of avoiding new introductions is becoming increasingly obvious.

HOST RANGE, HOST SUSCEPTIBILITY AND GEOGRAPHIC DISTRIBUTION

In an effort to consolidate 140 years of literature with regards to the geographical distribution and host range of *L. acicola*, a detailed list of these data has been compiled (Table 1). This shows that the pathogen has been reported in 31 countries and on 53

pine species and pine hybrids. The majority of the host records on native and non-native trees are from the Americas, followed by Europe. The pathogen has not been found in Africa, Australia or New Zealand and in South America it is known only in Colombia. Of the 69 reports of the pathogen (Table 1), 31 were made in the last decade (2009–2019). This suggests that incidences of the pathogen are most likely increasing.

In North America, the first report of *L. acicola* was in 1876 on native *Pinus echinata* as *Cryptosporium aciculum* (de Thümen, 1878). Since then, the pathogen has been reported in the USA on several susceptible species, including non-native *P. caribaea* and *P. pinea*, and native *P. elliottii*, *P. echinata*, *P. glabra*, *P. ponderosa*, *P. rigida*, *P. taeda* and *P. virginiana* (Hedgcock, 1929; Siggers, 1944; Sinclair and Lyon, 2005; Webster, 1930) as well as on regionally planted exotic species such as *P. attenuata*, *P. coulteri*, *P. muricata* and *P. sabiniana* (Siggers, 1944). *Pinus palustris* seedlings are the most severely affected, largely due to the grass stage associated with early growth and where BSNB can cause complete defoliation (Siggers, 1934). Here it can result in mortality reaching 50% and higher in the southeastern USA (Cordell *et al.*, 1990). New reports of *L. acicola* causing damage on *P. strobus* have emerged since 2005 in the northeastern USA and Canada and these have been attributed to changes in precipitation and climate in the regions (Broders *et al.*, 2015; Wyka *et al.*, 2017, 2018). *Lecanosticta acicola* is also recognized as a component of a complex of pathogens that cause white pine needle damage (WPND) in this region (Broders *et al.*, 2015). Additionally, the pathogen has been reported on *P. banksiana* and *P. contorta* var. *latifolia* in Canada (Laut *et al.*, 1966).

Lecanosticta acicola has been reported from 17 European countries (for a complete list of records see Table 1). The pathogen was first recorded in northern Spain in 1942 (Martínez, 1942), where it still occurs on *P. radiata* (Ortíz de Urbina *et al.*, 2017). In southwest Europe, *L. acicola* has caused severe defoliation of *P. radiata* × *P. attenuata*, leading to the felling of 100 ha in the 1990s (Lévy, 1996). *Lecanosticta acicola* is spreading through the valleys in the Alps in Switzerland (Holdenrieder and Sieber, 1995), Austria (Cech, 1997; Hintsteiner *et al.*, 2012), Italy (La Porta and Capretti, 2000) and Slovenia (Jurc and Jurc, 2010; Sadiković *et al.*, 2019), which can be attributed to high humidity in deep valleys or the proximity of lakes. In Europe, *L. acicola* often infects *P. mugo*, a susceptible species on which it has recently caused severe outbreaks in Austria (https://gd.eppo.int/reporting/article-5139). It also infects other pine species such as *P. sylvestris* and *P. nigra*. The pathogen has been recorded in several peat bog sites in southern Bavaria (Germany) and southern Bohemia (Czech Republic). These locations are naturally humid throughout the year and the susceptible pine species *P. mugo* and/or *P. uncinata* subsp. *uliginosa* can be heavily infected, leading to considerable mortality. Similarly, *L. acicola* was recorded in the Baltic states (Drenkhan and Hanso, 2009) and, most recently, also in Sweden (Cleary *et al.*, 2019). These records usually come from stands close to the sea or, very frequently, from botanical gardens or urban areas.

Other pine species such as *Pinus* × *rhaetica* and *P. ponderosa* have also been affected by *L. acicola* (Adamson *et al.*, 2015, 2018). *Lecanosticta acicola* has been present in Croatia on *P. halepensis* for more than 40 years (Milatović, 1976; Sadiković *et al.*, 2019). Interestingly, the pathogen was identified only at a single site in Ireland despite large-scale screening throughout the British Isles (Mullett *et al.*, 2018). From all these records, it is reasonable to conclude that *L. acicola* is spreading in Europe in native and non-native pine species, in plantations and natural forests, and associated with different climatic conditions.

In Asia, BSNB has been reported in China in plantations of non-native *P. thunbergii*, *P. elliottii* and *P. taeda* where the trees were severely damaged by the pathogen (Huang *et al.*, 1995), and on *P. caribaea*, *P. palustris*, *P. clausa* and *P. echinata* that were reported to be susceptible to infection (Li *et al.*, 1986). It was suggested that native pines such as *P. taiwanensis*, *P. fenzeliana* and *P. massoniana* were highly resistant to infection (Huang *et al.*, 1995; Li *et al.*, 1986). BSNB has been reported on native *P. thunbergii* in Japan (Suto and Ougi, 1998) as well as on native *P. thunbergii* in South Korea but the disease was not severe (Seo *et al.*, 2012).

Although some species of *Pinus* seem to not be susceptible to infection by *L. acicola*, the pathogen has the potential to overcome host resistance in a favourable environment and expand its host range, as is suggested for *D. septosporum* and *D. pini* (Drenkhan *et al.*, 2016). For example, *L. acicola* is rarely reported on native *P. sylvestris* in Europe. Considering the importance of *P. sylvestris* in Europe, it will be important to monitor the presence of the pathogen on this host. Only single incidences of *L. acicola* have been reported on *P. sylvestris* in Austria (Cech and Krehan, 2008), Slovenia (Jurc and Jurc, 2010) and most recently in Estonia (Adamson *et al.*, 2018) and Ireland (Mullett *et al.*, 2018). In contrast, *L. acicola* is an important pathogen of *P. sylvestris* grown as part of the Christmas tree industry since the 1960s in the USA (Skilling and Nicholls, 1974). This implies that under favourable conditions this host could be infected by the pathogen. Investigations on the impact of DNB on *P. sylvestris* revealed that there is high intraspecific variability of *P. sylvestris* in Europe and that susceptibility of the host to the pathogen varies between individuals (Perry *et al.*, 2016a,b) and this could also influence the potential importance of *L. acicola*. Unusually high humidity associated with climate change could increase pathogen pressure on *P. sylvestris* (Perry *et al.*, 2016a) and the single incidences in Europe should carefully be monitored. Caution must also be taken when planting susceptible exotic hosts alongside native forests, as this could influence the vulnerability of native forests (Piotrowska *et al.*, 2018).

Of the 69 reports of *L. acicola*, only 22 used DNA sequence comparisons for species verification. This is of concern as there might be an over- or underestimation of hosts affected by BSNB globally. In Central America, for example, *L. acicola* was reported based on identifications using morphological characters. Because the pathogen has not yet been confirmed as occurring in this region using DNA sequences (Quaedvlieg *et al.*, 2012; van der Nest *et al.*, 2019), those reports could be erroneous and may represent different species which could possibly cause new outbreaks if not contained in their native environment.

MOLECULAR DIAGNOSTICS AND FUTURE PROSPECTS

Molecular markers used for species identification

Three molecular methods are currently being used to accurately identify *L. acicola*. These include sequencing of various gene regions, an ITS-RFLP method and a conventional PCR that uses species-specific primers. The most common of these approaches is comparison of DNA sequences for the ITS gene region (Adamson *et al.*, 2015, 2018; Cleary *et al.*, 2019; Markovskaja *et al.*, 2011; Mullett *et al.*, 2018). However, the *TEF 1* (Fig. 3) and *BT 2* gene regions have been recommended to distinguish between species of the Mycosphaerellaceae (Quaedvlieg *et al.*, 2012). In order to accurately distinguish between different species of *Lecanosticta,* van der Nest *et al.* (2019) used a multi-gene phylogenetic approach using sequences for the ITS, *TEF 1, BT 1, MS204* and *RPB 2* gene regions. The outcome was the discovery of four new species, with the ITS and *TEF 1* proving to be the gene regions showing the best amplification success across all species. Pehl *et al.* (2004) developed an ITS-RFLP method to distinguish between *L. acicola*, *D. septosporum* and ten other plant pathogens. However, whether this method remains valid after the recognition of various new species (van der Nest *et al.*, 2019) will need to be established.

Another rapid method allowing for the identification of *L. acicola*, *D. septosporum* and *D. pini* is a conventional PCR that uses species-specific primers (Ioos *et al.*, 2010). These were developed to partially amplify the *TEF 1* gene for *L. acicola* and *D. pini*, and partially amplify the *BT 2* gene region in *D. septosporum* (Ioos *et al.*, 2010). Importantly, this method can be used to identify the pathogens directly from infected needles (Adamson *et al.*, 2015; Ortíz de Urbina *et al.*, 2017; Schneider *et al.*, 2019) and is now widely used for preliminary identification of *L. acicola* (Adamson *et al.*, 2018; Sadiković *et al.*, 2019). A multiplex qPCR was also recently developed to detect *L. acicola* as well as *Dothistroma* species from needles simultaneously using probe-labelled primers developed by Ioos *et al.* (2010) and Schneider *et al.* (2019), which could become more widely used once that technology is more easily available.

Population genetic studies

Knowledge regarding the population structure and diversity of pathogens such as *L. acicola* allow for an understanding of migration patterns as well various aspects of their invasion biology. Eleven polymorphic microsatellite markers and mating type primers have been developed for this purpose (Janoušek *et al.*, 2014). The first population genetic study using these markers revealed that two lineages of *L. acicola* were introduced into Europe, possibly on two separate occasions (Janoušek *et al.*, 2016). These results are similar to an earlier study where RAPD analysis of *L. acicola*, collected in the northern and southern parts of the USA and China, showed that the Chinese population originated from the southern USA and that the collection from the northern USA was unique (Huang *et al.*, 1995). A second population genetic study compared populations from Croatia and Slovenia and revealed four distinct populations with possible introductions from other sources within the two countries (Sadiković *et al.*, 2019). Currently available knowledge suggests a Northern American centre of origin for this pathogen (Huang *et al.*, 1995; Janoušek *et al.*, 2016; van der Nest *et al.*, 2019) but further sampling and analyses are required to support this hypothesis. In the population genetic study of Janoušek *et al.* (2016), the microsatellite markers amplified poorly for the *L. acicola* isolates from Mexico and Central America. A later study (van der Nest *et al.*, 2019) showed that these isolates were *L. variabilis*, a new and recently described species.

The study by Janoušek *et al.* (2014) showed that *L. acicola* is heterothallic and that two individuals, one with a *MAT1-1-1* idiomorph and the other with a *MAT1-2* idiomorph, are needed for sexual reproduction to occur. Consequently, to understand whether sexual recombination might occur in a region, it is important to have a knowledge of the mating type idiomorph distribution. Mating type primers that amplify the *MAT1-1-1* and *MAT1-2* idiomorphs and that tested positive for *Dothistroma* species as well as *L. acicola*, *L. guatemalensis* and *L. gloeospora* have been developed (Janoušek *et al.*, 2014). It is, however, not yet known whether these markers will amplify these gene regions for the other, newly described *Lecanosticta* species.

Janoušek *et al.* (2016) considered the global *L. acicola* population and showed that the ratio of mating type idiomorphs in Mississippi, Austria, France and Germany reflected sexual recombination in these regions/countries. In contrast, only asexual reproduction occurs in the Czech Republic and northern parts of America. Using the mating type markers of Janoušek *et al.* (2014), the distribution of MAT1 and MAT2 isolates was detected in studies with isolates from Croatia (Sadiković *et al.*, 2019), Estonia (Adamson *et al.*, 2015, 2018), Ireland, Portugal, Russia (Mullett

et al., 2018) as well as Spain (Ortíz de Urbina *et al.*, 2017). In Spain, both mating types were detected whereas only single mating types were detected in all other areas studied. However, in Estonia it was suggested that a second introduction of the pathogen occurred since only MAT1 was initially present but that later both mating types were detected in the same region (Adamson *et al.*, 2015). In populations with equal ratios of mating types or with both mating types present, sexual reproduction could occur, possibly giving rise to more virulent strains. This emphasizes a need to exercise caution and thus to prevent introduction of new strains into regions where the pathogen is already present.

Future prospects in the age of genomics

Canada's Michael Smith Genome Sciences Centre has recently released a full genome for a *L. acicola* isolate from France (https://www.ncbi.nlm.nih.gov/assembly/GCA_000504345.2#/def). This genome has not yet been annotated but provides a valuable resource for future studies. Many other genomes of Dothidiomycetes, which have been sequenced and annotated, are available for comparative purposes (de Wit *et al.*, 2012; Ohm *et al.*, 2012). Annotation of putative genes of the *L. acicola* genome, utilizing knowledge of these other genomes, will provide insights into questions regarding many aspects of the biology of *L. acicola*. Opportunities also now arise to sequence the genomes of other *Lecanosticta* spp. and to compare these in order to better understand their relative importance. It will also be possible to follow the *Dothistroma* example where a transcriptomic study considered which genes are expressed during various stages in the infection of *P. radiata* (Bradshaw *et al.*, 2016) and genome sequencing of global representatives of *D. septosporum* revealed that gene copy numbers could play a role in dothistromin production by the pathogen (Bradshaw *et al.*, 2019).

CONCLUSIONS

Lecanosticta acicola has been known in the southern USA for many decades. Consequently, its life cycle, mode of infection, host susceptibility and strategies to prevent infection, particularly on *P. palustris*, have been extensively studied in that region. Yet there is evidence to show that the pathogen, which now has an extensive host range, is spreading rapidly northwards. The reasons for this host range and geographical expansion require further study. Contemporary knowledge has also shown that there have been two introductions of *L. acicola* into Europe. Consequently, BSNB is becoming a disease of great concern in Europe, where it is increasingly being discovered on both non-native and native *Pinus* spp. There are many relevant hypotheses to explain the growing importance of BSNB and these include the effects of climate change, emergence of more aggressive strains of the pathogen and anthropogenic processes leading to new introductions. There is clearly a need for increased attention to and studies of *L. acicola*, particularly in Europe.

Recent studies have shown that there are eight species of *Lecanosticta* in addition to *L. acicola.* All of these other species appear to have a Mesoamerican origin. Much of the literature pertaining to *L. acicola* needs to be reconsidered given the fact that a single name has been widely used to refer to what we now know represents numerous cryptic species. *Lecanosticta acicola* identified based on DNA sequence comparisons has not been found in Central America, suggesting a North American centre of origin. Of the 69 reports of *L. acicola*, only 25 from 12 countries have been confirmed using DNA sequence-based tools (Table 1). Many reports of the pathogen could thus be erroneous and there is an urgent need to resolve this important question.

All the available knowledge regarding BSNB relates to studies on *L. acicola* and these are predominantly from the USA. Nothing is known regarding the relative importance of the remaining eight species of *Lecanostica*. At least some of these are most likely also important pathogens and their relative threat to global forests and forestry needs to be assessed. A concerted effort must be made to prevent their accidental introduction into new regions of the world and as part of this process DNA sequence-based techniques need to be routinely applied to allow for meaningful identification.

The development of new tools to study *Lecanosticta* spp. and BSNB provides many exciting opportunities to enhance our knowledge of this important group of pathogens. The population structure and diversity of *L. acicola* can now be easily studied in the USA as well as where new invasions occur in Europe, and at levels that were previously not possible. For example, application of the available microsatellite markers will enable a more comprehensive understanding of the pathogen as well as determination of its centre of origin.

Genome sequencing is rapidly becoming cheaper and more readily available, and an isolate of *L. acicola* is already available in the public domain for study. We envisage that all the species of *Lecanosticta* will be sequenced in the relatively near future and many isolates of some species will likely also be studied at this level. These studies, and others relating to the 'omics' level, will surely have a substantial impact on our understanding of a group of pathogens that is growing in importance and relevance. Overall, BSNB (including all species of *Lecanosticta*) has the potential to become a pine needle disease of global importance if proper preventative measures for the spread of the causal pathogens are not implemented.

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The aligned dataset used to draw Fig. 3 is deposited in TreeBASE (No. S24301).

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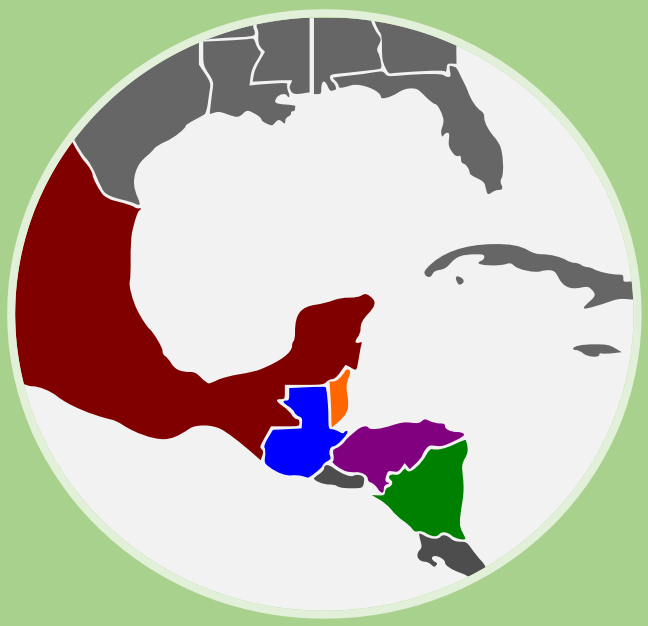
RESEARCH CHAPTER 1



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IMA Fungus

RESEARCH Open Access

Biodiversity of *Lecanosticta* pine-needle blight pathogens suggests a Mesoamerican Centre of origin

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Abstract

Lecanosticta acicola causes the disease known as brown spot needle blight (BSNB), on *Pinus* species. The pathogen is thought to have a Central American centre of origin. This was based on the morphological variation between isolates believed to represent *L. acicola* from native *Pinus* spp. Two species of *Lecanosticta*, *L. brevispora* and *L. guatemalensis*, have recently been described from Mexico and Guatemala respectively based on morphology and sequence-derived phylogenetic inference. However, the putative native pathogen, *L. acicola,* was not found in those areas. In this study, the species diversity of a large collection of *Lecanosticta* isolates from Central America was considered. Phylogenetic analyses of the *BT*1, ITS, *MS204*, *RPB2* and *TEF*1 gene regions revealed six species of *Lecanosticta*, four of which represented undescribed taxa. These are described here as *Lecanosticta jani* sp. nov. from Guatemala and Nicaragua, *L. pharomachri* sp. nov. from Guatemala and Honduras, *L. tecunumanii* sp. nov. from Guatemala and *L. variabilis* sp. nov. from Guatemala, Honduras, and Mexico. New host and country records were also found for the previously described *L. brevispora* and *L. guatemalensis*. *Lecanosticta acicola* was not found in any of the samples from Central America, and we hypothesize that it could be a northern hemisphere taxon. The high species diversity of *Lecanosticta* found in Mesoamerica suggests that this is a centre of diversity for the genus.

Keywords: Brown spot needle blight, *Lecanosticta*, Mesoamerica, *Pinus* pathogens, phylogeny

INTRODUCTION

Brown spot needle blight (BSNB) or Lecanosticta needle blight is an important needle disease on *Pinus* species. The disease is characterised by brown spots on necrotic yellow lesions at the points of infection and die-back of the needles from the apex, which often leads to premature defoliation (Ivory 1987). BSNB is caused by the fungal pathogen, *Lecanosticta acicola* (Siggers 1944). The fungus is a well-known pathogen in the USA and has also been recorded in Central America, Colombia, Europe as well as Asian countries including China, Japan and Korea. *Lecanosticta acicola* is regarded as an A2 quarantine pathogen in Europe and Colombia where it is present as well as an A1 quarantine pathogen in the rest of South America (COSAVE), Africa (IASPC) and the Eurasian Economic Union countries where it has yet to be recorded (https://gd.eppo.int/taxon/SCIRAC/categorization). Despite its quarantine status, *L. acicola* has been discovered in various new locations and on new hosts in Europe during the past decade (Jankovsky et al. 2009; Markovskaja et al. 2011; Anonymous 2012; Hintsteiner et al. 2012; Adamson et al. 2015; Janoušek et al. 2016; Ortíz de Urbina et al. 2017; Mullett et al. 2018; Cleary et al. 2019; Sadiković et al. 2019).

Siggers (1944) and Evans (1984) summarised the taxonomic and nomenclatural history of *Lecanosticta acicola*, which was complicated by the former system which allowed asexual and sexual morphs of the same species of fungi to be given separate scientific names (Kais 1971; Evans 1984). From 1972 to 2012, the name *Mycosphaerella dearnessii* was widely used for the causal agent of BSNB. It was, however, recently recognised that *Mycosphaerella* is polyphyletic and should be strictly used for

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fungi in *Ramularia* (Crous et al. 2007; Crous 2009). Following the One Fungus One Name (1F1N) convention (Hawksworth et al. 2011), the nomenclatural rules were changed in July 2011, and included in subsequent editions of the *International Code of Nomenclature for algae, fungi, and plants* (ICN) (Turland et al. 2018). *Lecanosticta* was taken up as the appropriate name, with *L. acicola* as type species of the genus (Crous et al. 2009a; Quaedvlieg et al. 2012).

Five species of *Lecanosticta* have been described: *Lecanosticta acicola, L. brevispora*, *L. guatemalensis* (Quaedvlieg et al. 2012), *L. gloeospora* (Evans 1984), and *L. longispora* (Marmolejo 2000). *Lecanosticta acicola* remains the best-known species and records suggest that it has a wide distribution in North and South America, Europe, and Asia (https://gd.eppo.int/taxon/SCIRAC/distribution). The remaining four species are known only from Mesoamerica (Evans 1984; Marmolejo 2000; Quaedvlieg et al. 2012). *Lecanosticta gloeospora* was described, based only on morphology, from disease symptoms on *Pinus pseudostrobus* from Iturbide, Nuevo León, Mexico (Evans 1984). It was subsequently reported on *P. pseudostrobus* collected in 1990 in Mexico (Marmolejo 2000). *Lecanosticta longispora* was originally described from *Pinus culminicola* in Nuevo León, Mexico, based on morphology (Marmolejo 2000). Quaedvlieg et al. (2012) redescribed and epitipified *L. longispora* based on DNA sequence and morphological data. Quaedvlieg et al. (2012) delineated *Mycosphaerella* species of quarantine significance in Europe, including isolates believed to be *L. acicola* from Central America. Those isolates were distinct taxa and were named *L. brevispora* and *L. guatemalensis* from *Pinus* sp. in Mexico and from *P. oocarpa* in Guatemala.

Names assigned to *Lecanosticta* species prior to 2012 were based only on morphological characteristics. Cryptic diversity in *Lecanosticta* is illustrated by *L. guatemalensis* (IMI281598), which was initially identified as *L. acicola* (Evans 1984; Quaedvlieg et al. 2012). Identifications made utilising only morphological characteristics should clearly be re-evaluated using DNA sequence data and phylogenetic inference.

Central America is believed to be the centre of origin of *L. acicola*. This hypothesis was first proposed by Evans (1984), when the fungus was isolated from native trees in pristine forests. In a recent phylogenetic study, high levels of diversity were found in the Translation Elongation 1-α gene region (*TEF*1) of isolates from Mexico and Guatemala (Janoušek et al. 2016). Furthermore, Central American isolates did not group in the same clade as isolates from Asia, Europe, and North America. Likewise, Janoušek et al. (2016) reported poor amplification of microsatellite regions that had been developed for *L. acicola* suggesting that the isolates could represent cryptic species. The present study emerged from an opportunity to collect pine needles infected with *Lecanosticta* spp. in Guatemala, Honduras and Nicaragua from 2010 to 2012. Specimens were identified based on DNA sequence comparisons and an attempt was made to confirm whether *L. acicola* occurs in Central America.

MATERIALS AND METHODS

Collections used in the study

Specimens prepared from ex-type cultures and other representatives of all known *Lecanosticta* species and closely related species (Quaedvlieg et al. 2012) were obtained from the culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands (CBS), and from the UK National Fungus Collection maintained by CABI Bioscience (Egham, UK: IMI). Living cultures or DNA of six isolates from Central America examined by Evans (1984), and believed to represent *L. acicola,* were also acquired from IMI (Table 1). Furthermore, isolates of *Dothistroma septosporum, D. pini, Phaeophleospora eugenia, P. gregaria,* and *Amycosphaerella africana* that represent genera in *Mycosphaerellaceae* closely related to *Lecanosticta* (Quaedvlieg et al. 2012) were included for comparative purposes. These cultures were obtained from CBS and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa (Table 1).

Pine needles, showing symptoms of brown spots or bands, were collected from *Pinus* species native to Central America from 2010 to 2012 in Guatemala, as well as from Honduras and Nicaragua in 2011 (Table 1). Conidiomata formed on the needles were aseptically excised, rolled onto 2% Dothistroma Sporulating Media (DSM: 5 g yeast extract (Biolab, Merck, Modderfontein, South Africa), 20 g malt extract (Biolab) and 15 g agar (BD Difco™, Sparks, MD) per litre of distilled water) with 100 mg/L streptomycin (Sigma-Aldrich, St Louis, MO) in order to release conidia from the conidiomata as described by Barnes et al. (2004). The isolated conidiomata were incubated for one to two days at 23 °C. The plates were examined using a dissection microscope and single germinating conidia were selected and replated onto 2% DSM. The single conidial isolates were grown for 4–6 wk. on a natural day light cycle, at 23 °C.

DNA extractions and sequencing

Fungal tissue was scraped from the surface of the cultures on 2% DSM with a sterile scalpel blade and lyophilized. The freeze-dried mycelium was homogenized using a Retsch MM301 mixer mill (Haan, Germany) and approximately 20 ng of the crushed mycelium was used as starting material for DNA extractions. DNA was extracted using a

Table 1 Details of isolates used in this study

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
Amycosphaerella africana	45395	CBS 110843	South Africa, Western Cape Province, Pampoenvlei	*Eucalyptus cladocalyx*	Nov 1994	Crous PW	KF901702	JX901653	MK015047	MK015515	MK015290
A. africana	45396	CBS 680.95	South Africa, Western Cape Province, Stellenbosch mountain	*E. viminalis*	Oct 1994	Crous PW	AY626981	KF903117	MK015048	MK015516	MK015291
Dothistroma pini	10930	CBS 116485	USA, Michigan, Montcalm County, Crystal Lake	*Pinus nigra*	2001	Adams G, Barnes I	AY808301	AY808266	AY808196	NA	MK015292
D. pini	**10951**	**CBS 116487**	**USA, Michigan, Montcalm County, Stanton**	***P. nigra***	**2001**	**Adams G, Barnes I**	**AY808302**	**AY808267**	**AY808197**	**NA**	**MK015293**
D. septosporum	**44656**	**CBS 140339**	**Russia, St. Petersburg, Park Sosnovka**	***P. sylvestris***	**Nov 2013**	**Drenkhan R, Musolin D, Adamson K**	**KU948400**	**MK015397**	**MK015049**	**MK015517**	**MK015294**
D. septosporum	44657	CBS 141531	Russia, St. Petersburg, Park Sosnovka	*P. sylvestris*	Nov 2013	Drenkhan R, Musolin D, Adamson K	KU948401	MK015398	MK015050	MK015518	MK015295
Lecanosticta acicola	9985	CBS 871.95	France	*P. radiata*	Apr 1995	Morelet M	GU214663	MK015399	MK015051	MK015519	MK015296
L. acicola	45426	CBS133790	Lithuania	*P. mugo*	2009	Markovskaja S, Kacergius A, Treigiene A	HM367708	JX901645	MK015052	MK015520	MK015297
L. acicola	**45427**	**CBS 133791**	**USA, New Hampshire, Blackwater**	***P. strobus***	**Jun 2011**	**Ostrofsky B**	**KC012999**	**KC013002**	**MK015053**	**MK015521**	**MK015298**
L. acicola	45428	CBS 322.33	USA	*P. palustris*	Feb 1933	Siggers PV	MK015156	MK015400	MK015054	MK015522	MK015299
L. acicola	50541		Lithuania, Curonian Spit, Juodkrante	*P. mugo*	Sep 2014	Markovskaja S	MK015157	MK015401	MK015055	MK015523	MK015300
L. acicola	50542		Lithuania, Curonian Spit, Juodkrante	*P. mugo*	Sep 2014	Markovskaja S	MK015158	MK015402	MK015056	MK015524	MK015301
L. brevispora	-[e]	1A.N5S2	Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015159	MK015403	–	–	–
L. brevispora	-[e]	1C.N1S3	Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015160	MK015404	MK015057	NA	NA
L. brevispora	-[e]	1C.N5S4	Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015161	MK015405	MK015058	MK015525	MK015302
L. brevispora	-[e]	1C.N6S2	Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015162	MK015406	–	–	–
L. brevispora	-[e]	1D.N1S3	Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015163	MK015407	MK015059	NA	NA
L. brevispora	-[e]	IB31.4a	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015164	MK015408	MK015060	MK015526	MK015303

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
L. brevispora	36894		Guatemala, Finca La Soledad (near Jalapa), Mataquescuintla	*P. pseudostrobus*	Oct 2010	Barnes I	MK015165	MK015409	MK015061	NA	MK015304
L. brevispora	37123		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015166	MK015410	NA	NA	MK015305
L. brevispora	42646		Honduras	*P. oocarpa*	–	–	MK015167	MK015411	MK015062	MK015527	MK015306
L. brevispora	42647		Guatemala, Lugar, La Soledad, Jalapa	*P. oocarpa*	Oct 2010	Barnes I	MK015168	MK015412	MK015063	MK015528	MK015307
L. brevispora	**45424**	**CBS 133601**	**Mexico**	***Pinus* sp.**	**Oct 2009**	**Yanes-Morales M**	**JX901763**	**JX901649**	**MK015064**	**MK015529**	**MK015308**
L. brevispora	46499		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015169	MK015413	–	–	–
L. brevispora	46500		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015170	MK015414	–	–	–
L. brevispora	46501		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015171	MK015415	MK015065	NA	NA
L. brevispora	46502		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015172	MK015416	–	–	–
L. brevispora	46503		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015173	MK015417	MK015066	MK015530	MK015309
L. brevispora	46504		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015174	MK015418	MK015067	MK015531	MK015310
L. brevispora	46505		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015175	MK015419	NA	NA	MK015311
L. brevispora	46506		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015176	MK015420	–	–	–
L. brevispora	46507		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015177	MK015421	–	–	–
L. brevispora	46508		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015178	MK015422	–	–	–
L. brevispora	46509		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015179	MK015423	–	–	–
L. brevispora	46510		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015180	MK015424	NA	NA	MK015312
L. brevispora	46511		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015181	MK015425	–	–	–
L. brevispora	46512		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015182	MK015426	–	–	–
L. brevispora	46807		Guatemala, Alta Verapaz,	*P. oocarpa*	Oct 2010	Barnes I	MK015183	MK015427	MK015068	MK015532	MK015313

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
			Santa Cruz Verapaz, near Tactíc								
L. brevispora	49291		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015184	MK015428	MK015069	NA	MK015314
L. brevispora	49292		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015185	MK015429	MK015070	MK015533	MK015315
L. brevispora	49293		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015186	NA	MK015071	MK015534	MK015316
L. brevispora	49294		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015187	NA	MK015072	MK015535	MK015317
L. brevispora	49295		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015188	NA	MK015073	MK015536	MK015318
L. brevispora	49296		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015189	MK015430	MK015074	MK015537	MK015319
L. brevispora	49297		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015190	MK015431	MK015075	MK015538	MK015320
L. brevispora	49298		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015191	MK015432	MK015076	MK015539	MK015321
L. brevispora	50523		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015192	MK015433	–	–	–
L. brevispora	50526		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015193	MK015434	MK015077	NA	NA
L. brevispora	50527		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015194	MK015435	NA	NA	MK015322
L. brevispora	50528		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015195	MK015436	MK015078	NA	NA
L. brevispora	50529		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015196	MK015437	–	–	–
L. brevispora	50530		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015197	MK015438	MK015079	MK015540	MK015323
L. brevispora	50531		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015198	MK015439	MK015080	MK015541	MK015324
L. brevispora	50532		Guatemala, Chimaltenango, Tecpán, Finca La Esperanza	*P. pseudostrobus*	Jun 2011	Barnes I	MK015199	MK015440	–	–	–
L. brevispora	51050		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015200	MK015441	NA	MK015542	MK015325

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
***L. gloeospora*[c]**	**42645**	**IMI 283812**	**Mexico, Nuevo León, Iturbide-Galeana**	***P. pseudostrobus***	**May 1983**	**Evans HC**	**KU948431**	**MK015442**	**MK015081**	**MK015543**	**MK015326**
L. guatemalensis	- [e]	IB30/2d	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015201	MK015443	–	–	–
L. guatemalensis	- [e]	IB32/1a	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015202	MK015444	–	–	–
L. guatemalensis	- [e]	IB32/2e	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015203	MK015445	MK015082	NA	NA
L. guatemalensis	- [e]	IB35/2e	Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015204	MK015446	MK015083	NA	NA
L. guatemalensis	- [e]	IB35/2j	Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015205	MK015447	–	–	–
L. guatemalensis	- [e]	IB35/9a	Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015206	MK015448	MK015084	NA	NA
L. guatemalensis[c]		IMI 275573	Honduras, Yoro	*P. oocarpa*	Oct 1980	Evans HC	MK015207	MK015449	NA	NA	NA
L. guatemalensis[c]		IMI 281563	Honduras	*P. caribaea*	May 1982	Evans HC	MK015208	NA	NA	NA	NA
L. guatemalensis[c]		IMI 281596	Nicaragua	*P. tecunumanii*	Nov 1981	Evans HC	MK015209	MK015450	NA	NA	NA
L. guatemalensis	- [e]	N3/1c	Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015210	MK015451	MK015085	MK015544	MK015327
L. guatemalensis	36811		Guatemala, Jalapa, Finca Forestal Soledad	*P. maximinoi*	Oct 2010	Barnes I	MK015211	MK015452	MK015086	NA	MK015328
L. guatemalensis	36812		Guatemala, Coban, San Juan Chamelco	*P. maximinoi*	Oct 2010	Barnes I	MK015212	MK015453	MK015087	MK015545	MK015329
L. guatemalensis	37121		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015213	MK015454	–	–	–
L. guatemalensis	37122		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015214	MK015455	MK015088	MK015546	MK015330
L. guatemalensis	37124		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015215	MK015456	–	–	–
L. guatemalensis	37126		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015216	MK015457	MK015089	MK015547	MK015331
L. guatemalensis	37127		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015217	MK015458	–	–	–
***L. guatemalensis*[c]**	**42206**	**IMI 281598**	**Guatemala**	***P. oocarpa***	**1983**	**Evans HC**	**JX901764**	**JX901650**	**MK015090**	**MK015548**	**MK015332**
L. guatemalensis	43890		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015218	MK015459	–	–	–
L. guatemalensis	43891		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015219	MK015460	MK015091	NA	NA

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
L. guatemalensis	43892		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015220	MK015461	MK015092	NA	NA
L. guatemalensis	43893		Guatemala, Chiquimula, San José la Arada	*P. oocarpa*	Oct 2010	Barnes I	MK015221	MK015462	MK015093	NA	NA
L. guatemalensis	43894		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015222	MK015463	MK015094	NA	NA
L. guatemalensis	43895		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015223	MK015464	MK015095	MK015549	MK015333
L. guatemalensis	45386		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015224	MK015465	–	–	–
L. guatemalensis	45387		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015225	MK015466	MK015096	MK015550	MK015334
L. guatemalensis	45391		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015226	MK015467	MK015097	NA	NA
L. guatemalensis	45392		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2011	Barnes I	MK015227	MK015468	MK015098	MK015551	MK015335
L. guatemalensis	45393		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015228	MK015469	–	–	–
L. guatemalensis	45394		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015229	NA	–	–	–
L. guatemalensis	46811		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015230	MK015470	MK015099	MK015552	MK015336
L. guatemalensis	46817		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015231	MK015471	MK015100	MK015553	MK015337
L. guatemalensis	46819		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015232	NA	–	–	–
L. guatemalensis	47108		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015233	MK015472	–	–	–
L. guatemalensis	49400		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015234	MK015473	MK015101	MK015554	MK015338
L. guatemalensis	49402		Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015235	MK015474	MK015102	MK015555	MK015339
L. guatemalensis	51052		Guatemala, Chiquimula, San José la Arada	*P. oocarpa*	Oct 2010	Barnes I	MK015236	MK015475	MK015103	MK015556	MK015340
L. guatemalensis	51142		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015237	MK015476	MK015104	MK015557	MK015341
L. jani	-[e]	267.44.N1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015238	MK015477	MK015105	MK015558	MK015342
L. jani	-[e]	267.47.N1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015239	MK015478	MK015106	MK015559	MK015343
L. jani	-[e]	267.47.N2	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015240	MK015479	MK015107	MK015560	MK015344
L. jani	-[e]	267.51.N2S1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015241	MK015480	NA	NA	MK015345
L. jani	-[e]	267.52.N1S1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015242	MK015481	MK015108	MK015561	MK015346

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
L. jani	- [e]	267.52.N2S1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015243	MK015482	MK015109	MK015562	MK015347
L. jani	- [e]	IB30/2b	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015244	MK015483	MK015110	MK015563	NA
L. jani	- [e]	IB35/3c	Guatemala, Chiquimula	*P. oocarpa*	Oct 2010	Barnes I	MK015245	MK015484	MK015111	MK015564	MK015348
L. jani	- [e]	IB13/2f	Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015246	MK015485	MK015112	MK015565	MK015349
L. jani	- [e]	N3/2c	Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015247	NA	MK015113	MK015566	MK015350
L. jani	36808		Guatemala, Jalapa, Finca Forestal Soledad	*P. maximinoi*	Oct 2010	Barnes I	MK015248	NA	MK015114	MK015567	MK015351
L. jani	36810		Guatemala, Jalapa, Finca Forestal Soledad	*P. maximinoi*	Oct 2010	Barnes I	MK015249	NA	MK015115	MK015568	MK015352
L. jani	37128		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015250	MK015486	MK015116	MK015569	MK015353
L. jani	38950	CBS 144446; PREM 62186	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015251	MK015487	MK015117	MK015570	MK015354
L. jani	**38958**	**CBS 144456; PREM 62185**	**Guatemala, Jalapa, Finca La Soledad, Mataquescuintla**	***P. oocarpa***	**Sep 2012**	**Barnes I**	**MK015252**	**MK015488**	**MK015118**	**MK015571**	**MK015355**
L. jani	38959		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015253	NA	NA	NA	NA
L. jani	38968		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015254	NA	MK015119	NA	NA
L. jani	45388		Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015255	NA	MK015120	MK015573	MK015356
L. jani	45389		Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015256	MK015489	MK015121	MK015574	MK015357
L. jani	47109		Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015257	MK015490	MK015122	MK015575	MK015358
L. jani	48830		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015258	NA	MK015123	MK015576	MK015359
L. jani	48831	CBS 144447; PREM 62187	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015259	MK015491	MK015124	MK015577	MK015360
L. jani	49401		Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015260	MK015492	NA	MK015578	MK015361
L. jani	51051		Guatemala	*P. maximinoi*	Oct 2010	Barnes I	MK015261	MK015493	MK015125	MK015579	MK015362
L. jani	51058		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015262	MK015494	MK015126	MK015580	MK015363
L. jani	51059		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. tecunumanii*	Sep 2012	Barnes I	MK015263	MK015495	MK015127	MK015581	MK015364
L. jani	51143		Nicaragua, Matagalpa	*P. oocarpa*	Jun 2011	Barnes I	MK015264	NA	MK015128	MK015582	MK015365

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
L. longispora	**45429**	**CBS 133602**	**Mexico**	***Pinus* sp.**	**Oct 2009**	**Yanes-Morales M**	**JX901766**	**JX901651**	**MK015129**	**MK015583**	**MK015366**
L. longispora	45430	CPC 17941	Mexico	*Pinus* sp.	Oct 2009	Yanes-Morales M	JX901765	JX901652	MK015130	MK015584	MK015367
L. pharomachri	- [e]	267.8A.N2S1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015265	MK015496	NA	NA	MK015368
L. pharomachri	- [e]	267.12.N1S2	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015266	NA	NA	NA	MK015369
L. pharomachri	- [e]	267.30.MD.N1	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015267	NA	NA	NA	MK015370
L. pharomachri	- [e]	267.30.MD.N2	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015268	MK015497	MK015131	NA	MK015371
L. pharomachri	- [e]	267.30.N4	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015269	MK015498	MK015132	MK015585	MK015372
L. pharomachri	37132		Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015270	MK015499	MK015133	MK015586	MK015373
L. pharomachri	37133		Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015271	MK015500	MK015134	MK015587	MK015374
L. pharomachri	37134		Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015272	MK015501	MK015135	MK015588	MK015375
L. pharomachri	**37136**	**CBS 144448; PREM 62188**	**Guatemala, Baja Verapaz, San Jerónimo, Salamá**	***P. tecunumanii***	**Oct 2010**	**Barnes I**	**MK015273**	**MK015502**	**MK015136**	**MK015589**	**MK015376**
L. pharomachri	38947	CBS 144695; PREM 62189	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015274	MK015503	MK015137	MK015590	MK015377
L. pharomachri	38974	CBS 144449; PREM 62190	Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015275	MK015504	MK015138	MK015591	MK015378
L. pharomachri	38975		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015276	NA	NA	NA	NA
L. pharomachri	38976		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015277	MK015505	MK015139	NA	MK015379
L. pharomachri	46810		Honduras	*P. oocarpa*	–	–	MK015278	MK015506	MK015140	MK015592	MK015380
L. pharomachri	46813		Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015279	MK015507	MK015141	MK015593	MK015381
L. pharomachri	51053		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015280	NA	MK015142	NA	MK015382
L. pharomachri	51054		Guatemala, Jalapa, Finca La Soledad, Mataquescuintla	*P. oocarpa*	Sep 2012	Barnes I	MK015281	MK015508	NA	NA	MK015383
L. tecunumanii	**46805**	**CBS 144450; PREM 62191**	Guatemala, Baja Verapaz, San Jerónimo, Salamá	***P. tecunumanii***	**Oct 2010**	**Barnes I**	**MK015282**	**MK015509**	**MK015143**	**MK015594**	**MK015384**
L. tecunumanii	46812	CBS 144452; PREM 62193	Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015283	MK015510	MK015144	MK015595	MK015385

Table 1 Details of isolates used in this study *(Continued)*

Species	CMW number[a]	Other collection number[b]	Sampling site (Country, Region, Location)	Host	Collection date	Collector	GenBank accession numbers[d]				
							ITS	*TEF1*	*BT1*	*MS204*	*RPB2*
L. tecunumanii	49403	CBS 144451; PREM 62192	Guatemala, Baja Verapaz, San Jerónimo, Salamá	*P. tecunumanii*	Oct 2010	Barnes I	MK015284	MK015511	MK015145	MK015596	MK015386
L. variabilis	36809	CBS 144455; PREM 62195	Guatemala, Jalapa, Finca Forestal Soledad	*P. maximinoi*	Oct 2010	Barnes I	MK015285	MK015512	MK015146	MK015597	MK015387
L. variabilis	37125	CBS 144454; PREM 62194	Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015286	KJ938446	MK015147	MK015598	MK015388
L. variabilis	37129		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015287	KJ938445	MK015148	MK015599	MK015389
***L. variabilis*[c]**	**42205**	**IMI 281561; CBS 144453; PREM 62196**	**Honduras, Santa Barbara, Lago Yojoa**	***P. caribaea***	**Oct 1980**	**Evans HC**	**MK015288**	**MK015513**	**MK015149**	MK015600	**MK015390**
L. variabilis	45390		Guatemala, Alta Verapaz, Santa Cruz Verapaz, near Tactíc	*P. oocarpa*	Oct 2010	Barnes I	MK015289	MK015514	MK015150	MK015601	MK015391
L. variabilis	45425	CBS 133789	Mexico	*Pinus* sp.	Nov 2009	Yanez-Morales M	JX901762	JX901648	MK015151	MK015602	MK015392
Phaeophleospora eugeniae	45432	CPC15159	Brazil, Vicosa, Paraiso	*Eugenia uniflora*	Mar 2008	Alfenas AC	FJ493189	JX901667	MK015152	MK015603	NA
P. eugeniae	45433	CPC 15143	Brazil, Vicosa, Paraiso	*E. uniflora*	Mar 2008	Alfenas AC	FJ493188	JX901666	MK015153	MK015604	NA
P. gregaria	45434	CBS 111166	South Africa, Western Cape Province, de Hoop Nature Reserve	*Eucalyptus cladocalyx*	Sep 1995	Wood A	JX901773	JX901664	MK015154	MK015605	MK015393
P. gregaria	45435	CBS 114662	South Africa, Western Cape Province, Devon Valley, Stellenbosch	*Eucalyptus* sp.	Jun 1995	Crous PW	DQ302953	JX901654	MK015155	MK015606	MK015394

[a]*CMW* Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa;
[b]*CBS* Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, *CPC* Personal collection of Pedro Crous housed at CBS, *IMI* The UK National Fungus Collection, CABI Bioscience, Egham, UK, *PREM* The dried herbarium collection of the South African National Collection of Fungi, Mycology Unit, Biosystematics Division, Plant Protection Institute, Agricultural Research Council, Pretoria, South Africa
[c]Cultures were collected by HC Evans in Central America
[d]'-'= was not amplified; 'NA'= amplification unsuccessful;
[e] = no viable culture available that could be submitted to CMW
Ex-type isolate of each species is indicated in bold

Zymo Research ZR Fungal/Bacterial DNA MiniPrep™ kit (Irvine, CA) and eluted into a final volume of 50 µl. The quality and quantity of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA). DNA concentrations were diluted to 20 ng/µl working stock for polymerase chain reaction (PCR) amplifications and stored at – 20 °C until further use.

The nuclear rDNA region encompassing the internal transcribed spacers (ITS) 1 and 2, along with the 5.8S rDNA region was amplified using primers ITS1 and ITS4 (White et al. 1990) and a portion of the translation elongation factor 1-α gene (*TEF*1) using primers EF1-728F (Carbone and Kohn 1999) and EF2 (O'Donnell et al. 1998) for all the isolates. The Beta-tubulin-2 gene region (*BT*2) was amplified using the primer pair T1 (O'Donnell and Cigelnik 1997) and β-Sandy-R (Stukenbrock et al. 2012) or the primers Bt2A and Bt2B (Glass and Donaldson 1995). The Beta-tubulin-1 gene region (*BT*1) was amplified using primers Bt1A and Bt1B (Glass and Donaldson 1995), the RNA polymerase II second largest subunit (*RPB*2) gene region using primers RPB2-5f2 (Sung et al. 2007) and RPB2-7cR (Liu et al. 1999) and the guanine nucleotide-binding protein subunit beta (*MS204*) using primers MS204F.cerato and MS204R.cerato (Fourie et al. 2015).

PCR reactions for each of the six regions contained 20 ng DNA, 2.5 µl 10x PCR reaction buffer, 2.5 mM $MgCl_2$, 400 nM of each primer, 200 µM of each dNTP and 1 U Faststart *Taq* DNA Polymerase (Roche Diagnostics, Indianapolis, IN). Reaction volumes were adjusted to 25 µl with sterile SABAX water (Adcock Ingram, Midrand, South Africa). PCR reactions were carried out on an Applied Biosystems® Veriti® 96 well Thermal cycler (Thermo Fisher Scientific, Waltham, MA). The cycling conditions for all six gene regions included an initial denaturation step at 95 °C for 4 min, 10 cycles consisting of 94 °C for 20 s (denaturation), a 45 s annealing step according to the primer pair annealing temperature (Table 2) and an elongation step of 45 s at 72 °C. This was followed by a further 25 cycles of 94 °C for 20 s, 45 s with a 5 s extension step per cycle at the indicated annealing temperature, a 72 °C extension for 45 s and a final step of 72 °C for 10 min. The annealing temperature was set at 56 °C for ITS, 52 °C for *TEF*1, 50 °C for *BT*1, 52 °C for *BT*2, 55 °C for *MS204* and 56 °C for *RPB*2. To visualise amplified products, 5 µl PCR products were stained with 1 µl GelRed™ nucleic acid gel stain (Biotium, Fremont, CA) and separated on 2% SeaKem® LE agarose gel (Lonza, Rockland, ME) for 20 min at 100 V and viewed under a UV light using the GelDoc™ EZ Imager (BioRad, Hercules, CA). PCR products were cleaned with a 6.65% G-50 Sephadex solution (Sigma-Aldrich, St Louis, MO) following the manufacturer's instructions using Centri-sep spin columns (Princeton Separations, Freehold, NJ).

The concentrations of the cleaned PCR products were determined using a NanoDrop ND-1000 spectrophotometer and 60–100 ng of DNA and products were sequenced in both the forward and reverse direction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an ABI PRISM 3500xl capillary auto sequencer (Thermo Fisher Scientific).

Forward and reverse sequences were aligned and consensus sequences generated in CLC Main workbench version 8.0 (CLC Bio, https://www.qiagenbioinformatics.com/products/clc-main-workbench/). All consensus

Table 2 Primers used for PCR amplification and sequencing in this study

Locus	Primer name	Direction	Primer sequence 5′ to 3′	Annealing temperature used (°C)	Amplification success	Reference
*BT*1	Bt1a	Forward	TTC CCC CGT CTC CAC TTC TTC ATG	50	87.4%	Glass and Donaldson 1995
	Bt1b	Reverse	GAC GAG ATC GTT CAT GTT GAA CTC	50		Glass and Donaldson 1995
*BT*2[a]	T1	Forward	AAC ATG CGT GAG ATT GTA AGT	52	–	O'Donnell and Cigelnik 1997
	β-Sandy-R	Reverse	GCR CGN GGV ACR TAC TTG TT	52		Stukenbrock et al. 2012
	Bt2a	Forward	GGT AAC CAA ATC GGT GCT GCT TTC	52	–	Glass and Donaldson 1995
	Bt2b	Reverse	ACC CTC AGT GTA GTG ACC CTT GGC	52		Glass and Donaldson 1995
*TEF*1	EF1-728F	Forward	CAT CGA GAA GTT CGA GAA GG	52	88.2%	Carbone and Kohn 1999
	EF-2	Reverse	GGA RGT ACC AGT SAT CAT GTT	52		O'Donnell et al. 1998
ITS	ITS1	Forward	GAA GTA AAA GTC GTA ACA AGG	56	100%	White et al. 1990
	ITS4	Reverse	TCC TCC GCT TAT TGA TAT GC	56		White et al. 1990
MS204	MS204F.cerato	Forward	AAG GGC ACC CTC GAG GGC CAC	55	71.7%	Fourie et al. 2015
	MS204R.cerato	Reverse	GAT GGT RAC GGT GTT GAT GTA	55		Fourie et al. 2015
*RPB*2	RPB2-5f2	Forward	GGG GWG AYC AGA AGA AGG C	56	82.7%	Sung et al. 2007
	fRPB2-7cR	Reverse	CCC ATR GCT TGY TTR CCC AT	56		Liu et al. 1999

[a]*BT*2 amplification success using all primer combinations was very low and abandoned

sequences generated in this study were deposited in GenBank that is hosted by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/genbank/) (Table 1).

Data analyses

Five datasets (*BT*1, ITS, *MS204*, *RPB*2 and *TEF*1) were generated and analysed individually. A partition homogeneity test (PHT) was performed with the software package PAUP* 4.0b10 (Swofford 2003) to test congruence between the five gene regions and a sixth dataset, where sequences were available for all five gene regions, was compiled and analysed. The *BT*1, ITS, *MS204* and *RPB*2 datasets included all of the sequences generated in this study and additional sequences available from GenBank (Table 1). The *TEF*1 dataset included all of the sequence data generated in this study as well as additional sequences representing 14 different *TEF*1 haplotypes of *L. acicola* (including possible cryptic species) (Janoušek et al. 2016) that were downloaded from GenBank (Table 3). Sequences for all datasets were aligned with the online version of MAFFT Version 7 (Katoh and Standley 2013; http://mafft.cbrc.jp/alignment/server/) using default settings. Aligned data were imported into MEGA 7.0.14 (Kumar et al. 2016) and manually checked and adjusted.

Three separate analyses were performed for each of the six datasets: Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI). The MP analysis were performed with the software package PAUP* 4.0b10 (Swofford 2003). Gaps were treated as a fifth character state. One thousand random stepwise addition heuristic searches were performed with tree-bisection-reconnection (TBR) as the branch-swapping algorithm. Uninformative characters were excluded and the consistency index (CI), homoplasy index (HI), rescaled consistency index (RC), retention index (RI) and tree length (TL) were determined for the resulting trees (Table 4). The confidence levels were estimated by performing 1000 bootstrap replicates.

In order to determine the ML and BI, the best fit substitution model for each of the data sets were determined using jModelTest 0.1.1 (Posada 2008). Maximum likelihood analysis was performed with the program PhyML 3.0 (Guindon et al. 2010). The confidence levels were estimated with 1000 bootstrap replicates.

MrBayes 3.1.2 (Ronquist et al. 2012) was used to determine the BI for each data set by applying the Markov Chain Monte Carlo (MCMC) method. For each dataset, four independent MCMC chains were randomly started and run for six million generations, applying the best substitution model determined by jModelTest 0.1.1. Trees were sampled every 100 generations. Burn-in values were determined using Tracer 1.6 (Rambaut et al. 2014) by comparing the log likelihoods. Trees sampled in the burn-in phase were discarded. The remaining trees were used to construct majority rule consensus trees and to determine posterior probabilities for the tree topology.

Morphological characterization

Cultures were grown on 2% Malt Extract Agar (MEA), Oatmeal Agar (OA) and Potato Dextrose Agar (PDA) (Crous et al. 2009b; Quaedvlieg et al. 2012) at 20 °C for 2 wk. in darkness in order to examine the morphology and colour of the cultures of each species. Cultures on MEA were used for microscopic measurements of the conidiophores, conidiogenous cells and conidia. Slides were mounted in SABAX water (Adcock Ingram, Midrand, South Africa) for microscopy and examined using a Zeiss Axioskop 2 Plus compound microscope (Zeiss, Oberkochen, Germany). Photographic images were captured with a Nikon DS-Ri2 camera with the NIS Element BR v4.3 software package (Nikon, Tokyo, Japan). Up to 50 measurements of each morphologically characteristic structure was taken for each ex-type isolate and ten measurements were made for each of the paratypes examined. The mean, standard deviation, minimum and maximum were calculated for each morphological structure and the measurements presented as (minimum–) (mean – standard deviation) – (mean + standard deviation) (–maximum) for the conidia and conidiogenous cells. For the conidiophores, the maximum observed length was indicated together with the width as (minimum–) (mean) (–maximum).

Temperature requirements for growth in culture was studied on representative isolates selected for each of the novel species. Four by four millimeter blocks of each culture were plated, in triplicate, onto the centres of 2% MEA plates per temperature (10, 15, 20, 25, and 30 °C) and incubated in darkness. The diameters of each colony were recorded weekly along perpendicular axes for 4 wk. The colour and shape of each colony was recorded after 2 wk. of growth at 20 °C. Culture colour was determined using Rayner's colour chart (Rayner 1970).

Accession of cultures and types

Holotype specimens of the new species, which are dried cultures, are deposited in the National Mycological Herbarium in Pretoria (PREM). Cultures are deposited in the culture collection (CBS) of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, and ex-type cultures, as well as all other isolates included in this study, are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa (Table 5).

Table 3 GenBank numbers of *Lecanosticta acicola TEF*1 haplotypes included in the *TEF*1 phylogenetic analysis (Fig. 2) as well as additional locations represented by the haplotypes

Species name assigned in this study[a]	GenBank Accession number	Country	State / Region	Location	Host	Date of collection	Collector / Supplier
Lecanosticta acicola	KJ938442	Japan	Shimane	Matsue, Hamanogi	*Pinus thunbergii*	Feb 2010	Suto Y
L. acicola	KJ938439	Mexico	Nuevo León	Iturbide, Bosque Escuela	*Pinus halepensis*	May 2010	Marmolejo JG
L. acicola	KJ938440	Mexico	Nuevo León	Iturbide, Bosque Escuela	*Pinus halepensis*	May 2010	Marmolejo JG
L. acicola	KJ938441	Mexico	Nuevo León	Iturbide, Bosque Escuela	*Pinus halepensis*	May 2010	Marmolejo JG
L. acicola	KJ938438	USA	Maine	York, Lyman	*Pinus strobus*	Jun 2011	Ostrofsky W
L. acicola	KJ938443	USA	Mississippi	Harrison County	*Pinus palustris*	Oct 2012	Bartlett B, Burdine C
L. acicola	KJ938444	USA	Mississippi	Harrison County	*Pinus palustris*	Oct 2012	Bartlett B, Burdine C
L. acicola	KJ938450	USA	Mississippi	Harrison County	*Pinus palustris*	Oct 2012	Bartlett B, Burdine C, Roberds J
L. acicola	KJ938451	USA	Mississippi	Harrison County	*Pinus palustris*	Oct 2012	Bartlett B, Burdine C
Lecanosticta variabilis	KJ938445	Guatemala	Alta Verapaz	Santa Cruz Verapaz, near Tactíc	*Pinus oocarpa*	Oct 2010	Barnes I
L. variabilis	KJ938446	Guatemala	Alta Verapaz	Santa Cruz Verapaz, near Tactíc	*Pinus oocarpa*	Oct 2012	Barnes I
L. variabilis	KJ938447	Mexico	Nuevo León	Piñal de los Amoles, Querétaro	*Pinus* sp.	2011	Kunte L
L. variabilis	KJ938448	Mexico	Nuevo León	Iturbide, Bosque Escuela	*Pinus halepensis*	May 2010	Marmolejo JG
L. variabilis	KJ938449	Mexico	Nuevo León	Galeana, Cerro del Potosí	*Pinus arizonica* var. *stormiae*	Apr 2010	Marmolejo JG
Countries, regions, locations and hosts represented by the above isolates[b]							
	the same as KJ938438	Austria	Lower Austria	Hollenstein an der Ybbs	*Pinus mugo*	Oct 2004	Kirisits T, Barnes I
	the same as KJ938438	Austria	Lower Austria	Opponitz	*Pinus mugo*	2010	Hintsteiner M
	the same as KJ938438	Austria	Lower Austria	Saimannslehen	*Pinus* sp.	2010	Hintsteiner M
	the same as KJ938438	Austria	Lower Austria	Sankt Gallen	*Pinus mugo*	2010	Hintsteiner M
	the same as KJ938438	Austria	Lower Austria	Steyer, Pestalozzistraße	*Pinus mugo*	2010	Hintsteiner M
	the same as KJ938438	Austria	Lower Austria	Waidehofen an der Ybbs	*Pinus mugo*	Aug 2010	Janoušek J
	the same as KJ938438	Austria	Upper Austria	Gmunden	*Pinus nigra*	Jun 2012	Kirisits T
	the same as KJ938438	Canada	Québec	Demers-Centre	*Pinus strobus*	Jun 2011	Harvey L
	the same as KJ938438	Canada	Québec	Lake Aberdeen	*Pinus strobus*	Jun 2011	Harvey L
	the same as KJ938438	Canada	Québec	Lake Pinseault	*Pinus strobus*	Jun 2011	Harvey L
	the same as KJ938438	Canada	Québec	Montréal	*Pinus mugo*	Jun 2011	Harvey R
	the same as KJ938438	Canada	Québec	Waltham	*Pinus strobus*	Jun 2011	Harvey L
	the same as KJ938442	China	Fujie		*Pinus elliottii*	1988	Zheng-Yu H
	the same as KJ938451	Colombia	Refocosta L-75	Villanueva, Casanare	*Pinus caribaea*	Mar 2011	Rodas C, Barnes I
	the same as KJ938438	Croatia		Zadar	*Pinus halapensis*	Sep 2009	Diminic D
	the same as KJ938438	Czech Republic	Southern Bohemia	Borkovická Blata	*Pinus uncinata* subsp. *uliginosa*	Oct 2011	Janoušek J
	the same as KJ938438	Czech Republic	Southern Bohemia	Červená Blata	*Pinus uncinata* subsp. *uliginosa*	Aug 2009	Dvořák M, Janoušek J
	the same as KJ938438	Estonia	Harju maakond	Tallin	*Pinus ponderosa*	Jul 2008	Cech T

Table 3 GenBank numbers of *Lecanosticta acicola TEF*1 haplotypes included in the *TEF*1 phylogenetic analysis (Fig. 2) as well as additional locations represented by the haplotypes *(Continued)*

Species name assigned in this study[a]	GenBank Accession number	Country	State / Region	Location	Host	Date of collection	Collector / Supplier
	the same as KJ938451	France	Pyrénées-Atlantiques		*Pinus radiata*	2012	Kersaudy E, loos R
	the same as KJ938438	Germany	Bavaria	Grassau	*Pinus mugo*	2000	Blaschke FR, Wulf
	the same as KJ938438	Germany	Bavaria	Murnau	*Pinus mugo*	Feb 2010	Nannig A
	the same as KJ938438	Germany	Bavaria	Murnauer Filze	*Pinus mugo*	Nov 2011	Nannig A
	the same as KJ938438	Germany	Bavaria	Pfrűhlmoos	*Pinus mugo*	Nov 2011	Nannig A
	the same as KJ938438	Italy	Brecia	Gardone	*Pinus mugo*	Jun 2008	Cech T
	the same as KJ938438	Lithuania	Klaipėdský kraj	Curonian Spit, Juodkrante	*Pinus mugo*	2010	Markovskaja S
	the same as KJ938438	Slovenia	Upper Carniola	Bled	*Pinus mugo*	Jul 2009	Jurc D
	the same as KJ938442	South Korea	Naju	Sanpo-myeon	*Pinus thunbergii*	2010	KACC, Seo ST
	the same as KJ938451	Spain	Cantabria	San Sebastián de Garabandal	*Pinus radiata*	Oct 2012	Jankovský L, Janoušek J
	the same as KJ938438	Switzerland	Canton St Gallen	Walensee	*Pinus mugo*	Oct 1999	Wulf
	the same as KJ938438	USA	Maine	Androscoggin, Leeds	*Pinus strobus*	Jun 2011	Ostrofsky W
	the same as KJ938438	USA	Maine	Piscataquis, Sangerville	*Pinus strobus*	Jun 2011	Weimer J
	the same as KJ938438	USA	Maine	York, Lyman	*Pinus strobus*	Jun 2011	Ostrofsky W
	the same as KJ938438	USA	Michigan	Wexford County, Springville Township	*Pinus sylvestris*	2011	Odonnell J
	the same as KJ938444	USA	Mississippi	Harrison County	*Pinus palustris*	Oct 2012	Bartlett B, Burdine C, Roberds J
	the same as KJ938438	USA	New Hampshire	Hillsboro, Fox State Park	*Pinus strobus*	Jun 2011	Weimer J
	the same as KJ938438	USA	New Hampshire	Merrimack, Black Water Reserve	*Pinus strobus*	Jun 2011	Weimer J
	the same as KJ938438	USA	New Hampshire	Merrimack, Hopkinton-Everett	*Pinus strobus*	Jun 2011	Weimer J
	the same as KJ938438	USA	Vermont	Washington, Waterbury	*Pinus strobus*	Jun 2011	Lackey J
	the same as KJ938438	USA	Vermont	Windsor, Bethel	*Pinus strobus*	Jul 2011	Munck I
	the same as KJ938438	USA	Wisconsin	Merrillan	*Pinus sylvestris*	Apr 2010	Stanosz G

[a]*Lecanosticta variabilis* was previously identified as *L. acicola* but is now defined as a new species
[b]Information adapted from Janoušek et al. (2016), Table S1

RESULTS

Fungal collections

Twenty-six isolates or DNA samples were obtained from culture collections to include in the study. An additional 127 isolates of putative *Lecanosticta* species were obtained from symptomatic needles collected from 36 different trees in Guatemala, Nicaragua and Honduras (Table 1). In Guatemala, 22 isolates were obtained from *Pinus oocarpa, P. maximinoi,* and *P. tecunumanii* needles that were collected in the Alta Verapaz District, 16 isolates were obtained from *P. oocarpa* needles collected in Chiquimula, 35 isolates from *P. pseudostrobus* needles collected in the Chimaltenango District in the Tecpán Municipality, eight isolates from *P. tecunumanii* needles collected in the Baja Verapaz District, 29 isolates from *P. tecunumanii* and *P. oocarpa* needles collected in the Jalapa District, and seven isolates from *P. maximinoi* needles in Coban and other regions (Table 1). Two isolates were obtained from *P. oocarpa* needles collected in Honduras and eight isolates were made from *P. oocarpa* needles collected in Matagalpa, Nicaragua.

DNA extraction and sequencing

The ITS and *TEF*1 regions were sequenced for all 153 isolates obtained and the *BT*1, *MS204* and *RPB*2 regions were sequenced for 127 representatives of all

Table 4 PCR amplification size, phylogenetic data and the substitution models used in the phylogenetic analysis for each gene region and for the combined datasets

	ITS	*TEF*1	*BT*1	*MS204*	*RPB2*	Combined datasets
Approximate amplicon size (bp)	550	520	420	760	940	–
Number of taxa analysed	153	147	111	91	105	76
Aligned characters (bp)	734	586	440	785	929	3344
Number of parsimony-uninformative characters	621	143	357	519	538	2438
Number of parsimony-informative characters	114	423	82	266	371	1121
Number of trees retained	108	396	1	2448	420	100
Consistency index	0.865	0.499	0.739	0.791	0.738	0.607
Homoplasy index	0.135	0.501	0.261	0.209	0.262	0.393
Rescaled consistency index	0.850	0.459	0.703	0.748	0.696	0.555
Retention index	0.982	0.919	0.951	0.946	0.943	0.914
Tree Length	163	1675	138	546	722	2642
Substitution model	TPM2uf + G	GTR + G	GTR + G	TVM + G	TrN + G	GTR + G

monophyletic groups identified in the generated ITS and *TEF*1 phylogenetic trees. The selected representatives included all of the closely related *Mycosphaerellaceae* isolates, all the isolates that did not group with known *Lecanosticta* species, and a selection of isolates that grouped with known *Lecanosticta* species (Table 1). PCR fragments of approximately 550 bp were generated for ITS, 520 bp for *TEF*1, 420 bp for *BT*1, 760 bp for *MS204* and 940 bp for *RPB*2. The amplification success of the *TEF*1, *BT*1, *MS204* and *RPB*2 gene regions varied for the isolates that were selected and the amplification success rate of *TEF*1 was 88.2%, *BT*1 was 87.4%, *MS204* was 71.7 and 82.7% for the *RPB*2 region (Table 2). The *BT*2 region did not amplify well across species of *Lecanosticta*. The amplification success rate and subsequent sequencing of the *BT*2 region using the T1 and β-Sandy-R primer pair, as well as Bt2a and Bt2b was very poor and further analysis of the *BT*2 region was abandoned.

Phylogenetic analyses

For the analyses, the datasets of the ITS region consisted of 153 taxa with 734 aligned nucleotides including gaps; the *TEF*1 dataset consisted of 147 taxa with 586 aligned nucleotides, the *BT*1 dataset consisted of 111 taxa with 440 aligned nucleotides; the *MS204* dataset consisted of 91 taxa with 785 aligned nucleotides, and the *RPB*2 dataset consisted of 105 taxa with 929 aligned nucleotides, all including gaps. The PHT test yielded a *P* value = 0.01 and therefore the five datasets were considered incongruent. However, it was previously argued that a P value > 0.01 did not reduce phylogenetic accuracy (Cunningham 1997) and a combined phylogenetic tree representing the five gene regions ITS, *TEF*1, *BT*1, *MS204* and *RPB*2 was constructed for presentation purposes (Fig. 1). The combined dataset consisted of 76 taxa with 3344 aligned nucleotides including gaps. Constant characters, parsimony-uninformative and informative characters, the consistency index (CI), homoplasy index (HI), rescaled consistency index (RC), retention index (RI) and tree length (TL) values for the maximum parsimony analyses are indicated in Table 4. For the parsimony analyses, 108 trees were retained for ITS, 396 for *TEF*1, 1 for *BT*1, 2448 for *MS204* and 420 for *RPB*2. The best fit substitution models for ML and BI were selected by Akaike Information Criterion (AIC) and are indicated in Table 4. A 10% burn-in value was selected in the BI analysis for each of the data matrices for each of the analyses. Because the MP, ML and BI analysis all resulted in similar tree topologies, the ML trees were selected and chosen for presentation (Figs. 1 and 2, Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3 and Additional file 4: Figure S4).

Phylogenetic analyses of the combined dataset (Fig. 1), ITS (Additional file 1: Figure S1), *TEF*1 (Fig. 2) and *MS204* (Additional file 3: Figure S3) consistently grouped the isolates sequenced in this study into seven distinct clades. The clades in Fig. 2 and Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3 and Additional file 4: Figure S4 are labelled according to the clades assigned in Fig. 1. In the case of *RPB*2 (Additional file 4: Figure S4) Clades 1–4, and 7 were also present but Clades 5 and 6 were not distinct from each other for this particular gene region. In the case of *BT*1 (Additional file 2: Figure S2), Clades 3, 5 and 6 could not be distinguished from each other. None of the isolates grouped with the types of *L. gloeospora* or *L. longispora*.

Forty-two of the isolates from Central America grouped in Clade 1 based on the ITS analysis (Additional file 1: Figure S1) and were identified as *Lecanosticta brevispora*. This was the most common species identified from the Central American collection and most isolates were from

Table 5 Specimens for which the morphology was examined for the description of *Lecanosticta jani, L. pharomachri, L. tecunumanii* and *L. variabilis*

Species	CMW number[a]	Status of specimen	Herbarium specimen[b]	Ex-type isolates[c]
Lecanosticta jani	CMW 38950[d]	Paratype	PREM 62186	CBS 144446
	CMW 38958[d]	Holotype	PREM 62185	CBS 144456
	CMW 48831[e]	Paratype	PREM 62187	CBS 144447
	CMW 51058[d]	Additional material examined		
	CMW 51059[d]	Additional material examined		
	CMW 51143[e]	Additional material examined		
	CMW47109[e]	Additional material examined		
Lecanosticta pharomachri	CMW 37136	Holotype	PREM 62188	CBS 144448
	CMW 38947	Paratype	PREM 62189	CBS 144695
	CMW 38974	Paratype	PREM 62190	CBS 144449
	CMW 38976	Additional material examined		
	CMW 51053	Additional material examined		
	CMW 51054	Additional material examined		
Lecanosticta tecunumanii	CMW 46805	Holotype	PREM 62191	CBS 144450
	CMW 46812	Paratype	PREM 62193	CBS 144452
	CMW 49403	Paratype	PREM 62192	CBS 144451
Lecanosticta variabilis	CMW 42205	Holotype	PREM 62196	CBS 144453, IMI 281561
	CMW 37125	Paratype	PREM 62194	CBS 144454
	CMW 36809	Paratype	PREM 62195	CBS 144455
	CMW 45425	Additional material examined	CBS H-21112	CBS 133789
	CMW 37129	Additional material examined		

[a]*CMW* Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; [b]The herbarium deposits are dried cultures that serve as holotype and paratype specimens. PREM = The dried herbarium collection of the South African National Collection of Fungi, Mycology Unit, Biosystematics Division, Plant protection Institute, Agricultural Research Council, Pretoria, South Africa; [c]The ex-type cultures are living cultures linked to the holotype and paratype specimens. CBS = The culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IMI = The UK National Fungus Collection maintained by CABI Bioscience, Egham, UK; [d] *Lecanosticta jani* cultures with the Type 2 morphology; [e] *Lecanosticta jani* cultures with the Type 1 morphology

Chimaltenango on *Pinus pseudostrobus*. The pathogen was also isolated from *P. oocarpa* needles near Jalapa as well as near Tactíc in Guatemala and in Honduras. This clade was well supported for all five of the gene regions analysed.

Twenty-seven isolates grouped into Clade 2 in the ITS analyses (Additional file 1: Figure S1) and represent an undescribed species. Clade 2 resolved into two subclades in the five gene analyses. Subclade 1 was mostly isolated from Chiquimula and Alta Verapaz in Guatemala on *P. oocarpa, P. maximinoi* and *P. tecunumanii* as well as from *P. oocarpa* in Nicaragua. Isolates collected in Jalapa in Guatemala mostly grouped into Subclade 2. However, the topology of isolates CMW 47109 (Subclade 1 on Additional file 1: Figure S1, Additional file 3: Figure S3, Additional file 4: Figure S4; Subclade 2 on Fig. 2), CMW 51059 (Subclade 1 on Additional file 1: Figure S1, Additional file 3: Figure S3, Additional file 4: Figure S4), IB30.2b (Subclade 1 on Additional file 1: Figure S1, Additional file 3: Figure S3; Subclade 2 on Fig. 2) and IB30.2b (Subclade 1 on Additional file 1: Figure S1, Additional file 3: Figure S3, Additional file 4: Figure S4; Subclade 2 on Fig. 2) changed in the two subclades depending on the gene region analysed (Fig. 2, Additional file 1: Figure S1, Additional file 3: Figure S3, Additional file 4: Figure S4). Furthermore, the two subclades were not well supported for the *BT*1 gene region. Therefore, the two subclades are treated here as representing a single species.

Clade 3 also represented an undescribed *Lecanosticta* species. This clade included 11 isolates from *P. oocarpa* in Jalapa, Guatemala, one isolate from *P. oocarpa* in Honduras, as well as five isolates collected from Baja Verapaz in Guatemala on *P. tecunumanii.* This clade had high bootstrap support for *TEF*1, *MS204* and *RPB*2 but was not well supported in the ITS and *BT*1 gene regions. Three isolates collected from different needles on a single *P. tecunumanii* tree in Baja Verapaz in Guatemala grouped together in Clade 4 and represent another undescribed species. With the exception of *BT*1, Clade 4 was statistically well supported in all the gene regions that were analysed.

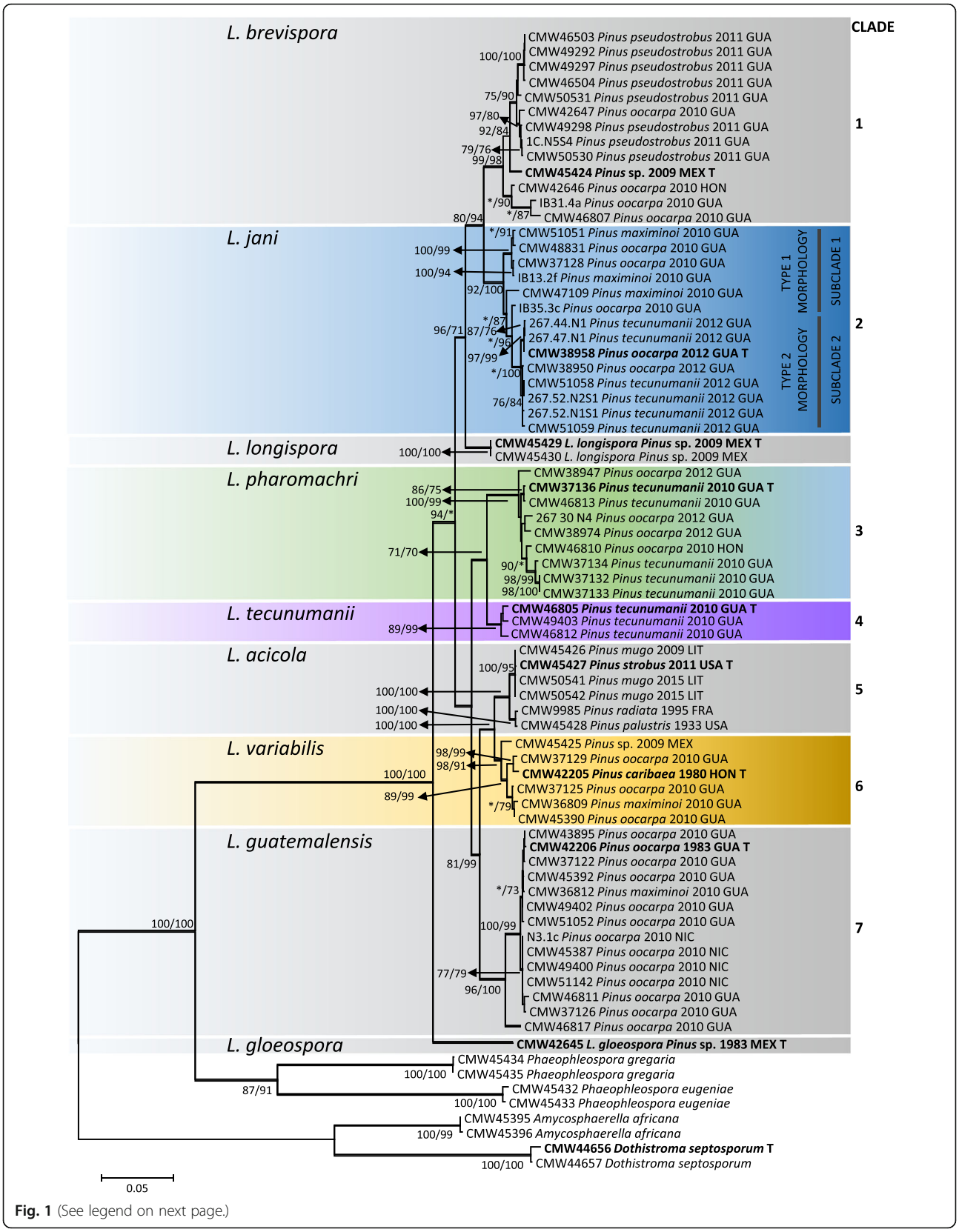

Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the combined data of the ITS, *TEF*1, *BT*1, *MS204* and *RPB2* gene regions. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma septosporum* was used as the outgroup taxa. The indicated clades are referred to in the text. All represented type species are indicated in bold and with a "T"

Clade 5 accommodated sequences representing nine of the 14 known *TEF*1 haplotypes of *L. acicola* identified by Janoušek et al. (2016). These *TEF*1 haplotypes represent isolates collected from North America (Canada, USA, and Mexico), South America (Colombia), Europe (Spain, France, Switzerland, Slovenia, Lithuania, Italy, Germany, Estonia, Czech Republic, Croatia, and Austria) and Asia (South Korea, Japan, and China) (Table 3). This clade was clearly distinct from other clades in the ITS, *TEF*1, *BT*1 and *MS204* phylogenetic analysis and statistically well supported in the ITS, *TEF*1, and *MS204* analyses. Clade 5 included the ex-type of *L. acicola* and therefore is that species. None of the isolates from Central America obtained in the present study grouped with this clade in any of the gene regions analysed.

The remaining five assigned *L. acicola TEF*1 haplotypes considered by Janoušek et al. (2016), grouped together in Clade 6. This was together with an isolate obtained from *P. caribaea* in Honduras collected in 1983 (Evans 1984), four isolates obtained in the present study from Guatemala on *P. oocarpa* and *P. maximinoi,* and an isolate previously identified as *L. acicola* from Mexico on an unknown *Pinus* species (Quaedvlieg et al. 2012). In the present study, Clade 6 is treated as a novel taxon. The ITS, *TEF*1, *BT*1 and *MS204* gene regions clearly distinguish Clades 5 and 6, however, *RPB*2 was not effective in resolving these two groups.

The second most abundant species collected in this study was *Lecanosticta guatemalensis*, represented by Clade 7 in the phylogenetic analyses. This clade was well supported in all five gene regions that were analysed. A total of 37 isolates from our collection grouped together with *L. guatemalensis* based in the ITS and *TEF*1 analyses. *Lecanosticta guatemalensis* was identified on *P. maximinoi* and *P. oocarpa* in various regions of Guatemala, as well as on *P. oocarpa* in Nicaragua. Isolates that had previously been collected in Nicaragua and Honduras and that were identified as *L. acicola* by Evans (1984) based on morphological characteristics also grouped with *L. guatemalensis* in the present study.

TAXONOMY

Using phylogenetic analyses, 51 of the *Lecanosticta* isolates obtained from Guatemala, Honduras and Nicaragua, one isolate obtained from CBS, and one isolate obtained from IMI, were found to include four undescribed species. These are described below as follows:

Lecanosticta jani van der Nest, M.J. Wingf. & I. Barnes, **sp. nov.**

MycoBank MB 826875. (Fig. 3)

Etymology: The name is derived from Janus, the Roman god of gates and doorways having two faces or sides, and refers to the variable culture morphology ranging from light pink and fluffy to dark olive green and mucoid.

Diagnosis: *Lecanosticta jani* can be distinguished from the closely related *L. brevispora* by the distinct globose basal cells on the conidiophores that are mostly observed on MEA.

Type: **Guatemala**: Jalapa, Finca la Soledad, Mataquescuintla, on needles of *Pinus oocarpa*, 20 Sept 2012, *I. Barnes* (PREM 62185 – holotype; CMW 38958 = CBS 144456 – ex-type culture).

Description: *Sexual morph* unknown. *Conidiomata* isabelline to vinaceous brown on MEA. *Conidiophores* subcylindrical, often with a swollen globose basal cell, densely aggregated, honey to hyaline, smooth to verruculose, unbranched or branched at base, often encased in a yellow to light brown mucoid sheath, to 82 µm in length, 4.5–7.0 µm diam. *Conidiogenous cells* terminal, integrated, subcylindrical, honey to hyaline, smooth to verruculose, proliferating several times percurrently with visible annelations near apex, septate or aseptate, (8.5–)16.5(– 24.0) × (3.0–)4.5(– 6.5) µm. *Conidia* solitary, sub-cylindrical to narrowly fusoid-ellipsoidal, with subobtusely rounded apex, base truncate, brown, verruculose, frequently with mucoid sheath, two distinct sizes with conidial type one more abundant than conidial type two. *Conidial type 1*: 1–2-septate, base (1.5–)2.0–2.5(– 3.5) µm diam, (9.5–)14.5–21.5(– 30.0) × (2.0–)2.5–3.5(– 4.0) µm. *Conidial type 2*: 1–3-septate, base (1.5–)2.0–2.5(– 3.0) µm diam, (26.5–)30.5–37.0(– 38.0) × (2.0–)2.5–3.0(– 3.5) µm.

Culture characteristics: Colonies with two distinct morphologies. One type (Type 1), flat to somewhat erumpent, spreading with flat to fluffy aerial mycelium. A second type (Type 2) erumpent, mucoid and shiny, with irregular form and undulate to filiform edges. On MEA, the surface of Type 1 isolates pale to rosy vinaceous, reverse flesh to peach coloured. Type 2

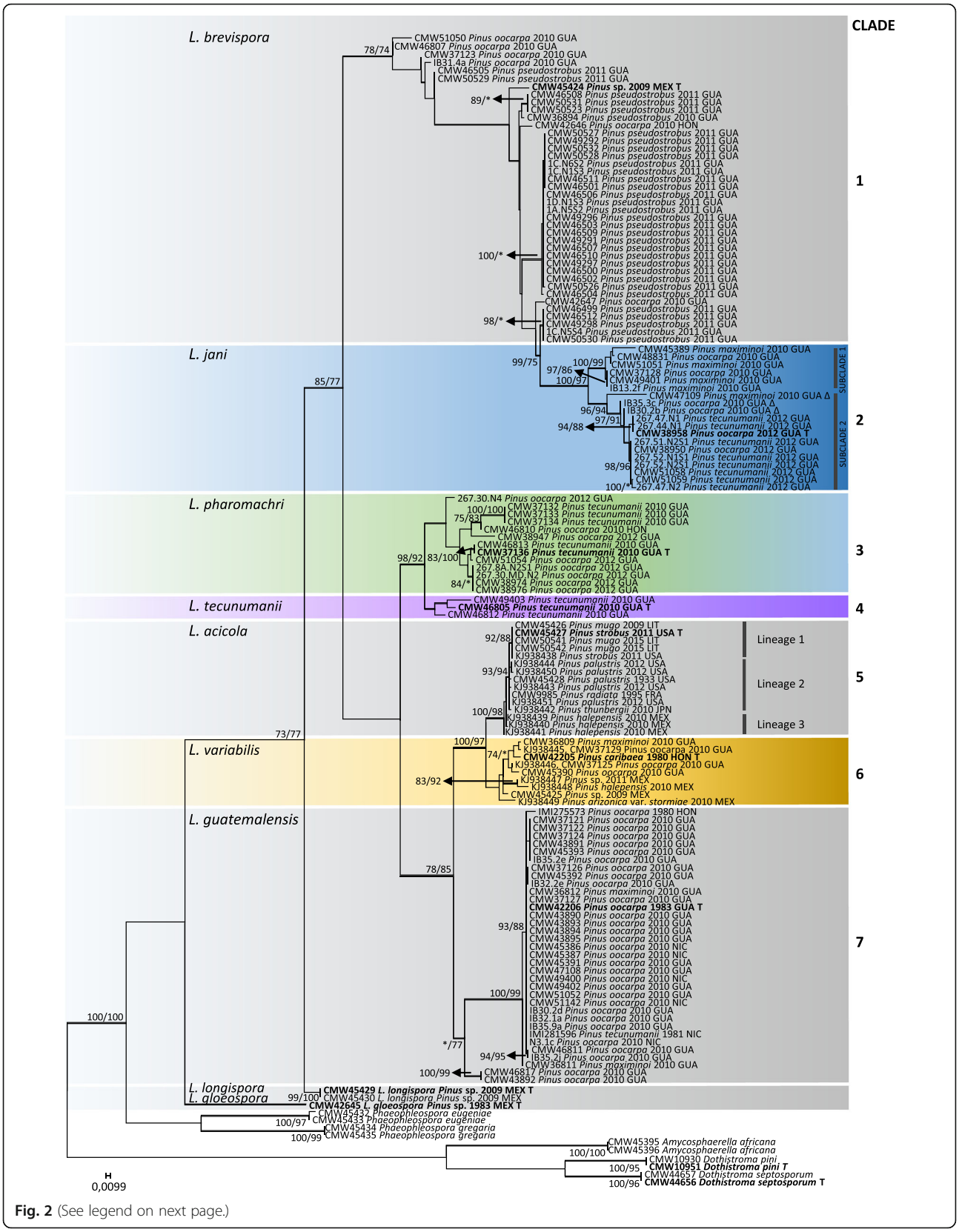

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the *TEF*1 region. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma* species were used as the outgroup taxa. All represented type species are indicated in bold and with a "T". Clades indicated on the left correspond with the clades in Fig. 1. Within the *L. jani* clade a "Δ" next to the isolate indicates that the isolate either exhibits Type 2 morphology and groups with Subclade 1, or, exhibits Type 1 morphology and groups with Subclade 2

isolates citrine to isabelline, reverse olivaceous to fuscous black (Fig. 3). On PDA, Type 1 surface rosy vinaceous to peach in centre with dark brown edge, isabelline in reverse. Type 2, surface dark olivaceous with fuscious black centres and tufts of isabelline mycelium at edges, dark isabelline in reverse. On OA, Type 1 surface dirty white to pale vinaceous, fluffy mycelia to flat growth. Type 2 surface flat with smooth edge, fuscious black in centre at the point of inoculation with light apricot surrounding mycelium. *Growth characteristics:* optimal growth temperature for Type 1 isolates 25 °C, after 4 wk., colonies at 10, 15, 20, 25 and 30 °C reached maximum of 10.5, 22, 32, 32 and 10 mm respectively, with mean growth rate of 2.1, 5.1, 6.9, 7 and 1.8 mm / wk. respectively. Type 2 isolates optimal growth temperature 20 °C, after 4 wk., colonies at 10, 15, 20, 25 and 30 °C reached maximum of 12.5, 17, 29.5, 22 and 4.5 mm, with mean growth of 2.1, 3.3, 5.5, 5 and 1 mm / wk. respectively.

Notes: *Lecanosticta jani* resolved in a distinct clade (Clade 2, Figs. 1 and 2, Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Figure S3 and Additional file 4: Figure S4) based on all five gene regions considered. This clade divides into two subclades that were mostly represented by isolates obtained from Alta Verapaz and Chiquimula in Guatemala as well as in Nicaragua in subclade 1 and isolates obtained from Jalapa in Guatemala in subclade 2. Jalapa isolates all had the Type 2 morphology and the dark colour was associated with conidial production. Type 1 isolates produced few spores after 2 wk. The optimal growth temperature and growth rates were different for the two isolate types. However, the topology of some isolates changed between the two subclades depending on the gene region that is analysed and therefore the subclades are treated as one species. The morphological variation suggests that the two types could represent two ecotypes.

Additional material examined: **Guatemala:** Alta Verapaz, Santa Cruz Verapaz, near Tactíc, on needles of *Pinus oocarpa*, 21 Oct 2010, *I. Barnes* (culture CMW47109); *loc. cit. I. Barnes* (PREM 62187; CMW 48831 = CBS 144447 – culture); Jalapa, Finca la Soledad, Mataquescuintla, on needles of *Pinus oocarpa*, 20 Sept 2012, *I. Barnes* (PREM 62186, CMW 38950 = CBS 144446 – culture); Jalapa, Finca la Soledad, Mataquescuintla, on needles of *Pinus tecunumanii*, 20 Sept 2012, *I. Barnes* (cultures CMW 51058, CMW 51059). -**Nicaragua:** Matagalpa, on needles of *Pinus oocarpa*, 20 June 2011, *I. Barnes* (culture CMW 51143).

Lecanosticta pharomachri van der Nest, M.J. Wingf. & I. Barnes, **sp. nov.**

MycoBank MB 826876. (Fig. 4)

Etymology: The epithet refers to the Resplendid Quetzal (*Pharomachrus mocinno*), which is the national bird of Guatemala and the spirit bird/companion of Tecún Umán; a Guatemalan legend.

Diagnosis: *Lecanosticta pharomachri* is distinguished from the other taxa in the genus by all five gene regions investigated but especially by sequences of *TEF*1, *MS204* or *RPB*2. Conidia are also larger than those of *L. guatemalensis* and similar to *L. acicola* but differ from these species in that the conidia are frequently surrounded by a thick mucoid sheath and are mostly straight.

Type: **Guatemala:** Baja Verapaz, San Jerónimo, Salamá, on needles of *Pinus tecunumanii*, Nov 2010, *I. Barnes* (PREM 62188 – holotype; CMW 37136 = CBS 144448 – ex-type cultures).

Description: *Sexual morph* not observed. *Conidiomata* dark vinaceous brown on MEA. *Conidiophores* subcylindrical to cylindrical, densely aggregated, vinaceous brown to hyaline, smooth to verruculose, unbranched or branched at base, often encased in a light brown mucoid sheath, to 45 μm in length, 2.5–4.0 μm diam. *Conidiogenous cells* terminal, integrated, subcylindrical to cylindrical, luteus brown to hyaline, smooth to verruculose, surrounded by mucilage, holoblastic, proliferating several times percurrently with visible annelations near apex, septate or aseptate, (6.5–)9.5–13.5(– 16.0) × (1.5–)2.0–2.5(– 3.0) μm. *Conidia* released in a greenish olivaceous to honey mass, solitary, straight to slightly curved, cylindrical, with subobtusely rounded apex, base truncate, guttulate, hyaline to light brown, verruculose, frequently with thick mucoid sheath, 1–3-septate, base (1.5–)2.0–3.0(– 3.5) μm diam, (21.0)25.0–34.0(– 49.0) × (2.5–)3.0–4.0(– 5.0) μm. Germ tubes observed between conidia as

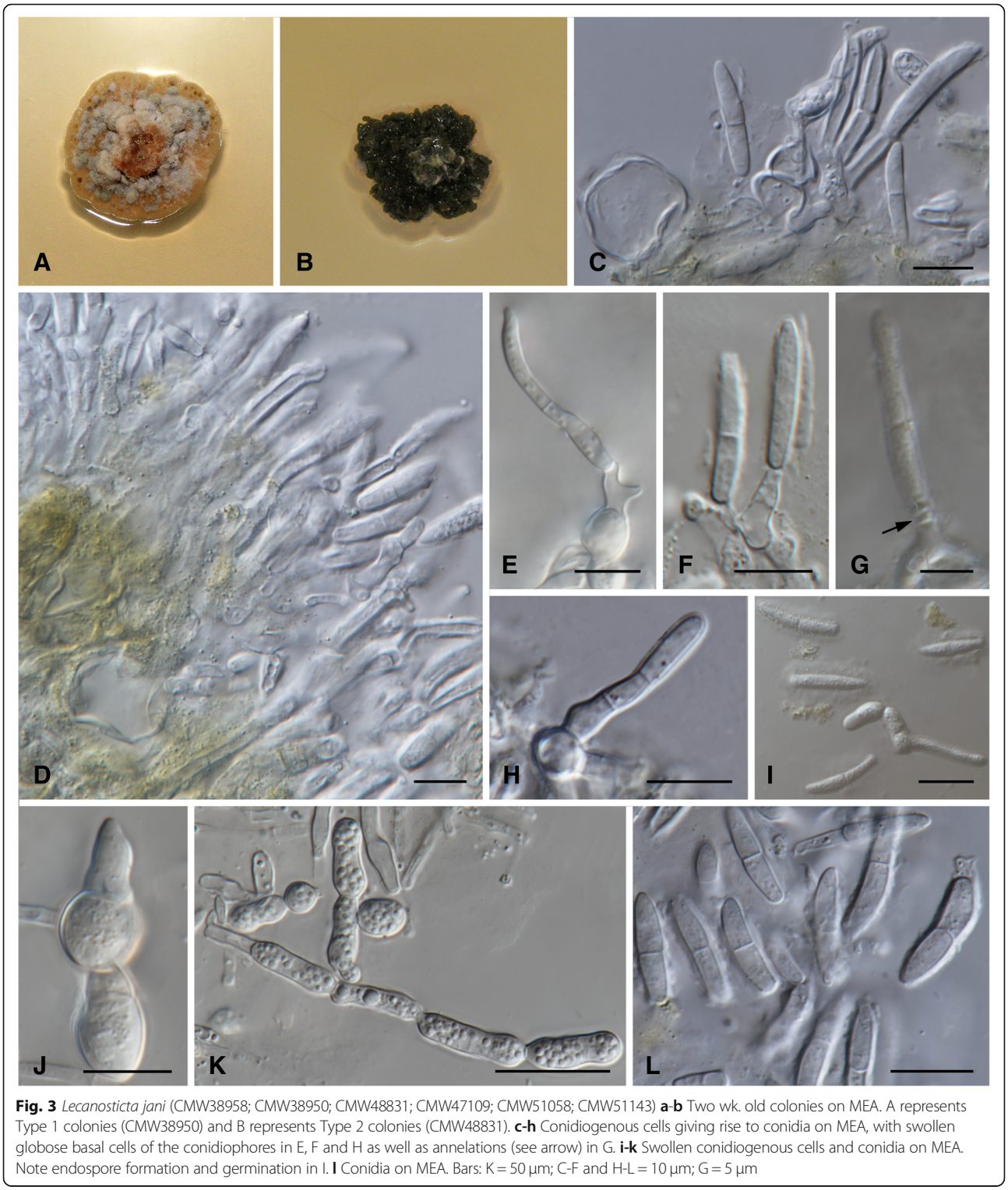

Fig. 3 *Lecanosticta jani* (CMW38958; CMW38950; CMW48831; CMW47109; CMW51058; CMW51143) **a**-**b** Two wk. old colonies on MEA. A represents Type 1 colonies (CMW38950) and B represents Type 2 colonies (CMW48831). **c-h** Conidiogenous cells giving rise to conidia on MEA, with swollen globose basal cells of the conidiophores in E, F and H as well as annelations (see arrow) in G. **i-k** Swollen conidiogenous cells and conidia on MEA. Note endospore formation and germination in I. **l** Conidia on MEA. Bars: K = 50 µm; C-F and H-L = 10 µm; G = 5 µm

well as conidial budding - secondary conidia sometimes produced from apical cell, 0–2-septate.

Culture characteristics: Colonies flat to erumpent, form irregular with undulate edge, spreading with fluffy aerial mycelium at centers. On MEA, surface apricot to cinnamon with isabelline and rosy buff mycelial mat at centers, reverse isabelline to dark brick in centre with cinnamon to apricot edges. On PDA, surfaces rosy to pale vinaceous with light isabelline to greenish white

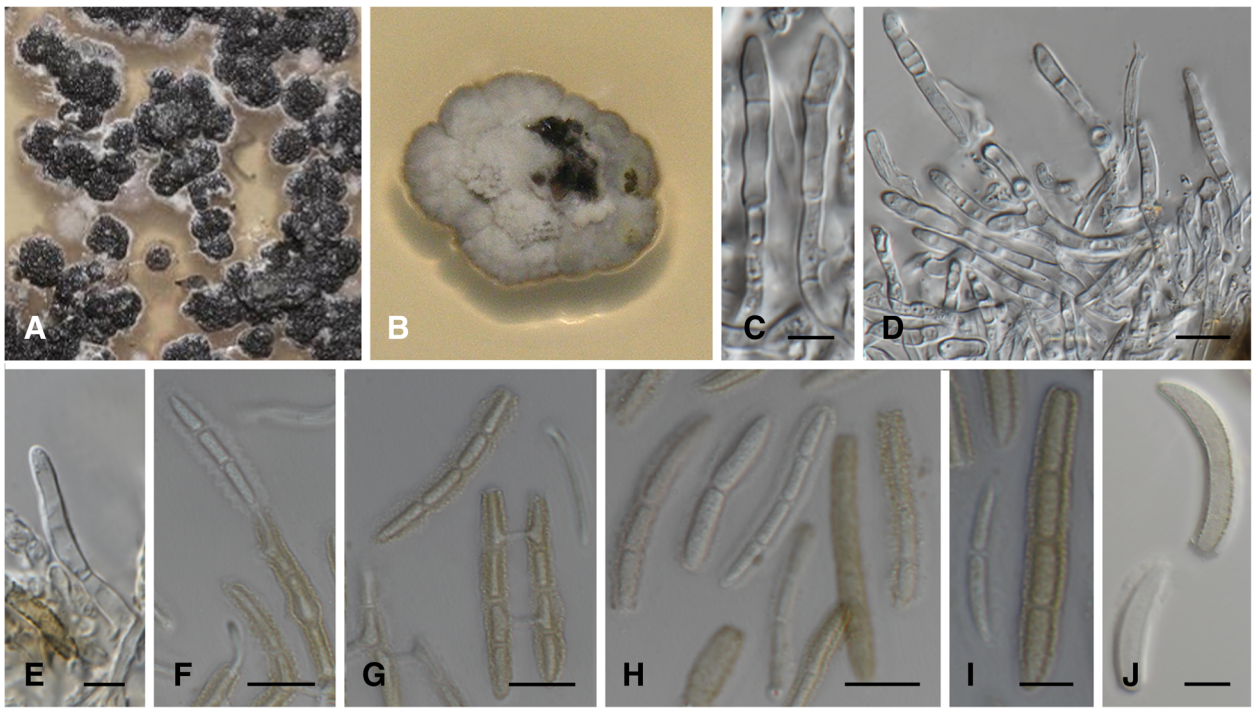

Fig. 4 *Lecanosticta pharomachri* (CMW 37136; CMW38947). **a**, **b** Two wk. old colonies on MEA. **c**-**e** Conidiogenous cells giving rise to conidia on MEA. **f**, **g** Conjugation tube formation between conidia as well as conidia bearing smaller conidial cells. **h-j** Variation in conidia on MEA. Bars: D, F-H and J = 10 µm; C, E and I = 5 µm

edges, reverse isabelline with cream edges. On OA, surface dirty white to isabelline to dark brown, fluffy mycelium to flat growth. *Growth characteristics:* optimal growth temperature 20 °C, after 4 wk., colonies at 10, 15, 20, 25, and 30 °C reaching a maximum of 9, 17, 18.5, 18.5 and 8.5 mm diam, with mean growth rates of 1.9, 3.6, 4.6, 4.4, and 1.9 mm / wk. respectively.

Notes: Some of the isolates, including the ex-type strain, produced a luteus exudate that diffused into MEA after 4–6 wk. Conjugation tubes were reported previously in *L. acicola* cultures as well as in needles (Siggers 1950; Crosby 1966). Conjugation tubes were also observed in this species (Fig. 4g) in the present study. Endospores as described by Crosby (1966) were also observed in some conidia.

Additional material examined: **Guatemala**: Jalapa, Finca la Soledad, Mataquescuintla, on needles of *Pinus oocarpa*, 20 Sept 2012, *I. Barnes* (cultures CMW 38976, CMW 51053 and CMW 51054); *loc. cit.*, *I. Barnes* (PREM 62189; CMW 38947 = CBS 144695 – culture; PREM 62190, CMW 38974 = CBS 144449 – culture).

Lecanosticta tecunumanii van der Nest, M.J. Wingf. & I. Barnes, **sp. nov.**

MycoBank MB 826877. (Fig. 5)

Etymology: Name refers to the Guatemalan legend, Tecún Umán, and *Pinus tecunumanii*, the host plant from which the holotype was collected.

Diagnosis: *Lecanosticta tecunumanii* is distinguished from the other taxa by the ITS, *TEF*1, *MS204* and *RPB*2 gene regions. Morphologically, it is distinct in having only 1-septate conidia after 2 wk. of incubation on MEA, but 2-septate and 3-septate conidia are occasionally observed in older cultures.

Type: **Guatemala**: Baja Verapaz, San Jerónimo, Salamá, on needles of *Pinus tecunumanii*, Oct 2011, *I. Barnes* (PREM 62191 – holotype; CMW 46805 = CBS 144450 – ex-type cultures).

Description: *Sexual morph* not observed. *Conidiomata* isabelline to visaceous brown on MEA. *Conidiophores* cylindrical, densely aggregated, hyaline to pale yellow-brown, smooth to slightly verruculose, unbranched or branched at base, to 120 µm in length, 2.0–5.0 µm diam. *Conidiogenous cells* terminal or indeterminate, integrated or discrete, cylindrical, hyaline to honey, smooth to verruculose, proliferating several times percurrently with visible

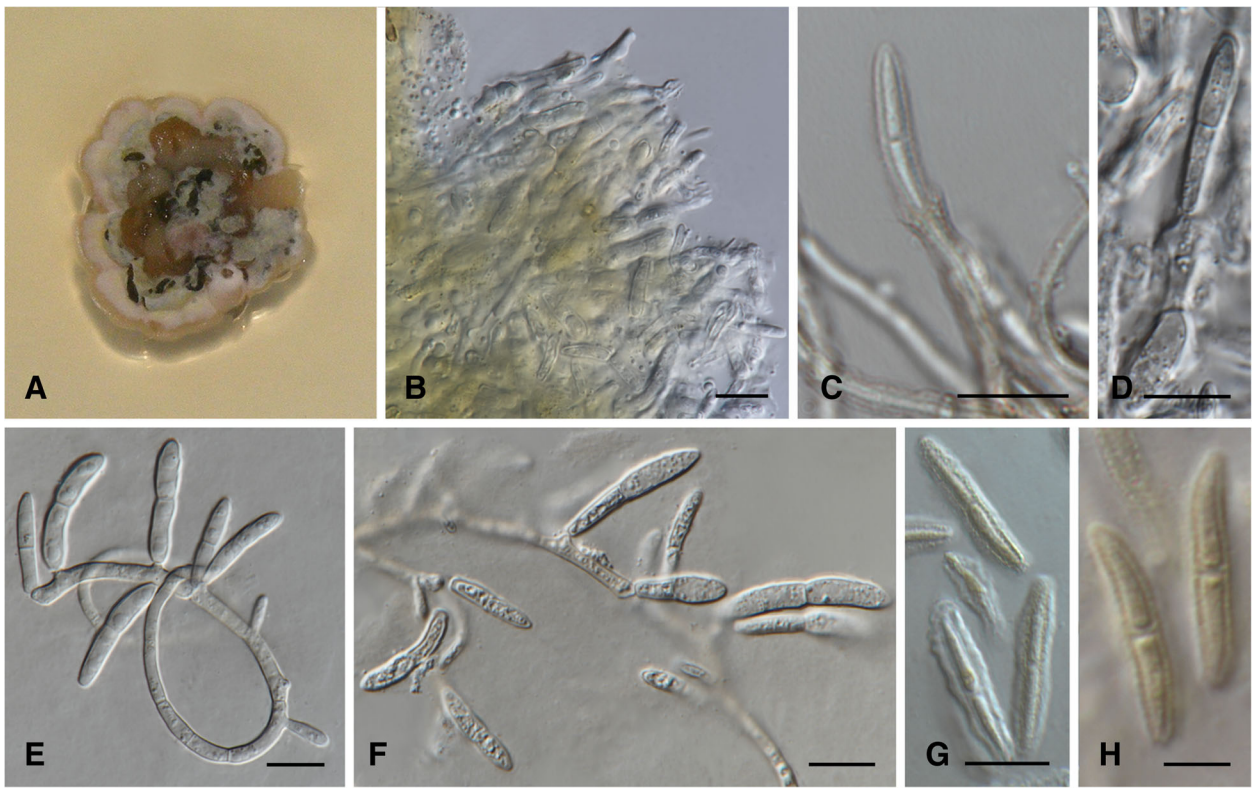

Fig. 5 *Lecanosticta tecunumanii* (CMW46805; CMW46812). **a** Two wk. old colony on MEA**. b-d** Conidiogenous cells giving rise to conidia on MEA. **e-f** Micronematous conidiogenesis observed on MEA with conidia. **g-h** Uniseptate conidia with or without a mucoid sheath observed on MEA. Bars: B-G = 10 μm; H = 5 μm

annelations near apex or micronematous, septate or aseptate, (5.0–)7.0–14.5(– 15.5) × (1.5–)2.0–2.5(– 3.0) μm. Micronematous cells (6–)10.5–18.5(– 27.0) × (2.0–)2.0–2.5(– 3.0) μm. *Conidia* solitary, straight to slightly curved, subcylindrical to fusiform, with subobtusely rounded or sharply pointed apex, base truncate, guttulate, smooth to granulate, hyaline to cream buff to light brown, occasionally enclosed in mucoid sheath, 1-septate, base (1.5–)1.5–2.0(– 2.0) μm diam., (14.5–)16.0–21.0(– 24.0) × (2.0–)2.5–3.0(– 3.5) μm.

Culture characteristics: Colonies somewhat erumpent, spreading with flat to fluffy aerial mycelium. On MEA, surface olivaceous to isabelline with rosy buff mycelial tufts, reverse isabelline. On PDA, surface rosy vinaceous to peach in centre with a dark brown edge, isabelline in reverse. On OA, surface dirty white to pale vinaceous, fluffy mycelia to flat peach growth. *Growth characteristics*: optimal growth temperature 25 °C, after 4 wk., colonies at 10, 15, 20, 25, and 30 °C reached maximum of 9, 15.5, 24, 24, and 4.5 mm, with mean growth of 2.2, 3.8, 5.3, 5.7, and 1.1 mm / wk. respectively.

Notes: Micronematous conidiogenesis (Fig. 5E - F), observed more frequently than distinct conidiophores in culture.

Additional material examined: **Guatemala**: Baja Verapaz, San Jerónimo, Salamá, on needles of *Pinus tecunumanii*, Oct 2011, *I. Barnes* (PREM 62192, CMW 49403 = CBS 144451 – culture; PREM 62193, CMW 46812 = CBS 144452 – culture).

Lecanosticta variabilis van der Nest, M.J. Wingf. & I. Barnes, **sp. nov.**

MycoBank MB 826878. (Fig. 6)

Etymology: The epithet refers to the variable size and shape of the conidia.

Diagnosis: *Lecanosticta variabilis* is distinguished from the closely related species, *L. acicola*, by either ITS, *TEF*1 or *MS204*. Morphologically, it is distinguished from other species with the exception of *L. acicola* by the diffusion of sulphur-yellow to cinnamon metabolite into PDA and a luteus to sienna coloured metabolite

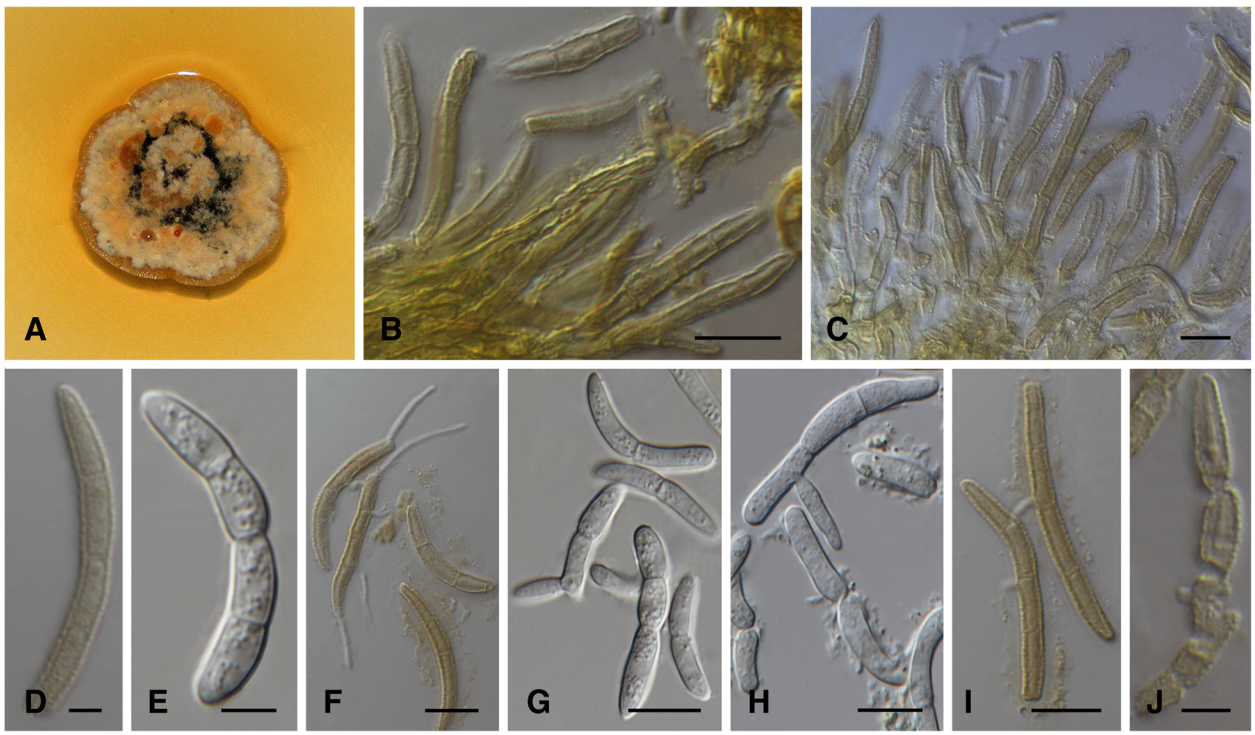

Fig. 6 *Lecanosticta variabilis* (CMW42205; CMW37125). **a** Colony on MEA with luteus exudate diffusing into medium. **b-c** Conidiogenous cells giving rise to conidia on MEA. **d-h** Various conidial shapes and sizes on MEA. **f** Germinating conidia on MEA. **g-h** Swollen conidial cells giving rise to smaller conidia. **i** Conjugation tube formation between two conidia. **j** Conidium disintegrating on MEA. Bars: B-C, F-I = 10 µm; E, J = 5 µm; D = 2,5 µm

produced on MEA within 2 wk. This species also has smaller conidia than those of *L. acicola*.

Type: **Honduras**: Santa Barbara, on needles of *Pinus caribaea*, 1980, *H.C. Evans*, (PREM 62196 – holotype; CMW 42205 = IMI 281561 = CBS 144453 – ex-type culture).

Description: *Sexual state* not observed. *Conidiomata* olivaceous to vinaceous brown on MEA. *Conidiophores* cylindrical, extending in densely aggregated palisade, hyaline to honey to pale vinaceous brown, smooth to verruculose, unbranched or branched at base, septate or aseptate, often encased in granular yellow to light brown mucoid sheath, length up to 60 µm, 2.0–5.0 µm diam. *Conidiogenous cells* terminal, integrated, subcylindrical to cylindrical, hyaline to light brown, smooth to verruculose, proliferating several times percurrently with visible annelations near apex, septate or aseptate, (4.5–)5.5–10.5(– 12.0) × (1.5–)2.0–3.5(– 5.0) µm. *Conidia* three different conidial types. All three types solitary, smooth to verruculose, subhyaline to honey to light brown, often enclosed in granular light luteus mucoid sheath. Type 1 straight to strongly curved, subcylindrical to cylindrical, subobtusely rounded apex, truncate, 1–4-septate, base (1.5–)2.0–2.5(– 3.0) µm diam. (22–)25.0–34.0(– 43.0) × (2.0–)2.5–3.0(– 3.5) µm. Type 2 slightly curved, cylindrical with both apex and base rounded, 0–2-septate, (14.5–)15.5–19.5(– 22.0) × (2.0–)2.5–3.0(– 3.5) µm. Type 3 buds from larger conidia (see notes) or from conidiogenous cells, hyaline, fusiform to cylindrical with subobtusely rounded apex and base, 0–1-septate, (10.0–)11.0–14.0(– 15.5) × (2.0–)2.0–2.5(– 3.0) µm.

Culture characteristics: Colonies flat to somewhat erumpent, spreading, with sparse aerial mycelium, surface folded, with smooth, lobate margins. On MEA, surface isabelline with patches of pale luteus to dark olivaceous green, reverse olivaceous to fuscous black. Mucoid yellow to peach to yellow-green exudate present. Luteus to sienna coloured metabolite diffusing into medium. On PDA, surface isabelline in centre, rosy buff in outer region, dark olivacous-brown on edges and isabelline in reverse. Sulphur yellow to cinnamon coloured metabolite diffuses into media. On OA, surface dirty white with diffuse umber outer region. *Growth characteristics*: optimal growth temperature 25 °C, after 4 wk., colonies at 10, 15, 20, 25 and 30 °C reached maximum of 11.5, 21, 31, 31.5 and 22.5 mm,

with mean growth of 2.2, 4.5, 6.1, 6.9 and 3.6 mm / wk. respectively.

Notes: The cells in the conidia often swell and break off, forming endospores as described in *L. acicola* (Siggers 1950; Crosby 1966; Evans 1984). Secondary conidia were commonly produced in cultures of this species, similar to those previously described for *L. acicola* specimens examined directly from needles (Evans 1984).

Additional material examined: **Guatemala**: Alta Verapaz, Santa Cruz Verapaz, near Tactíc, on needles of *Pinus oocarpa*, 21 Oct 2010, *I. Barnes* (PREM 62194, CMW 37125 = CBS 144454 – culture); *loc. cit., I. Barnes* (culture CMW 37129); Jalapa, Finca Forestal Soledad, on needles of *Pinus maximinoi*, 21 Oct 2010, *I. Barnes* (PREM 62195, CMW 36809 = CBS 144455 – culture). **–Mexico**: on needles of a *Pinus* sp., 30 Nov 2009, *M. de Jesús Yáñez-Morales* (CBS H-21112; culture CMW45425 = CPC 17822 = CBS 133789);

DISCUSSION

Four novel species of *Lecanostica* from infected pine needles collected in Central America are reported and named as *L. jani*, *L. pharomachri*, *L. tecunumanii*, and *L. variabilis*. There are now nine species described in the genus and these can be distinguished based on a phylogenetic inference for multiple gene regions. The two previously described species, *L. brevispora* and *L. guatemalensis,* were also found in this study and they provide new host and country records. The well-known pine pathogen, *L. acicola*, was not found on any of the samples collected from five *Pinus* spp. in seven regions of Central America considered in this study. This suggests that the species is not native in that region.

Results of the present study support the view of Quaedvlieg et al. (2012) that a combination of the ITS and *TEF*1 should be used as barcoding loci to distinguish between species of *Lecanosticta* and other closely related species. Additionally, statistically well supported clades were obtained in this study using the *MS204* gene region. However, genus-specific primers should ideally be designed to increase the amplification success rate for this gene region in *Lecanosticta*. Although the *BT*2 gene was also proposed as a possible barcoding region that could be used to distinguish between *Lecanosticta* species and other species of *Mycosphaerellaceae* (Quaedvlieg et al. 2012), it amplified poorly in the present study. The *BT*1 gene region distinguished most of the species, but not *L. pharomachri* and *L. variabilis* and provided low statistical support at all nodes.

The results of this study support the view of Evans (1984) that *Lecanostica* species are comprised of morphotypes or ecotypes. Based on phylogenetic analyses, we were able to define lineages for species also supported by morphological characteristics. The *TEF*1 sequences were highly variable but several well supported clades and subclades were observed within species (Fig. 2). These clades possibly represent additional new species but we lacked sufficient cultures and support to describe them. The clade with the most diversity in terms of unique *TEF*1 haplotypes, Clade 1, was *L. brevispora* (represented by 22.1% of *TEF*1 haplotypes in the genus) and this species was also represented by the largest number of isolates. High haplotype diversity was observed in the *L. jani* (16.1% of *TEF*1 haplotypes) and *L. pharomachri* (10.3% of *TEF*1 haplotypes) clades and different lineages were observed in the *L. acicola* (13.2% of *TEF*1 haplotypes), *L. guatemalensis* (17.6% of *TEF*1 haplotypes), and *L. variabilis* (13.2% of *TEF*1 haplotypes) clades. The other gene regions, especially *MS204* and *RPB*2 were also highly variable in terms of distinguishing haplotypes. *RPB*2 is however, not recommended to distinguish between *L. acicola* and *L. variabilis* as these two species form paraphyletic groups in the tree for this gene region.

The paleo-geographic region that includes Mexico and extends into Central America is regarded as one of three centres of diversity of *Pinus* species (Farjon 1996). Pine needles that were sampled from Central America in this study were symptomatic but serious disease was not observed. This suggests that *Lecanosticta* species have co-speciated with their native pine hosts in this region. Of the nine known species, *L. gloeospora* and *L. longispora* have been identified only in Mexico and *L. brevispora* and *L. variabilis* have been identified in both Mexico and Central America. *Lecanosticta guatemalensis, L. jani, L. pharomachri* and *L. tecunumanii* are currently known only from Central America.

Lecanosticta acicola has been redefined in this study. All isolates from Central America that had previously been identified as *L. acicola,* based on morphological characteristics, are now treated as different species. This is based on newly available DNA sequence data and phylogenetic analyses emerging from this study as well as that of Quaedvlieg et al. (2012). *L. acicola* is, however, still considered as present in Mexico.

Based on *TEF*1 analyses, *L. acicola* resolves in three lineages. Janoušek et al. (2016) used microsatellites to show that a lineage of *L. acicola* from the northern USA was introduced into Central and Northern Europe, and a lineage from the southern USA was introduced into France, Spain, and Colombia. Similarly, Huang et al. (1995) reported that *L. acicola* was introduced into China from the southern part of the USA. Our analyses of the *TEF*1 sequences of isolates from the northern

parts of the USA, Lithuania, and a representative sequence for Central and Northern Europe and Canada (KJ938438, Table 3), formed one distinct lineage with *L. acicola* (Fig. 2). All isolates from the southern parts of the USA, as well as representative sequences for Asia, France, Spain, and Colombia (Table 3), formed a second distinct lineage in the clade accommodating *L. acicola* (Fig. 2). The third lineage included only isolates from Mexico, which suggests that isolates in this lineage have remained in their area of origin and have not been introduced elsewhere. Because this Mexican lineage had strong bootstrap support separating it from the other two lineages, it could represent a further new species. Only *TEF*1 data are currently available for the Mexican collections (downloaded from GenBank) and other gene regions would need to be sequenced and analysed to determine whether this really represents a further novel taxon.

Evans (1984) first speculated that Central America could be the centre of origin of *Lecanosticta*. The phylogenetic analyses conducted in the present study showed that there is a high diversity of species and lineages for this genus in Central America, which supports Evans' hypothesis. This is the first study where all known species of *Lecanosticta* have been delineated based on DNA sequence data and phylogenetic analysis, and it has led to the recognition of additional new taxa from Central America and Mexico. Eight of the nine species of *Lecanosticta* have been reported only from this region, and our results consequently represent strong support for a Mesoamerican *Lecanosticta* centre of diversity and likely origin. Population genetic analyses for the most common of these species will serve to provide additional support for this hypothesis.

CONCLUSIONS

Phylogenetic inference based on DNA sequence data including new collections from Mexico and Central America revealed four novel species and reaffirmed the identity of the five previously described taxa. The most important of these species is the well-known pine pathogen *L. acicola* that was redefined as a North American taxon and for which at least three distinct lineages can be distinguished using the *TEF*1 gene region. New regions of occurrence and host range emerged for *Lecanosticta* spp. with eight of the nine species occurring in Mesoamerica. This suggests that Mesoamerica is the most likely centre of origin for *Lecanosticta*. *Lecanosticta acicola* was best known as the causal agent of the important brown spot needle blight of *Pinus palustris* in the southeastern USA but it has more recently spread within the USA and Europe where it has become an increasingly important pathogen of numerous *Pinus* spp. The other species of *Lecanosticta,* including those newly described, are of unknown importance but it seems likely that some of them could pose a threat to *Pinus* spp. if they were introduced into new environments in the future. The fact that various Mesoamerican *Pinus* spp. are increasingly being used for plantation development in the Southern Hemisphere implies that extreme caution should be applied not to introduce *Lecanosticta* spp. together with germplasm needed for future planting programmes.

Additional files

Additional file 1: Figure S1. Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the ITS region. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma* species were used as the outgroup taxa. All represented type species are indicated in bold and with a "T". Clades indicated on the left correspond with the clades in Fig. 1. Within the *L. jani* clade a "Δ" next to the isolate indicates that the isolate exhibits Type 2 morphology but it groups with Subclade 1 or exhibits Type 1 morphology but groups with Subclade 2. (PPTX 61 kb)

Additional file 2: Figure S2. Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the *BT*1 region. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma* species were used as the outgroup taxa. All represented type species are indicated in bold and with a "T". Clades indicated on the left correspond with the clades in Fig. 1. (PPTX 54 kb)

Additional file 3: Figure S3. Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the *MS204* region. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma septosporum* was used as the outgroup taxa. All represented type species are indicated in bold and with a "T". Clades indicated on the left correspond with the clades in Fig. 1. Within the *L. jani* clade a "Δ" next to the isolate indicates that the isolate exhibits Type 2 morphology but it groups with Subclade 1 or exhibits Type 1 morphology but groups with Subclade 2. (PPTX 55 kb)

Additional file 4: Figure S4. Maximum likelihood tree representing the five known and four novel species of *Lecanosticta* generated from the *RPB*2 region. MP bootstrap support (> 70%) are indicated first, followed by ML bootstrap values (MP/ML, * = insignificant value). Bold branches indicate BI values > than 0.95. *Dothistroma* species were used as the outgroup taxa. All represented type species are indicated in bold and with a "T". Clades indicated on the left correspond with the clades in Fig. 1. Within the *L. jani* clade a "Δ" next to the isolate indicates that the isolate exhibits Type 2 morphology but it groups with Subclade 1 or exhibits Type 1 morphology but groups with Subclade 2. (PPTX 61 kb)

Abbreviations

1F1N: One Fungus One Name; AIC: Akaike Information Criterion; BI: Bayesian inference; BSNB: Brown spot needle blight; *BT*1: Beta-tubulin-1 gene region; *BT*2: Beta-tubulin-2 gene region; CA: California; CBS: The culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CI: Consistency index; CMW: The culture collection of the Forestry and Agricultural Biotechnology Institute; COSAVE: El Comité de Sanidad Vegetal; CPC: Personal collection of Pedro Crous housed at CBS; DSM: Dothistroma Sporulating Media; FABI: Forestry and Agricultural Biotechnology Institute; HI: Homoplasy index; IASPC: Inter-African Phytosanitary Council; ICN: International Code of Nomenclature for algae, fungi, and plants; IMI: The UK National Fungus Collection maintained by CABI Bioscience, Egham, UK; ITS: Internal transcribed spacers; MA: Massachusetts; MB: MycoBank; MCMC: Markov Chain Monte Carlo; MD: Maryland; ME: Maine; MEA: Malt Extract Agar; ML: Maximum likelihood; MO: Missouri; MP: Maximum parsimony; *MS204*: The guanine nucleotide-binding protein subunit beta; NCBI: National Centre for Biotechnology Information; NJ: New Jersey;

OA: Oatmeal Agar; PCR: Polymerase chain reaction; PDA: Potato Dextrose Agar; PHT: Partition homogeneity test; PREM: The dried herbarium collection of the South African National Collection of Fungi; RC: Rescaled consistency index; RI: Retention index; *RPB2*: RNA polymerase II second largest subunit; TBR: Tree-bisection-reconnection; *TEF1*: Translation elongation factor 1-α gene; TL: Tree length

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Availability of data and materials
All data generated in this study are included in this published article and its supplementary files. The datasets analysed are available from the corresponding author on reasonable request.

Authors' contributions
Acquisition of sample material was performed by PO and IB. Fungal isolations were done by IB. Data collection and all analyses were performed by AvdN. Funding acquisition was done by IB and MJW. IB and MJW supervised the project. AvdN wrote the original draft, and review and editing was performed by AvdN, IB MJW and PO. All authors read and approved the manuscript.

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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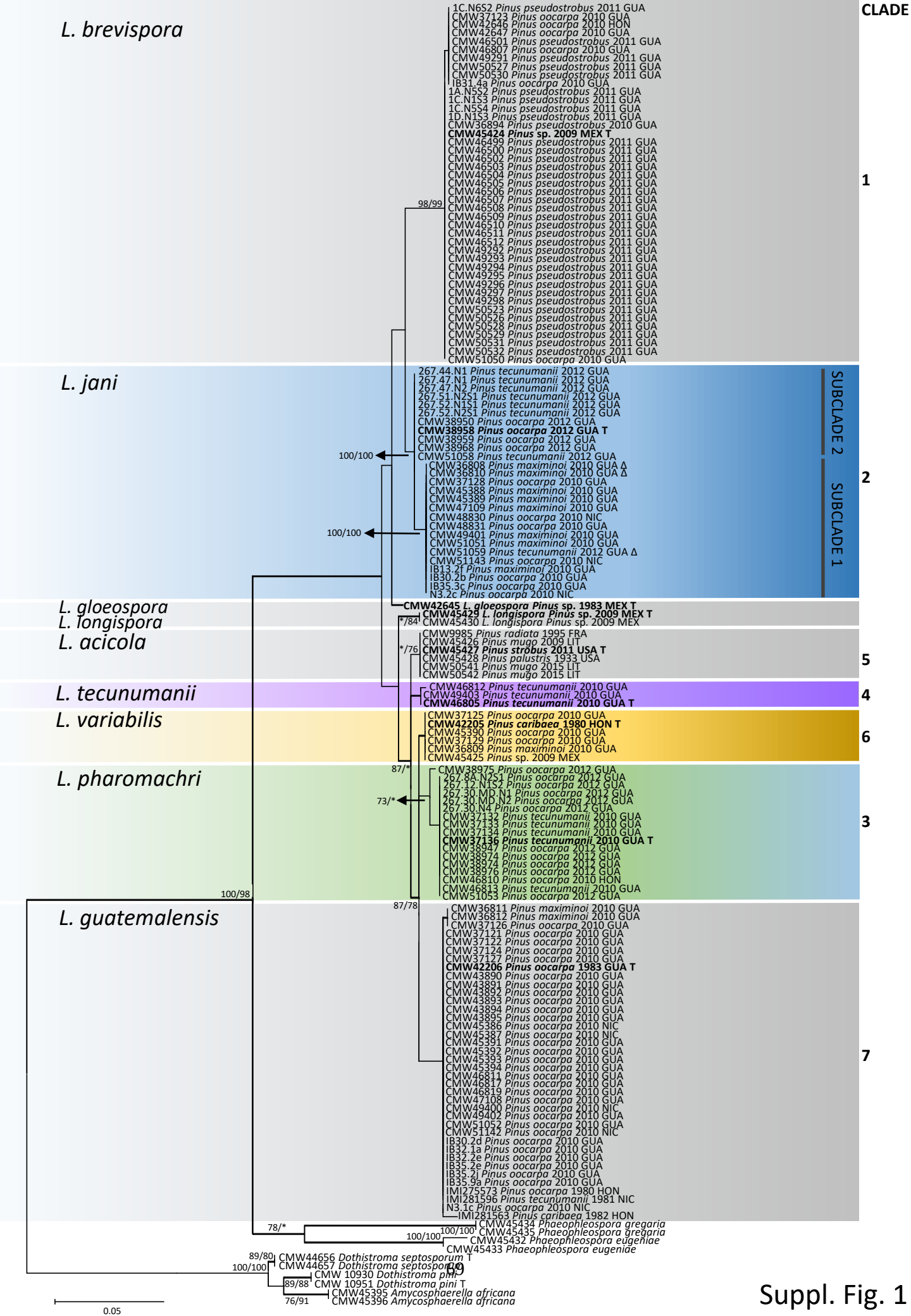
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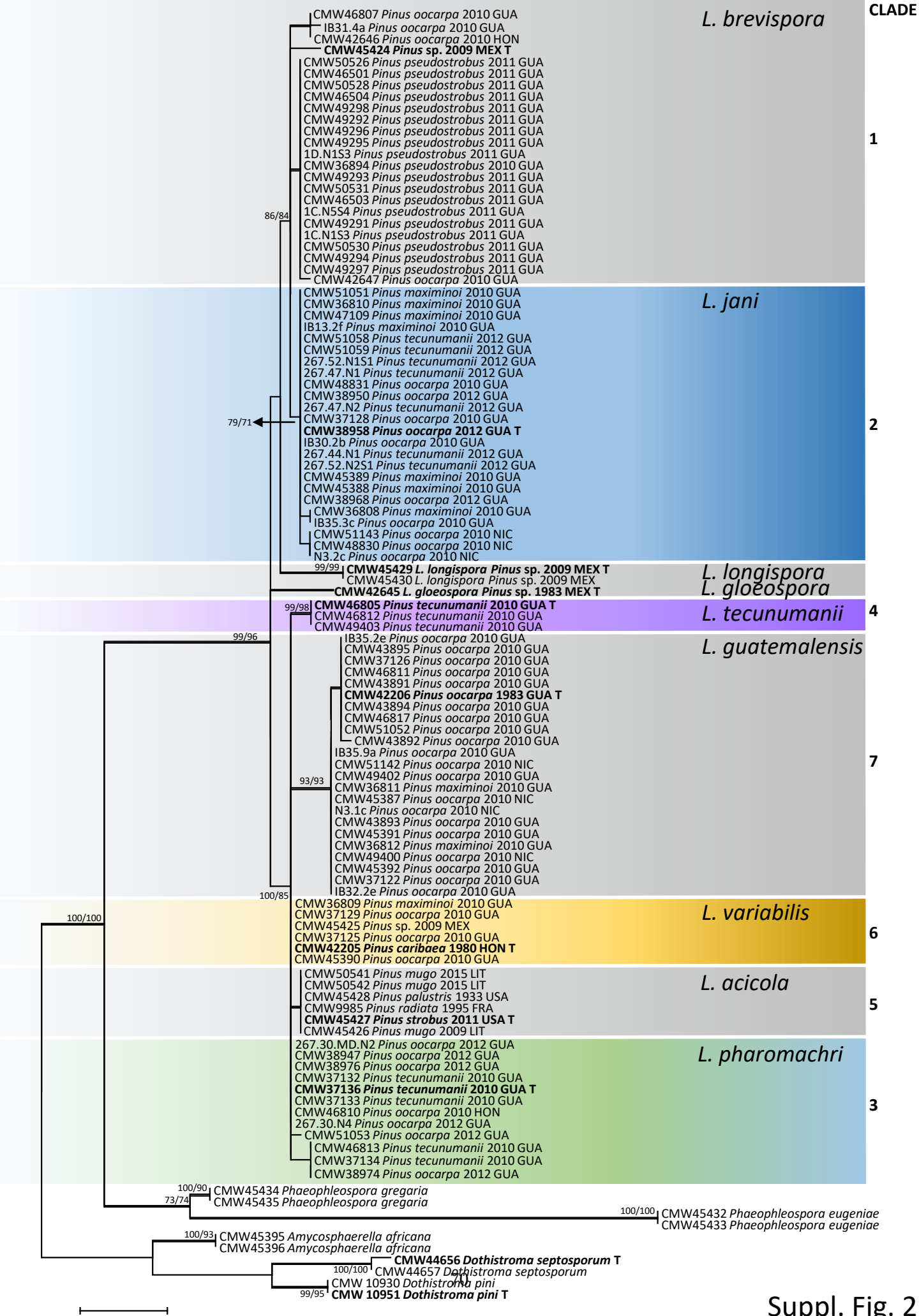
Suppl. Fig. 1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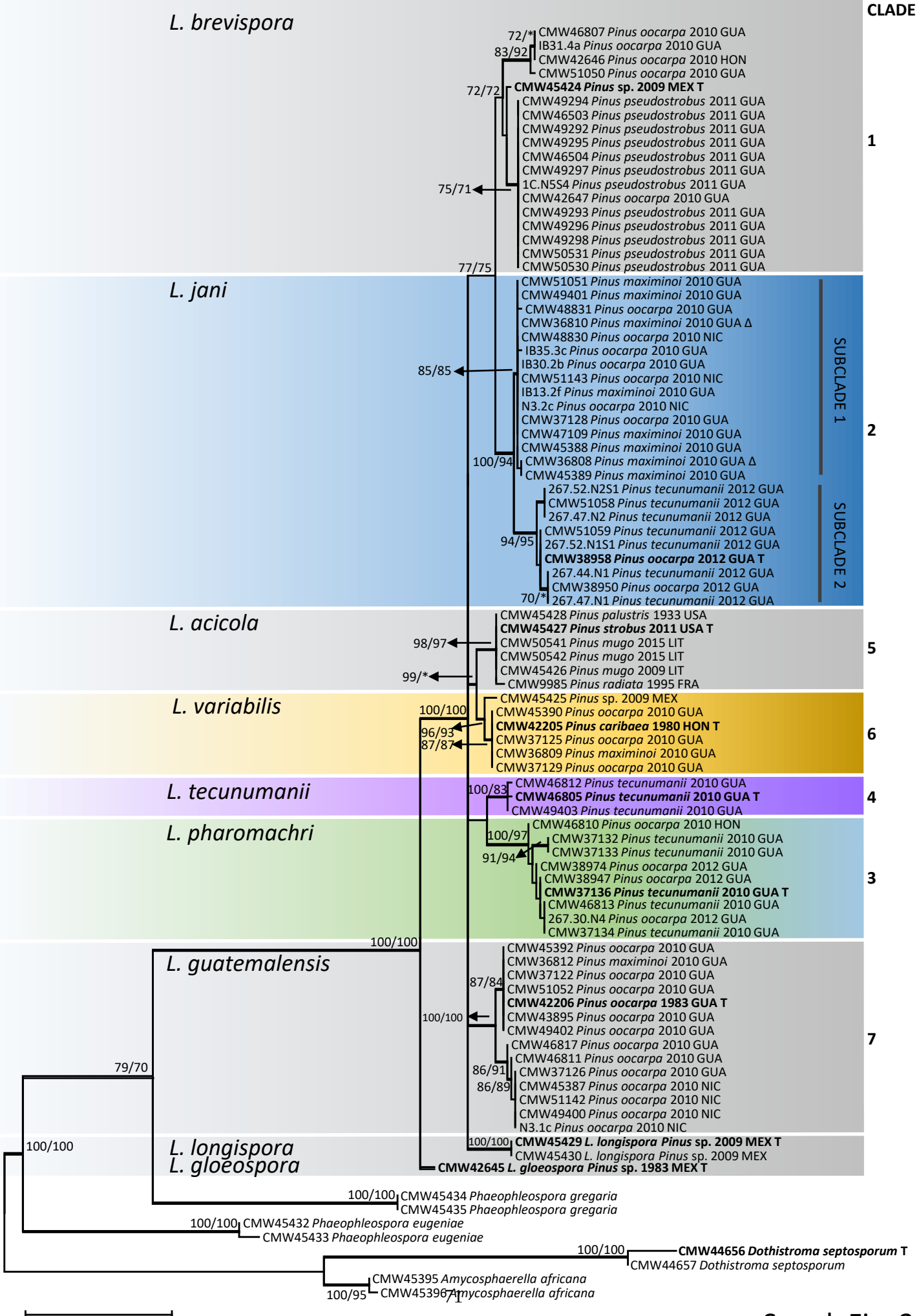
Suppl. Fig. 2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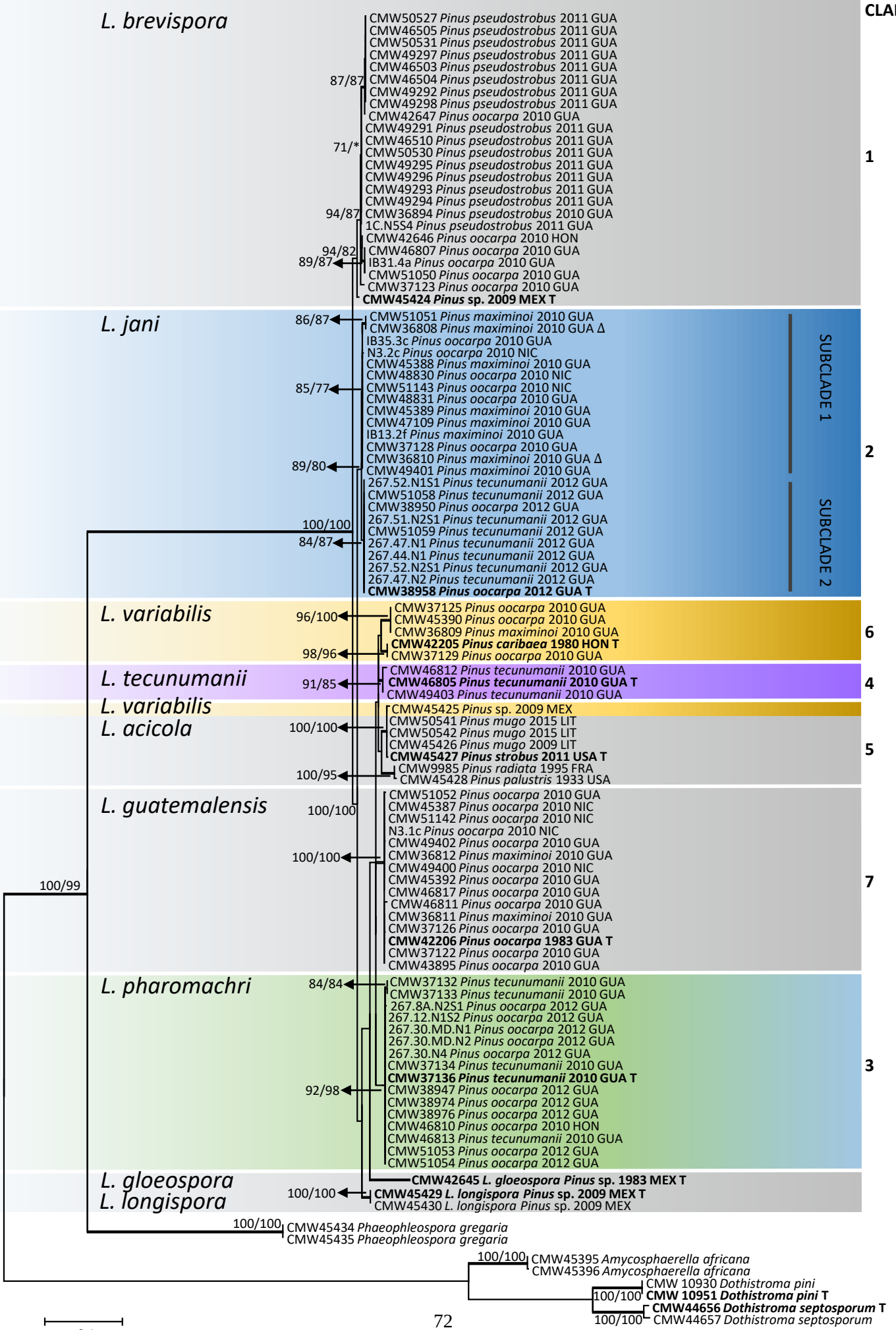
Suppl. Fig. 3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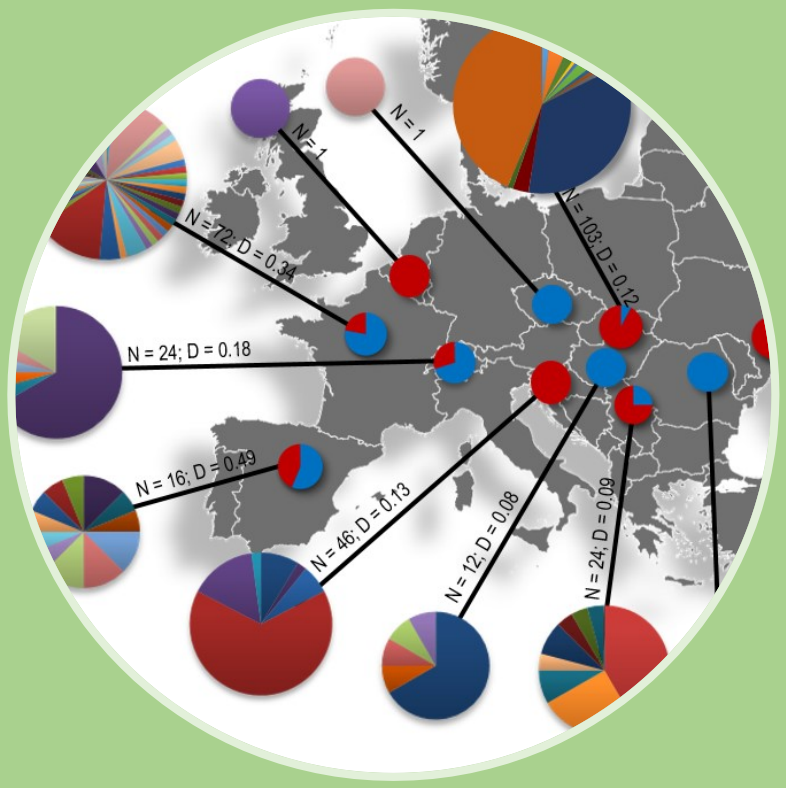
CMW51054 Pinus oocarpa 2012 GUA
3
L. gloeospora
L. longispora
CMW42645 L. gloeospora Pinus sp. 1983 MEX T
100/100
CMW45429 L. longispora Pinus sp. 2009 MEX T
CMW45430 L. longispora Pinus sp. 2009 MEX
100/100
CMW45434 Phaeophleospora gregaria
CMW45435 Phaeophleospora gregaria
100/100
CMW45395 Amycosphaerella africana
CMW45396 Amycosphaerella africana
CMW 10930 Dothistroma pini
100/100
CMW 10951 Dothistroma pini T
CMW44656 Dothistroma septosporum T
100/100
CMW44657 Dothistroma septosporum
0.1

Suppl. Fig. 4

RESEARCH CHAPTER 2



Population structure and diversity of the needle pathogen *Dothistroma pini* suggests human mediated movement in Europe

Abstract

Dothistroma needle blight is an important disease of *Pinus* species that can be caused by one of two distinct pathogens; *Dothistroma septosporum* and *D. pini*. *Dothistroma septosporum* has a wide geographic distribution and is relatively well-known. In contrast, *D. pini* is known only from the USA and Europe, and there is a distinct lack of knowledge regarding its population structure and genetic diversity. The recent development of 16 microsatellite markers for *D. pini* provided an opportunity to investigate the diversity, structure and mode of reproduction for populations collected over a period of 12 years, on eight different hosts in Europe. In total, 345 isolates from Belgium, the Czech Republic, France, Hungary, Romania, Western Russia, Serbia, Slovakia, Slovenia, Spain, Switzerland and Ukraine were screened with microsatellite and species-specific mating type markers. A total of 109 unique multilocus haplotypes were identified and structure analyses suggested that the populations are influenced by location rather than host. Populations from France and Spain displayed the highest level of genetic diversity followed by the population in Ukraine. Both mating types were detected in most countries, with the exception of Hungary, Russia and Slovenia. Evidence for sexual recombination was supported only in the population from Spain. The observed population structure and several shared haplotypes between non-bordering countries provides good evidence that the occurrence of *D. pini* in Europe has been strongly influenced by human activity in Europe.

1. Introduction

Dothistroma needle blight (DNB) is recognized as one of the most important diseases of *Pinus* spp., both in planted and native forests, worldwide. The disease has a long history of having damaged plantations in the Southern Hemisphere dating back to the 1960s (Gibson 1972), but during the course of the last three decades, it has also increased in severity and incidence in the Northern Hemisphere (Welsh et al. 2009; Drenkhan and Hanso 2009; Boroń et al. 2016; Drenkhan et al. 2016; Ghelardini et al. 2020). Dothistroma needle blight has been reported on 110 taxa, of which 96 are in the genus *Pinus* (Drenkhan et al. 2016; Jánošíková-Hečková et al. 2018) and reports of the disease on new hosts and in new geographical regions are increasing (Jánošíková-Hečková et al. 2018; Matsiakh et al. 2018; Mullett et al. 2018; Ondrušková et al. 2018; Mesanza et al. 2020; EPPO 2019).

For many years, the identity of the causal agents of DNB was confused and strongly debated (Barnes et al. 2016). This was due to a single distinct symptom (red bands on infected needles) and taxonomy reliant on morphological characteristics of the associated pathogen. Almost 110 years after the first description of DNB in France (Vuillemin 1896), it was conclusively shown that two distinct species can cause this disease. These include *Dothistroma septosporum* (Dorogin) M. Morelet and *D. pini* Hulbary that are most effectively distinguished based on DNA-based identification (Barnes et al. 2004; Barnes et al. 2016). In an attempt to consolidate existing knowledge, an extensive collaboration of pathologists participating in the DIAROD (Determining Invasiveness And Risk Of *Dothistroma:* DIAROD, COST Action FP1102) project documented, as far as possible, the geographic distribution, hosts and mating type distribution of these two *Dothistroma* spp. (Drenkhan et al. 2016).

Dothistroma septosporum has been the most extensively studied of the two DNB pathogens. This is at least in part due to its accidental introduction into various countries of the Southern

Hemisphere where it became one of the most important constraints to plantation forestry based on non-native *Pinus radiata* (Gibson 1972). *Dothistroma septosporum* has now been recorded in both the Southern and Northern Hemispheres in 48 countries (Drenkhan et al. 2016; Mullett et al. 2018; Matsiakh et al. 2018; Ghelardini et al. 2020) and its population structure and diversity in many of these areas is well understood (Adamson et al. 2018; Barnes et al. 2014b; Capron et al. 2020; Drenkhan et al. 2013; Mullett et al. 2015; Mullett et al. 2021; Oskay et al. 2020). Several genomes of the pathogen have been sequenced and population genomics studies (Ennos et al. 2020), as well as investigations considering factors affecting its pathogenicity have been conducted (Bradshaw et al. 2019; Guo et al. 2020). In contrast, very little is known regarding the biology or ecology of *D. pini*.

Dothistroma pini is known only in the Northern Hemisphere where it has been recorded in 17 countries on 18 different *Pinu*s hosts as well as *Picea abies* (Drenkhan et al. 2016; Jánošíková-Hečková et al. 2018; Matsiakh et al. 2018; Mullett et al. 2018; Ondrušková et al. 2018, Wartalska et al. 2021). The pathogen was first described on non-native *Pinus nigra* J.F. Arnold collected in Michigan (1960s), Minnesota and Nebraska in the USA (Barnes et al. 2004). At that time, it was thought to be restricted to the North American continent. Since then, *D. pini* has been reported in four additional states of the USA (Barnes et al. 2014a; Mullett et al. 2018).

Dothistroma pini was first discovered in Europe when it was found in the Ukraine and Russia in 2008 on non-native *Pinus nigra* subsp. *pallasiana* (Lamb.) Holmboe (Barnes et al. 2008b). However, molecular analysis of herbarium samples collected in France have shown that the pathogen has been present on the European continent at least since 1907 (Fabre et al. 2012). Since the first molecular identification of *D. pini* in Europe in 2008, the pathogen has also been confirmed as present in Belgium (Schmitz et al. 2013), Czech Republic (Bergová and Kryštofová 2014), France (Ioos et al. 2010), Georgia (Matsiakh et al. 2018), Germany (EPPO 2019), Hungary (Barnes et al. 2011), Montenegro (Lazarević et al. 2017), Poland (Wartalska

et al. 2021), Romania (Barnes et al. 2016), Serbia (Pap et al. 2015), Slovenia (Piškur et al. 2013), Slovakia (Ondrušková et al. 2017), Spain (Iturritxa et al. 2015) and Switzerland (Queloz et al. 2014).

Very little is known regarding the genetic diversity and population structure of *D. pini.* In a preliminary study testing 16 microsatellite markers developed for *D. pini* (Siziba et al. 2016), high levels of genetic diversity were found in populations of the pathogen in France, at least indicating the presence of the pathogen in that country for many years. In contrast, populations in other European countries such as Slovakia had low genetic diversity and strong signals of clonality, which suggests that *D. pini* was introduced into Slovakia (Adamčíková et al. 2021).

Collections of *D. pini* made over a 12-year period, and including those obtained while documenting the presence of both this species and *D. septosporum* in Europe by the DIAROD consortium, has resulted in a collection of 345 isolates. This collection provided an opportunity to expand on previous, relatively small-scale studies (Adamčíková et al. 2021; Siziba et al. 2016), and to more comprehensively consider the population structure and diversity of *D. pini* in Europe. The aims of this study were thus to (1) investigate the population diversity and structure of the pathogen including countries or specific locations where the pathogen has been reported in Europe, and (2) determine its mode of reproduction and likely means of dispersal in Europe.

2. Materials and Methods

2.1 Sample collection, fungal isolations and identifications

Pine needles that displayed DNB symptoms were collected between 2008 and 2019 from 31 locations in 11 countries of Europe (Table 1, Fig. 1). Additionally, the data generated for the eight locations in Slovakia by Adamčíková *et al.* (2021) were incorporated in this study. For most samples, isolations were made from the collected samples as described by Barnes *et al*.

(2004). Single germinating conidia were selected and plated onto 2% Dothistroma Sporulating Media (DSM: 5 g yeast extract (Biolab, Merck, Modderfontein, South Africa), 20 g malt extract (Biolab) and 15 g agar (BD Difco™, Sparks, MD) per litre of distilled water with 100 mg/l streptomycin (Sigma-Aldrich, St Louis, MO). The plates were incubated for 4-6 weeks at 23°C under natural day/night light cycles. All isolates are either maintained as cultures or freeze-dried material in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) in Pretoria, South Africa (Appendix 1).

Fungal tissue was freeze dried and DNA extracted using a Zymo Research ZR fungal/Bacterial DNA MiniPrep™ kit (Irvine, CA) as described by van der Nest *et al*. (2019b). The identity of the isolates was determined by amplifying and sequencing the internal transcribed spacers (ITS) 1 and 2 and the 5.8S rDNA region with the ITS1 and ITS4 primers (White et al. 1990) and using the protocols described in Barnes *et al.* (2004). The PCR amplicons were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the product was run on an ABI PRISM 3500xl capillary auto sequencer (Thermo Fisher Scientific).

CLC Main workbench version 8.0 (CLC Bio, https://www.qiagenbioinformatics. com/products/clc-main-workbench/) was used to create consensus sequences using the forward and reverse sequences of the ITS region for each isolate. All consensus sequences were compared in a BLAST analysis against the GenBank database (NCBI; http://www.ncbi.nlm.nih. gov/genbank/) to confirm the identity of each isolate. To determine the ITS haplotype for each confirmed isolate of *D. pini,* sequences were compared to those reported in Barnes *et al*. (2016) and Mullett *et al*. (2018) using MEGA 7.0.14 (Kumar et al. 2016).

2.2 Microsatellite amplification and haplotype determination

Sixteen labelled microsatellite markers (Siziba et al. 2016) were used to amplify all isolates considered in this study. An additional marker (Doth_A; Barnes *et al*. (2008a)) was included as an internal diagnostic marker. PCR reactions were performed as described by Adamčíková *et al.* (2021). When multiple bands were observed, the protocol was repeated but the $MgCl_2$ was reduced to 0.9 µl and the SABAX water volume adjusted accordingly (Table 2). PCR reactions were carried out on an Applied Biosystems® Veriti® 96 well Thermal cycler (Thermo Fisher Scientific, Waltham, MA). The fragments were amplified using the same cycling conditions described by Barnes *et al.* (2014b) with primer pair annealing temperatures provided in Table 2. The amplified products were visualized by staining 5 ul of each of the products with 1 ul GelRed nucleic acid gel stain (Biotium) and then separating the fragments on 2% SeaKem LE agarose gel (Lonza) for 15 min at 90 V. The fragments were viewed under a UV light using an GelDoc EZ Imager (BioRad).

PCR products were pooled in two panels for fragment analysis as described by Siziba *et al*. (2016) and with adjusted dilutions as indicated in Table 2. In preparation for analysis, one µl of the pooled product was added to 0.14 µl GENESCAN^{TM} -500 LIZ® (Life Technologies, Applied Biosystems, Warrington, UK) size standard and 12 µl formamide. An ABI PRISM 3500xl capillary auto sequencer (Thermo Fisher Scientific) was used for fragment analyses of the prepared reactions. Alleles sizes were scored using GENEMAPPER® Software version 5.0 (Applied Biosystems, Foster City, CA).

Alleles scored for each marker were combined to obtain a multilocus haplotype (MLH) for each isolate. Individual isolates were considered clones if they had the same combination of alleles for each marker analysed. The R package *poppr* (Kamvar et al. 2014) was used to

determine the number of MLHs in the dataset. Two datasets were generated for further analyses; the data set that had not been clone-corrected included all individuals and the clone-corrected data set contained single representatives of each unique multilocus haplotype per population. Populations consisted of isolates from a particular country.

2.3 Genetic diversity

The R package *poppr* (Kamvar et al. 2014) was used to calculate the number of multilocus haplotypes (MLH), the expected number of MLHs based on rarefaction (Hurlbert 1971), the Shannon-Wiener Index (Shannon 2001), the Stoddart and Taylor's Index (Stoddart and Taylor 1988), the Simpson's Index (Simpson 1949) and genotypic evenness (Grünwald et al. 2003) for the populations using the non-clone-corrected dataset, as well as the genetic diversity (Nei 1978) per population using the clone-corrected dataset. The clonal fraction was calculated as in Barnes *et al.* (2014b). Furthermore, allelic richness (A_R) and private allelic richness (PA_R) were determined using ADZE (Szpiech et al. 2008) that uses rarefaction to allow for comparisons between populations with varying sample sizes. Calculations were standardized corresponding to the country with the smallest population size (Russia, N=6). A minimum spanning network using Bruvo's genetic diversity (Bruvo et al. 2004) comparing the multilocus haplotypes over 16 microsatellite loci was also drawn using the *ismn* function in the *poppr* package.

2.4 Population structure

The clone-corrected dataset was used to determine the most likely number of population clusters based on microsatellite allele sizes for all the individuals using STRUCTURE 2.3.4 (Falush et al. 2003). The program assigns individuals to clusters (K) using a Bayesian clustering algorithm. Thirty independent runs of K = 1–20 were performed, with a burn-in

value of 100 000 and 500 000 iterations. An admixture model with correlated allele frequencies was selected with no additional priors such as information on the host or location.

The optimal number of clusters was estimated with StructureSelector (Li and Liu 2018). StructureSelector implements the Evanno method that includes delta(K) and LnP(K) (Evanno et al. 2005) with the additional four Peuchmaille methods (MAXMEAK, MAXMEDK, MEDMEDK and MEDMEAK) that provide a more accurate estimate of K in populations with uneven sizes (Puechmaille 2016). In order to implement the Puechmaille methods, countries were assigned as populations in the dataset and the analysis was repeated twice. First a threshold of 0.5 was selected and second a threshold of 0.8 was selected to apply more stringent assignment of individuals into clusters. After the optimal K was determined, isolates were assigned into the optimal K clusters with a final STRUCTURE run with thirty independent runs, a burn-in value of 100 000 and 1 000 000 iterations. CLUMPAK (Kopelman et al. 2015) was used to converge all 30 runs of the optimal K and the output was visualized using the DISTRUCT program (Rosenberg 2004). Both CLUMPAK and DISTRUCT were implemented using the StructureSelector website (https://lmme.qdio.ac.cn/StructureSelector/).

The *adegenet* package in R studio (Jombart and Ahmed 2011) was used to perform discriminant analysis of principal components (DAPC) (Jombart et al. 2010) to additionally visualize the population genetic structure of the European samples. The *find.clusters* function was used to determine the optimal number of clusters by assessment of Bayesian information criterion (BIC). The optimal number of principal components retained in the analysis was determined by cross-validation using the *xvalDapc* function.

An Analysis of Molecular Variance (AMOVA) test was implemented in GENALEX version 6.5 (Peakall and Smouse 2012). The test was used to evaluate if there was genetic differentiation among and within groups according to hosts species, countries and locations.

One thousand permutations of the dataset were used to test significance. The null hypothesis of no genetic difference was rejected at $P < 0.05$.

2.5 Mating type determination and random mating

The mating type of the *D. pini* isolates was determined by using the primers of Groenewald *et al.* (2007) or in some cases the primer set of Janoušek *et al.* (2014). Each reaction consisted of 2 µl template DNA (20 ng/µl concentration), 0.08 µl Faststart Taq DNA polymerase, 0.25 µl of each of the primers as specified by either Groenewald *et al.* (2007) or Janoušek *et al.* (2014), 0.6 µl of a mix of 200 mM dNTPs, 1.5 µl of 2.5mM $MgCl_2$, 1.25 µl 10x PCR reaction buffer and the volume was adjusted to 12.5 µl with sterile SABAX water.

PCR reactions were carried out on an Applied Biosystems® Veriti® 96 well Thermal cycler (Thermo Fisher Scientific, Waltham, MA). The cycling conditions for all microsatellite fragments included an initial denaturation step at 95°C for 4 min, 10 cycles consisting of 94°C for 20 s, a 45 s annealing step with the temperature set according to the protocols by Groenewald *et al.* (2007) or Janoušek *et al.* (2014), and an elongation step of 45 s at 72°C. This was followed by a further 25 cycles of 94°C for 20 s, 45 s with a 5 s extension step per cycle at the annealing temperature, a 72°C extension for 45 s and a final extension step of 72°C for 30 min. The amplified products were visualized by staining 10 ul of each product with GelRed™ nucleic acid gel stain. The fragments were separated on 2% SeaKem® LE agarose gel for 50 min at 90 V and viewed under a UV light using the GelDoc™ EZ Imager (BioRad, Hercules, CA). When using the Groenewald *et al.* (2007) primers, isolates that had an amplicon size of 820 bp were assigned as *MAT1-1* and those with a size of 480 bp were assigned as *MAT1-2*. The Janoušek *et al.* (2014) primer sets produced amplicon sizes of 634 bp for *MAT1-1-1* and 323 bp for *MAT1-2*.

The possibility of sexual recombination was investigated using three methods. An exact binomial test, using two-tailed P-values (http://www.biostathandbook.com/exactgof.html) was used to test if the mating type ratios deviated from a 1:1 ratio (at $P < 0.05$) in the non-clone-corrected dataset, which provides evidence of random mating. The index of association (I_A) (Brown et al. 1980; Smith et al. 1993) and rBarD (Agapow and Burt 2001) was used to test for linkage disequilibrium in the 16 microsatellite loci with both datasets using the R-package *poppr* (Kamvar et al. 2014). The null hypothesis of alleles at different loci having no linkage due to sexual mating was rejected when $P < 0.05$.

3. Results

3.1 Sample collection, fungal isolations and identification

A total of 345 cultures included in this study were obtained from collections made in Europe. All of these isolates screened with the Doth_A marker (Siziba et al. 2016) produced an allele size of 111 bp and were thus confirmed as *D. pini.* These included representatives from 12 (Belgium, Czech Republic, France, Hungary, Romania, Western Russia, Serbia, Slovakia, Slovenia, Spain, Switzerland and Ukraine) of the 16 European countries where *D. pini* has been reported. The isolations were made from plant material obtained from 10 different *Pinus* species or sub-species with *P. nigra* being the most common of these (Table 1).

Three of the six known *D. pini* ITS haplotypes (Barnes et al. 2016; Mullett et al. 2018) were identified in the collection of isolates (Table 1). Individuals having the ITS Haplotype 1 were the most abundant and were present in eight of the twelve countries (Czech Republic, France, Hungary, Slovakia, Slovenia, Spain, Switzerland, Ukraine) including 25 different locations. ITS Haplotype 2 was the second most abundant and was present in eight of the twelve countries (France, Romania, Western Russia, Serbia, Slovakia, Spain, Switzerland, Ukraine) and at 20 different locations. ITS Haplotype 4 individuals were present at nine locations in five countries

(Belgium, France, Serbia, Slovakia and Spain). All three haplotypes were present in France, Spain and Slovakia.

3.2 Microsatellite amplification and haplotype determination

A total of 109 alleles were detected across the 16 polymorphic microsatellite loci. The number of alleles at each locus ranged from 2 at DP-MS4 and DP-MS18 to 19 at DP-MS12 (Table 2). Isolates from Spain, Ukraine and Russia had the highest percentage (87.5%) of polymorphic loci (Appendix 1) and those from Hungary had the lowest percentage (31.2%) of polymorphic loci (excluding countries for which only single isolates were available).

A total of 109 unique multilocus haplotypes (MLH's) were identified in the 345 isolates analysed (Table 3, Fig. 2, Appendix 1) of which eight MLH's occurred in multiple, often non-bordering countries (Fig. 3). Some individuals sharing the same microsatellite MLH in different populations were of opposite mating type or of different ITS haplotypes, which suggests that they were not true clones. For example, multilocus haplotype 52 (Fig. 3) occurred in isolates from four countries (Hungary, Slovakia, Slovenia and Ukraine) and at seven different locations, covering a distance of approximately 1500 km. This MLH was represented by individuals with the *MAT1-1* idiomorph in Ukraine and the *MAT1-2* idiomorph in the other three countries. The second most commonly occurring MLH (MLH 56, Fig. 3) was shared by individuals from Souesmes (France), Diszel (Hungary) and Arborétum Mlyňany, Gabčikovo, Jahodna and Trstice in Slovakia. All of these individuals were of ITS Haplotype 1 but the individuals in Slovakia were *MAT1-2* and the isolates from France and Hungary were *MAT1-1*. The population from Russia included an individual having ITS Haplotype 2 that shared MLH 47 (Fig. 3) with an ITS Haplotype 1 individual in Hungary (1150 km apart).

3.3 Genetic diversity

Collections from France had the greatest number of MLHs and this was followed by isolates from the Ukraine. When considering populations with a sample size of six and higher, Hungary had the fewest MLHs (five) followed by Slovenia and Switzerland, which had six each (Table 3). When comparing the approximate number of haplotypes that would be expected for the largest shared sample size (N=6) based on rarefaction (eMLG), the genotypic richness was the highest in the France population (8.52) followed by that from Spain (8.50). The populations from Slovenia and Switzerland had the lowest genetic diversity (3.45 and 3.57 respectively) (Table 3). The Slovenian and Slovakian populations had the highest clonal fractions (0.87 and 0.86) followed by those from Switzerland 0.75 (Table 3). The lowest clonal fraction was found in populations from Russia (0) followed by those from Spain (0.25) and France (0.43). For populations collected within France, the clonal fraction ranged from 0 (Nueng-sur-Beuvron) to 0.61 (Villefranche-sur-Cher). In isolates from Slovenia, the clonal fraction also ranged from 0 (Ribnica) to 0.90 (Panovec). The clonal fraction of 0.55 in Ukraine was due to the high clonal fraction (0.67) in Tsjurupinsk (Table 4). The genetic diversity of isolates from all locations is summarized in Table 4.

Varying levels of genotypic diversity and genotypic richness were observed for the isolates considered in this study (Table 3). Populations from France followed by Spain displayed the highest level of genetic diversity and richness, based on the Simpson index (H), Stoddart Taylor's index (G) and allelic richness (A_R) and rarefaction of MLGs. The genotypic evenness (E.5) observed in the populations from Russia and Spain were the closest to having equal abundance. Using Nei's unbiased gene diversity, the Russian population had the highest gene diversity (0.546) followed by those from Spain (0.494), Ukraine (0.379) and France (0.344). This could be due to the uneven sample sizes obtained at the different locations because the algorithm does not correct for small population sizes. Populations from Slovenia and

Switzerland had the lowest genotypic diversity and genotypic richness. Countries for which only single isolates were available (i.e. Romania, Belgium and the Czech Republic) were not considered in the analyses.

The population from Spain had the highest number of private alleles (16.22%) followed by those from Slovakia (6.31%) and France (5.41%). Populations from Serbia, Slovenia and Switzerland had the lowest number of private alleles (0.90%). Within Slovakia, private alleles were from Arboretum Mlynana, Jahodna, Kosice and Zvolen and in France the private alleles were only from Souesmes (Table 4).

3.4 Population structure

There was no consensus between different methods of determining the optimal number of clusters in the STRUCTURE analysis. The Evanno ΔK supported 19 (K=19) clusters, which indicates that this method failed to detect population structure. LnP(K) suggested K=10 as the optimal scenario. The four Peuchmaille methods suggested that 5 - 8 clusters are most likely the optimal number of clusters depending on the threshold that was set (Fig. 4). The STRUCTURE barplots for K=2 to K=9 for the major modes are illustrated in Figure 5. The barplots for K = 5 - 8, together with the geographical distribution of the clusters are represented in Figure 6. In order to conduct the DAPC analysis, the *find.clusters* function in the *adegenet* package in R was used and this showed that K resides between 8 and 12. After several runs, K=10 was proposed as the optimal scenario (Fig. 7).

For both the K=8 and K=10 scenario, the DAPC (Fig. 7) and STRUCTURE analysis (Fig. 6) indicated that three or four major genetic groups reside between bordering countries in Western, Central, and Eastern Europe. Within these clusters, several smaller genetic groups were observed. The STRUCTURE analysis showed that populations in Western Europe (Belgium, Czech Republic, France and Switzerland) share a major cluster. In Central Europe,

one cluster was shared between Hungary, Slovakia and Slovenia and a second genetic cluster was shared between Slovakia and Serbia. In Eastern Europe, isolates from Romania, Russia and Ukraine shared a cluster. Several smaller scattered genetic groups also resided among the populations and the Slovenian population, as well as the Spanish population, included unique genetic clusters.

The DAPC clusters (Fig. 7) were mostly correlated with the geographic groups indicated by the STRUCTURE analysis with a Western group containing Cluster 1 (France, Spain, Switzerland), Cluster 3 (Czech Republic, France, Ukraine), Cluster 5 (Belgium, France and Spain) and Cluster 10 (France, Spain and Switzerland). A Central European group accommodated Cluster 4 (France, Hungary, Russia, Slovakia and Slovenia), Cluster 8 (Serbia and Slovakia) as well as a unique cluster (Cluster 2) having only individuals from Slovenia. The DAPC also indicated an Eastern European group with Cluster 6 (Russia, Ukraine), Cluster 7 (Romania, Russia and Ukraine) as well as Cluster 9 (Russia and Ukraine). The four distinct geographic groups suggested by both the STRUCTURE analysis and DAPC were also evident in a haplotype network drawn using Bruvo's genetic distance (Fig. 8)

The AMOVA results (Table 5) indicated significant population differentiation according to country (variance among individuals 47%, variance among countries 53%) and even more so by location within countries (variance among individuals 41%, variance among countries 59%). Although this explained less of the variance found among populations, AMOVA also strongly supported the grouping by host species (27% between species and 73% among individuals).

3.5 Mating type determination and random mating

The mating types were successfully amplified for all but two isolates, both from Slovakia (Table 6). Both mating type idiomorphs were detected in isolates from France, Serbia,

Slovakia, Spain, Switzerland and Ukraine (Table 6). However, in Nueng-sur-Beuvron in France only *MAT1-1* individuals were detected and in Serbia only *MAT1-2* individuals were present in isolates from Subotička Sand. Similarly, although both mating types were present in the Slovakian collections, either *MAT1-1* or *MAT1-2* individuals were detected at each of the 10 locations sampled in these countries. In Ukraine, the population from Nova Zburivka included only one individual that was *MAT1-2* and in Mykolaiv Kinburn, only *MAT1-1* individuals were detected. Although both mating types were found in these countries, random mating was statistically supported only in the populations from Spain, Switzerland and Ukraine as well as in the sub-populations from Souesmes and La Bouyale in France, Deliblatski Pesak in Serbia, and Hola Prystan, Tsjurupinsk and Mykolaiv Kinburn in Ukraine. In isolates from the Czech Republic, Hungary, Romania and Russia only *MAT1-1* individuals were present and in those from Belgium and Slovenia only *MAT1-2* individuals were present (Table 6, Appendix 1).

Testing linkage disequilibrium using the clone-corrected data set, with the index of association and rbarD, provided evidence for sexual recombination only in the population from Spain (P-value of 0.804). Analysis of the non-clone-corrected dataset also supported evidence of sexual recombination in Serbia (P-values of 0.281). This result is however not plausible as the data for both Deliblatski Pesak and Subotička Pescara in Serbia were pooled for this analysis and therefore do not reflect that single mating types were observed at each of these locations.

4. Discussion

This study provided the first available insights into the population structure and genetic diversity of *D. pini* in Europe. Even though extensive sampling was conducted in the area over a 12-year period, due to the low incidence of *D. pini*, sampling was relatively unstructured and sample sizes were relatively small. This was also emphasized in reports in Switzerland (Dubach

et al. 2018) as well as Spain (Ortíz de Urbina et al. 2017) where *D. pini* was less frequently detected than *D. septosporum.* Nonetheless, it was clear that *D. pini* is not new to the European continent.

Based on population structure analyses, the *D. pini* populations considered in this study grouped in four main geographic clusters including one in Western Europe, two in Central Europe, and one in Eastern Europe. Variable population diversity was observed between countries, with France, Spain and Ukraine having the highest levels of genetic diversity and the presence of both mating types. This suggests that *D. pini* has most likely been present in those countries for a long period of time. In contrast, there were populations that were clonal and with one mating type such as in Slovakia and Slovenia, suggesting new introductions. Additionally, the presence of the same MLHs over long distances suggests that human-mediated movement of *D. pini* is taking place in Europe.

Both mating types of *D. pini* were present in many populations considered in this study, but evidence for sexual recombination was supported only in the population from Spain. The fact that some isolates of the same MLH's were of different mating type, suggests that sexual recombination could be occurring in other European populations of *D. pini*. This is not unusual and has been found in pathogens such as *Teratosphaeria destructans* (Havenga et al. 2021) as well as *Verticillium dahliae*, a clonally reproducing pathogen, having individuals of opposite mating types that were indicative of cryptic or ancestral sexual recombination events (Milgroom et al. 2014; Short et al. 2014).

Dothistroma pini has a limited host range and is currently confined to a particular latitudinal geographical range both in Europe as well as in North America. The majority of the isolates in the present study were from several sub-species of *P. nigra* with few collections from *P. coulteri, P. jeffreyi, P. mugo, P. ponderosa, P. schwerinii* and *P. sylvestris.* Many of the single isolates from hosts other than *P. nigra* were from urban areas or arboreta and not from the

native ranges of the host trees. This suggests that *D. pini* is most likely not native to Europe. This is in contrast to the more commonly occurring *D. septosporum* that is hypothesised to be native to the *P. sylvestris* forests in Northern Europe (Adamson et al. 2018), Eastern Europe and Western Asia (Mullett et al. 2021).

The results of this study suggest that *D. pini* is not native to Europe and have provided no clues to its possible centre of origin of the pathogen. The only other area of the world where *D. pini* is known to occur is North America (Barnes et al. 2004; Barnes et al. 2014a; Mullett et al. 2018). Dothistroma needle blight is widespread in the USA and has been reported in 35 states (Drenkhan et al. 2016; Mullett et al. 2018). However, most of the reports were from the time before *D. septosporum* and *D. pini* were conclusively separated based on phylogenetic inference in 2004 (Barnes et al. 2004). Thus, the presence of *D. pini* has been confirmed in only seven states in the Central regions of the USA (Barnes et al. 2004; Barnes et al. 2014a; Mullett et al. 2018) and *D. septosporum* in four states (Barnes et al. 2004; Barnes et al. 2016). The availability of techniques to discriminate between the two species with relative ease (Barnes et al. 2004; Ioos et al. 2010; Schneider et al. 2019; Aglietti et al. 2021; Myrholm et al. 2021; Barnes et al. 2008a; Groenewald et al. 2007) should simplify efforts to collect isolates known to be those of *D. pini* in the future. This will facilitate an opportunity to conclusively show whether the pathogen is native to North America. The extensive data assembled in the present study will provide a solid foundation for the comparisons that would be needed to achieve that goal.

An intriguing question pertaining to DNB is why *D. septosporum* has spread from the Northern Hemisphere to many Southern Hemisphere countries but that the closely related *D. pini* has not done so. This could be related to host range where *D. septosporum* has mainly been a problem on *P. radiata* in the Southern Hemisphere (Gibson 1972; Barnes et al. 2014b; Drenkhan et al. 2016), although it has recently emerged as a serious constraint in plantation of *P. tecunumanii*

in Colombia (Rodas et al. 2016). Both *Dothistroma* species have relatively wide host ranges and as greater numbers of *Pinus* spp. are being tested and propagated in Southern Hemisphere countries, it seems plausible to suggest that *D. pini* poses an important threat to these resources. Based on experience with *D. septosporum* as well as the increasingly important pine needle pathogen *Lecanosticta acicola* (van der Nest et al. 2019a), and apparently *D. pini* as was found in this study, there is good reason to emphasize the importance of quarantine when moving pine germ plasm between countries and continents.

5. References

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Table 1. *Dothistroma pini* collections from Europe used in this study.

Country	Isolates per country	Isolates per location	Locality	Collectors	Year collected	Hosts	Plantation type	ITS Hap 1/Hap 2/Hap 4
Belgium	**1**	**1**	**-**	**Mullett MS**	**-**	**-**	**Unknown**	**-/-/1**
Czech Republic	**1**	**1**	**Bohemia, Chodská Lhota**	**Bergová E**	**2013**	***P. jeffreyi***	**Public greenery**	**1/-/-**
		9	La Bouyale	Barnes I, Mullett MS	2012	*P. nigra* subsp. *laricio*	Plantation	7/2/-
		29	La Ferté-Imbault	Barnes I, Mullett MS	2012	*P. nigra* subsp. *laricio*	Urban greenery	22/3/3 (1 missing data)
		2	Neung-sur-Beuvron	Barnes I, Mullett MS	2012	*P. nigra* subsp. *laricio*	Plantation	-/-/2
		14	Souesmes	Barnes I, Mullett MS	2012	*P. nigra* subsp. *laricio*	Plantation	14/-/-
		18	Villefranche-sur-Cher	Barnes I, Mullett MS	2012	*P. nigra* subsp. *laricio*	Plantation	17/-/1
France	**72**							**60/5/6** (1 missing data)
Hungary	**12**	**12**	**Diszel**	**Barnes I**	**2007**	***P. nigra***	**Pine stand next to road**	**12/-/-**
Romania	**2**	**2**	**Botoșani**	**Costache C**	**2015**	***P. nigra***	**Unknown**	**-/2/-**
		1	Kamensky district, Rostov oblast	Bulgakov TS	2006	*P. pallasiana*	Forest plantation	-/1/-
		3	Krasnosulinsky district, Donskoye forestry	Timur SB	2007	*P. pallasiana, P. mugo, P. nigra*	Forest plantation	-/3/-
		2	Tarasovsky district, Gorodishchenskoye forestry	Timur SB	2007	*P. pallasiana*	Forest plantation	-/2/-
Russia	**6**							**-/6/-**
		17	Deliblatski Pesak, Susara	Keca N	2014	*P. nigra*	Plantation	-/6/11
		7	Subotička Pescara	Sadikovic D	2014	*P. nigra*	Natural regeneration	-/2/5
Serbia	**24**							**-/8/15**
		41	Arboretum Mlynany	Adamcikova K, Ondruskova E, Heckova Z	2015	*P. coulteri, P. jeffreyi, P. nigra, P. ponderosa, P. schwerinii*	Arboretum	37/2/2
		3	Banská Belá	Adamcikova K, Ondruskova E, Heckova Z	2015	*P. nigra*	Urban greenery	-/3/-
		6	Gabčíkovo	Heckova Z, Adamcikova K	2015	*P. nigra*	Forest plantation	6/-/-

Country	Isolates per country	Isolates per location	Locality	Collectors	Year collected	Hosts	Plantation type	ITS Hap 1/Hap 2/Hap 4
		25	Jahodná	Heckova Z, Adamcikova K	2015	*P. nigra*	Forest plantation	25/-/-
		5	Košice	Adamcikova K	2015	*P. ponderosa*	Arboretum	5/-/-
		1	Kováčová	Adamcikova K, Ondruskova E, Heckova Z	2015	*P. mugo*	Urban greenery	-/1/-
		1	Ľubochňa	Adamcikova K, Ondruskova E, Heckova Z	2015	*P. sylvestris*	Natural regeneration	1/-/-
		1	Sečovce	Adamcikova K, Ondruskova E, Heckova Z	2015	*P. mugo*	Urban greenery	-/1/-
		17	Trstice	Adamcikova K	2015	*P. nigra*	Plantation/nursery	17/-/-
		3	Zvolen	Adamcikova K	2015	*P. jeffreyi*	Urban greenery	2/-/1
Slovakia	**103**							**93/7/3**
		2	Dutovlje (Karst)	Jurc D, Hauptman T	2013	*P. nigra*	Unknown	2/-/-
		2	Hruševica (Karst)	Jurc D, Hauptman T	2013	*P. nigra*	Unknown	2/-/-
		29	Panovec	Piškur B, Jurc D	2013	*P. nigra*	Unknown	29/-/-
		4	Pivka	Sadikovic D, Hauptman T	2013	*P. nigra*	Natural regeneration	4/-/-
		4	Prebold	Jurc D	2013	*P. nigra*	Unknown	4/-/-
		1	Radenci	Hauptman T	2015	*P. nigra*	Unknown	1/-/-
		2	Ribnica	Piškur B, Jurc D	2013	*P. nigra*	Unknown	2/-/-
		2	Škocjan	Jurc D, Hauptman T	2013	*P. nigra*	Unknown	2/-/-
Slovenia	**46**							**44/-/-**
		15	Aragon	Mullett MS	2017	*P. nigra* subsp. *nigra*	Plantation	9/2/4
		1	Boixar	Mullett MS	2017	*P. nigra* subsp. *nigra*	Plantation	-/1/-
Spain	**16**							**9/3/4**
Switzerland	**24**	**24**	**Weesen, Walensee**	**Holdenrieder O**	**2012/2013**	***P. nigra***		**7/17/-**
		9	Kherson, Hola prystan	Davydenko K	2013	*P. nigra* subsp. *pallasiana*	Forest plantation	-/9/-
		1	Kherson, Nova Zburivka	Davydenko K	2013	*P. nigra* subsp. *pallasiana*	Forest plantation	-/1/-
		21	Kherson, Tsjurupinsk	Usichenko AC, Davydenko K	2013	*P. nigra* subsp. *pallasiana*	Forest plantation	3/18/-
		5	Mykolaiv Kinburn	Davydenko K	2013	*P. nigra* subsp. *pallasiana*	Forest plantation	-/5/-
		2	Kinburg Peninsula	Davydenko K	2013	*P. nigra* subsp. *pallasiana*	Forest plantation	1/1/-
Ukraine	**38**							**4/34/-**
Total:	**345**							

Table 2. *Dothistroma pini* microsatellite PCR annealing temperatures, $MgCl_2$ adjustments, and dilutions for fragment analysis for each microsatellite marker.

Marker name	Optimum annealing temp (°C)	Reduced $MgCl_2$ for PCR amplification	Dilution ratio (µl) for fragment analyses	Panel number in fragment analyses	Number of alleles[1] per microsatellite marker
Doth_A	57	Yes	2/100	1	1
DP-MS1	62	Yes	2/100	1	13
DP-MS2	57	Yes	2/100	1	3
DP-MS4	60	No	1.5/100	1	2
DP-MS5	58	Yes	1/100	1	6
DP-MS6	58	No	1.6/200	2	4
DP-MS7	62	No	1.6/200	2	10
DP-MS8	60	Yes	2/200	2	4
DP-MS9	60	Yes	1.4/200	2	6
DP-MS10	58	Yes	0.8/100	1	3
DP-MS11	58	Yes	1/100	1	12
DP-MS12	60	Yes	2/200	2	19
DP-MS13	59	Yes	2/100	1	13
DP-MS15	60	No	2/200	2	4
DP-MS16	60	Yes	1.4/200	2	3
DP-MS17	60	No	1.2/200	2	6
DP-MS18	59	Yes	1.2/200	2	2

[1]Determined from 345 isolates analysed in Europe.

Table 3. Summary diversity statistics of *Dothistroma pini* isolates within populations by country in Europe.

Country[*]	**N**[1]	**MLH**[2]	**eMLH**[3]	**CF**[4]	**Total no of alleles**	**Unique alleles**	**A_R**[5]	**PA_R**[6]	**H**[7]	**G**[8]	**Lambda**[9]	**E.5**[10]	**D**[11]
Belgium	1	1	1.00 ± (0.000)	N/A	16	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Czech Republic	1	1	1.00 ± (0.000)	N/A	16	0	N/A	N/A	0.000	1.000	0.000	N/A	N/A
France	72	41	8.52 ± (1.074)	0.43	52	6	1.936 ± (0.215)	0.161 ± (0.054)	3.343	18.51	0.946	0.642	0.344
Hungary	12	5	6.00 ± (0.674)	0.58	23	2	1.236 ± (0.106)	0.066 ± (0.063)	0.674	1.589	0.708	0.623	0.079
Romania	2	2	2.00 ± (0.000)	0.00	18	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Russia	6	6	6.00 ± (0.000)	0.00	42	1	2.563 ± (0.288)	0.318 ± (0.133)	1.792	6.00	0.833	1.000	0.546
Serbia	24	8	5.58 ± (1.044)	0.67	24	1	1.231 ± (0.090)	0.140 ± (0.070)	1.814	4.36	0.771	0.655	0.087
Slovakia	103	15	4.11 ± (1.149)	0.86	35	5	1.323 ± (0.121)	0.080 ± (0.058)	1.610	3.26	0.693	0.564	0.116
Slovenia	46	6	3.45 ± (0.888)	0.87	30	1	1.361 ± (0.099)	0.079 ± (0.035)	1.122	2.17	0.539	0.564	0.132
Spain	16	12	8.50 ± (0.797)	0.25	59	18	2.562 ± (0.279)	0.701 ± (0.174)	2.426	10.67	0.906	0.937	0.494
Switzerland	24	6	3.57 ± (0.932)	0.75	29	1	1.490 ± (0.142)	0.138 ± (0.076)	1.099	2.09	0.521	0.543	0.184
Ukraine	38	17	6.67 ± (1.257)	0.55	49	3	2.019 ± (0.137)	0.031 ± (0.016)	2.365	6.94	0.856	0.616	0.379
Total	**345**	**109**	**8.18 ± (1.181)**	**0.316**	**109**	**39**			**3.724**	**18.00**	**0.944**	**0.420**	**0.425**

[*]Due to small sample sizes (N<6) in 26/39 of the locations, summary statistics were determined by country.
[1]N = Total number of isolates.
[2]Number of multilocus haplotypes. Equivalent to samples that have been clone-corrected.
[3]The number of expected MLG at the smallest sample size ≥ 10 based on rarefaction ± standard error.
[4]CF: Clonal Fraction = 1 – [MLH/N].
[5]Allelic richness ± standard error (Szpiech *et al.,* 2008). The smallest country sample size considered was 6.
[6]Privale allelic richness ± standard error (Szpiech *et al.,* 2008). The smallest country sample size considered was 6.
[7]H: Shannon-Wiener Index of MLG diversity (Shannon, 2001).
[8]G: Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor, 1988).
[9]Lambda: Simpson's Index (Simpson, 1949). – provides and estimation of the probability that two randomly selected genotypes are different. 0 = no genotypes different. 1 = all genotypes are different.
[10]E.5: Genotypic evenness, (Grünwald *et al.,* 2003).
[11]D = Nei's (1978) gene diversity.

Table 4. Summary diversity statistics of *Dothistroma pini* isolates within locations in Europe.

Country	Location	N[1]	MLH[2]	CF[3]	Total no. of alleles	Unique alleles
Belgium	Unknown	**1**	**1**	**N/A**	**16**	**0**
Czech Republic	Chodská Lhota	**1**	**1**	**N/A**	**16**	**0**
	La Bouyale	9	4	0.56	25	0
	La Ferté-Imbault	29	25	0.14	41	0
	Nueng-sur-Beuvron	2	2	0.00	23	0
	Souesmes	14	12	0.14	41	5
	Villefranche-sur-Cher	18	7	0.61	32	0
France		**72**	**41**	**0.43**	**52**	**6**
Hungary	Diszel	**12**	**5**	**0.58**	**23**	**2**
Romania	Botoșani	**2**	**2**	**0.00**	**18**	**1**
	Kamesky district	1	1	N/A	16	0
	Krasnosulinsky district	3	3	0.00	27	1
	Tarasovsky district	2	2	0.00	28	0
Russia		**6**	**6**	**0.00**	**42**	**1**
	Deliblatski Pesak, Susara	17	6	0.65	24	1
	Subotička Pescara	7	3	0.57	18	0
Serbia		**24**	**8**	**0.67**	**24**	**1**
	Arboretum Mlynany	41	6	0.85	28	2
	Banská Belá	3	1	0.67	16	0
	Gabčíkovo	6	2	0.67	17	0
	Jahodná	25	4	0.81	19	1
	Košice	5	1	0.80	16	1
	Kováčová	1	1	N/A	16	0
	Ľubochňa	1	1	N/A	16	0
	Sečovce	1	1	N/A	16	0
	Trstice	17	2	0.88	17	0
	Zvolen	3	2	0.25	22	0
Slovakia		**103**	**15**	**0.86**	**35**	**5**
	Dutovlje (Karst)	2	1	0.50	16	0
	Hruševica (Karst)	2	1	0.50	16	0
	Panovec	29	3	0.90	18	0
	Pivka	4	3	0.25	21	0
	Radenci	1	1	N/A	16	1
	Prebold	4	1	0.75	16	0
	Ribnica	2	2	0.00	18	0
	Škocjan	2	1	0.50	16	0
Slovenia		**46**	**6**	**0.87**	**30**	**1**
	Aragon	15	11	0.27	53	12
	Boixar	1	1	N/A	16	5
Spain		**16**	**12**	**0.25**	**59**	**18**
Switzerland	Walensee	**24**	**6**	**0.75**	**29**	**1**
	Kherson, Hola prystan	9	7	0.22	38	1
	Kherson, Nova Zburivka	1	1	N/A	16	0
	Kherson, Tsjurupinsk	21	7	0.67	31	0
	Mykolaiv Kinburn	5	4	0.20	38	2
	Kinburg Peninsula	2	2	0.00	22	0
Ukraine		**38**	**17**	**0.55**	**49**	**3**
Total		**345**	**109**	**0.316**	**109**	**39**

Grey highlights with bold text represent the totals per country. Due to small sample sizes (N<6) in 26/39 of the locations, summary statistics were determined by country and are summarized in Table 3.

[1]N = Total number of isolates.

[2]Number of multilocus haplotypes. Equivalent to samples that have been clone-corrected.

[3]CF: Clonal Fraction = 1 – [MLH/N].

Table 5. Hierarchical analysis of molecular variance (AMOVA) of *Dothistroma pini* populations, grouped by countries, by locations and by host species.

Source of variation	**df**	**Sum of squares**	**Mean squares**	**Estimate of variance**	**Total variation (%)**	**P-value**
Among Countries	8	1127.55	140.94	1.96	53%	0.01
Among Individuals grouped by country	334	1179.76	3.53	1.77	47%	
Within Individuals	343	0.00	0.00	0.00	0%	
Total	685	2307.32		3.72	100%	
Among Locations	27	1372.24	50.82	2.05	59%	0.01
Among Individuals grouped by location	309	886.36	2.87	1.43	41%	
Within Individuals	337	0.00	0.00	0.00	0%	
Total	673	2258.59		3.48	100%	
Among Hosts	7	507.88	72.55	0.98	27%	0.01
Among Individuals grouped by hosts	333	1791.66	5.38	2.69	73%	
Within Individuals	341	0.00	0.00	0.00	0%	
Total	681	2299.54		3.67	100%	

Table 6. Mating type ratios and index of association tests for the *Dothistroma pini* populations collected in Europe.

	Mating type ratios[a]					Linkage disequilibrium – Index of association[b]					
						Non-clone-corrected data			Clone-corrected data		
Country	**MAT1-1**	**MAT1-2**	**Could not determine**	**Expected ratio**	**P-value (two tailed test)**	**I_A**	**rbarD**	**P**	**I_A**	**rbarD**	**P**
Belgium	0	1		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Czech Republic	1	0		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
France	56	16		36	< 0.0001	1.450	0.128	0.0010	0.413	0.036	0.001
Hungary	12	0		6	0.001	-0.055	0.014	0.613	-0.511	0.128	0.970
Romania	2	0		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Russia	6	0		3	0.031	1.885	0.150	0.001	1.885	0.150	0.002
Serbia	6	18		12	0.023	**0.095**	**0.019**	**0.281**	-0.265	-0.053	0.818
Slovakia	8	93	2	52	< 0.0001	3.685	0.514	0.001	2.492	0.319	0.001
Slovenia	0	46		23	< 0.0001	5.265	0.685	0.001	3.151	0.398	0.001
Spain	**9**	**7**		**8**	**0.804**	0.974	0.077	0.001	**0.226**	**0.018**	**0.144**
Switzerland	17	7		**10**	**0.115**	5.540	0.794	0.001	4.179	0.604	0.001
Ukraine	19	19		**19**	**1.000**	7.099	0.548	0.001	4.977	0.384	0.001

Statistically non-significant values are highlighted in bold (P > 0.05) and indicate random mating is supported by the test.

[a]Mating type ratios are indicated per country using the non-clone-corrected dataset.

[b]The index of association tests were conducted per country using both datasets.

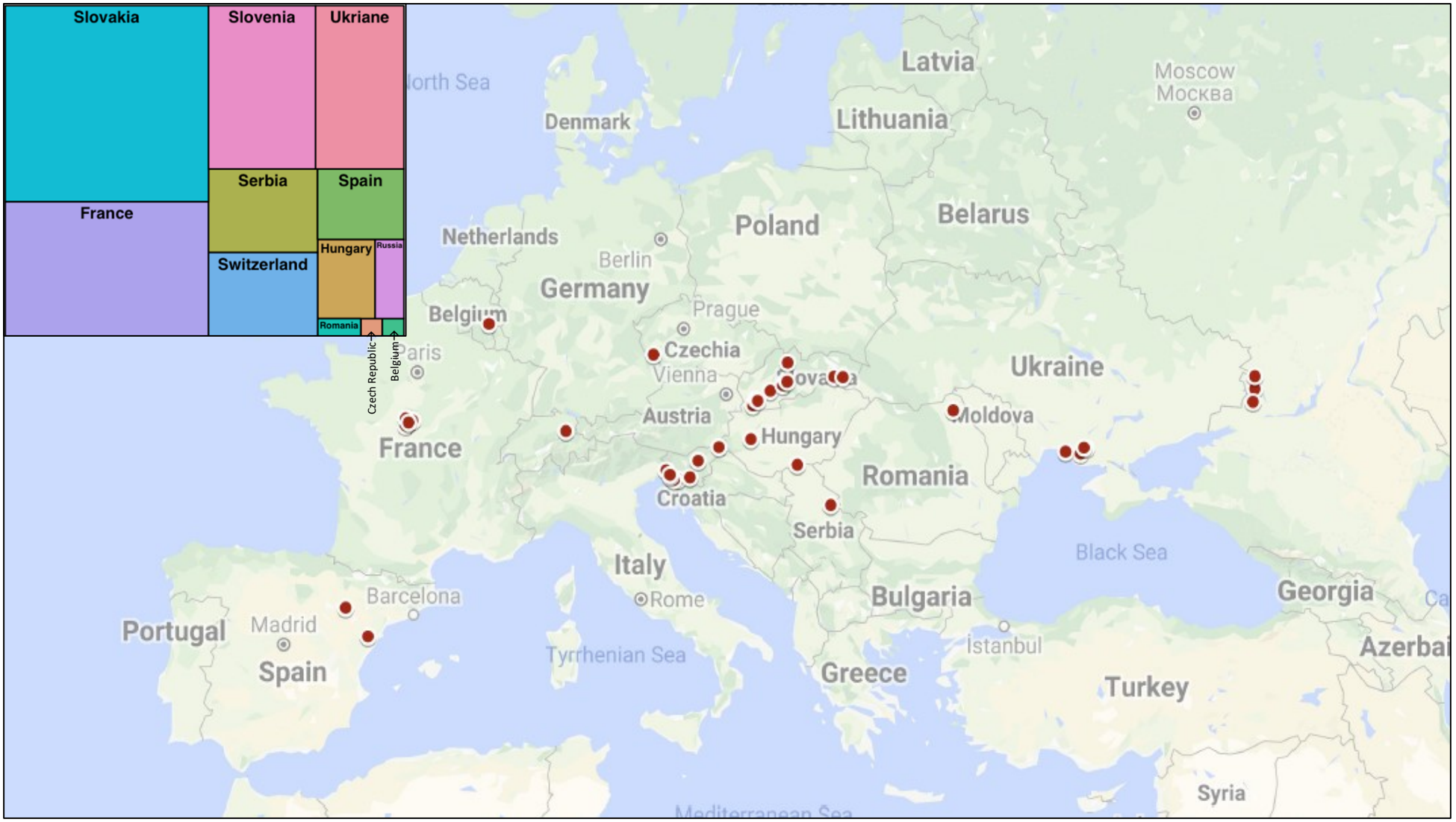

Figure 1. The 39 sampling locations of *D. pini* in Europe. The insert indicates the proportion of isolates obtained per country in relation to other countries.

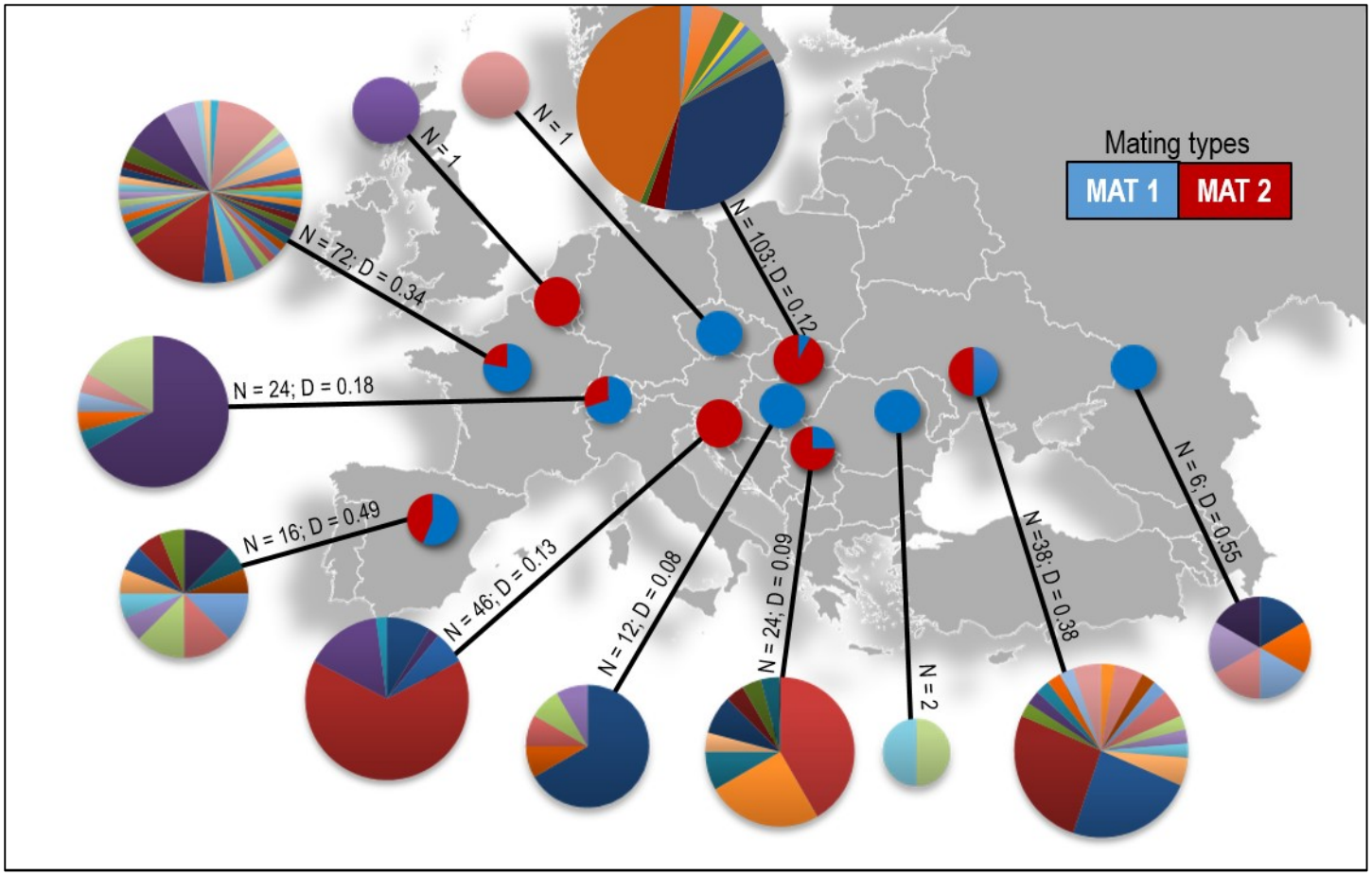

Figure 2. Microsatellite haplotype diversity and mating type ratios of *D. pini* in each of the sampled countries in Europe. The size of each MLH pie chart is proportional to the number of samples per country where Belgium N=1 and Slovakia N=103. N = number of isolates, D = Nei's genetic diversity.

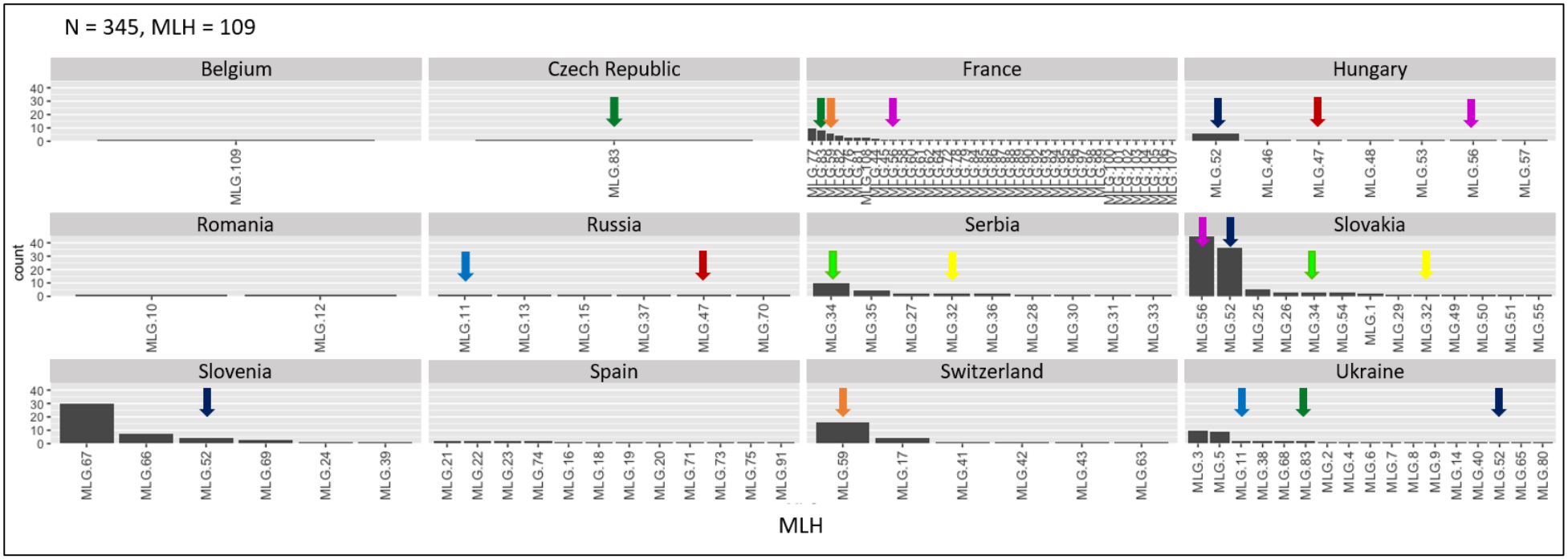

Figure 3. The 109 multilocus haplotypes (MLH) derived from microsatellite data in each country and shared between countries. Eight MLHs, indicated by coloured arrows are shared between multiple, often non-bordering countries. France contains the highest number of MLHs, followed by Ukraine. MLH 52 (navy blue) occurred in four countries (Hungary, Slovakia, Slovenia and Ukraine) in 7 locations, covering a distance of approximately 1500 km. The single individual from the Czech Republic shared MLH 83 (dark green) with isolates from different locations in France and 1480 km away as well as two isolates from Hola Prystan in Ukraine. MLH 56 (purple) was the second most occurring MLH and was shared by individuals in Souesmes (France), Diszel (Hungary) and Arborétum Mlyňany, Gabčikovo, Jahodna and Trstice in Slovakia. All of these individuals were of ITS Haplotype 1 but the individuals in Slovakia were *MAT1-2* idiomorphs and the isolates from France and Hungary were *MAT1-1* idiomorphs. MLH47 (red) occurred in the Russian population (ITS Haplotype 2 individual) and 1150km apart in the Hungarian population (ITS Haplotype 1 individual). MLH 11 (light blue) occurred in the population in Russia as well as in Hola Prystan in Ukraine (620 km apart). MLH 34 (lime green) was present in both Arborétum Mlyňany and Zvolen (Slovakia) as well as Delibratski Pesak and Subotička Pescara (Serbia), while individuals of MLH 32 (yellow) were detected in Kováčová (Slovakia) and Subotička Pescara (Serbia). MLH 59 (orange) was found in both Selles-Saint-Denis and La Ferté-Imbault (France) as well as in the population from Switzerland.

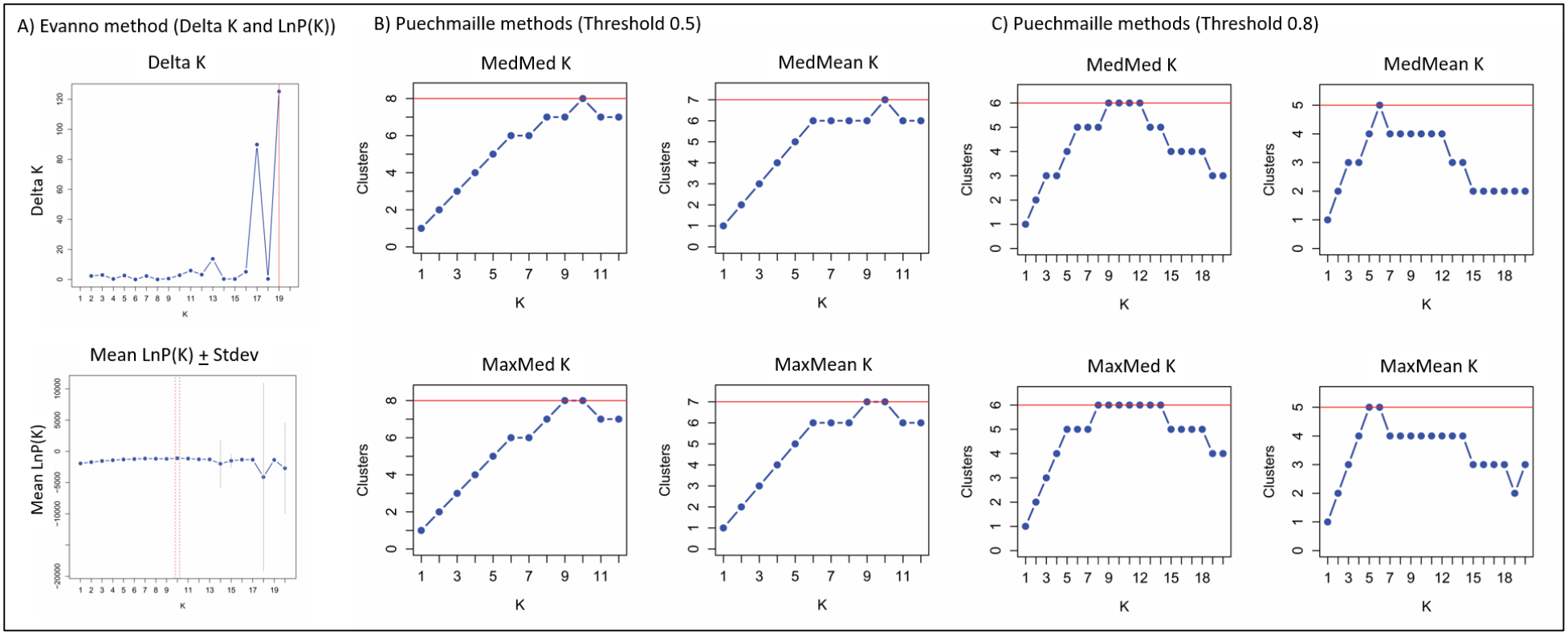

Figure 4. The optimum number of clusters determined using STRUCTURESELECTOR. (A) The Evanno method (Delta K and LnP(K), suggested K= 19 and K = 10 respectively. When a threshold of 0.5 was set, the Puechmaille methods (B) determined the most optimal number of clusters as 7 or 8 and at a threshold of 0.8 (C), the most optimal number of clusters were determined to be 5 or 6.

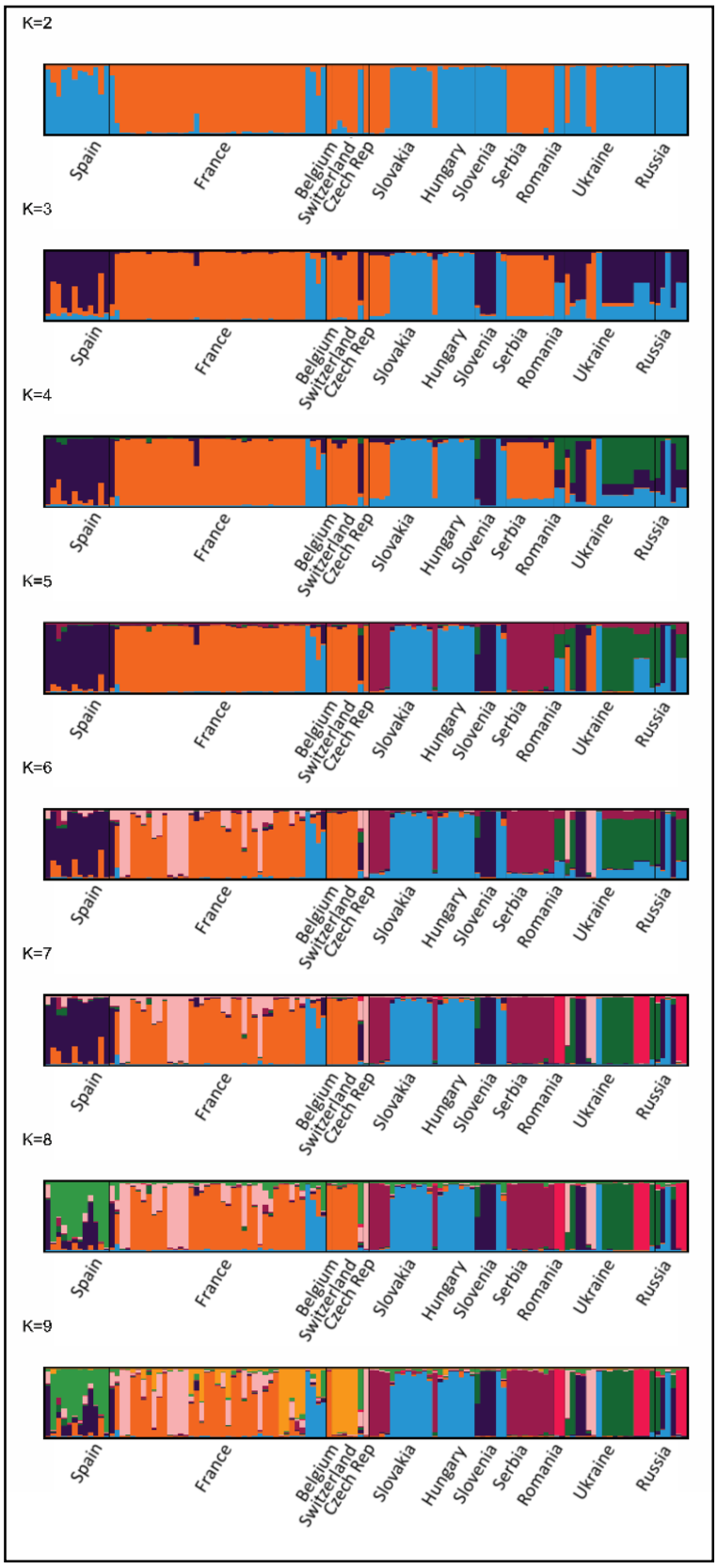

Figure 5. STRUCTURE results of *D. pini* populations per country using the clone-corrected dataset. The structure bar plots show the results for the major clustering modes from K=2 to K=9. The bar plots are divided according to geographical location.

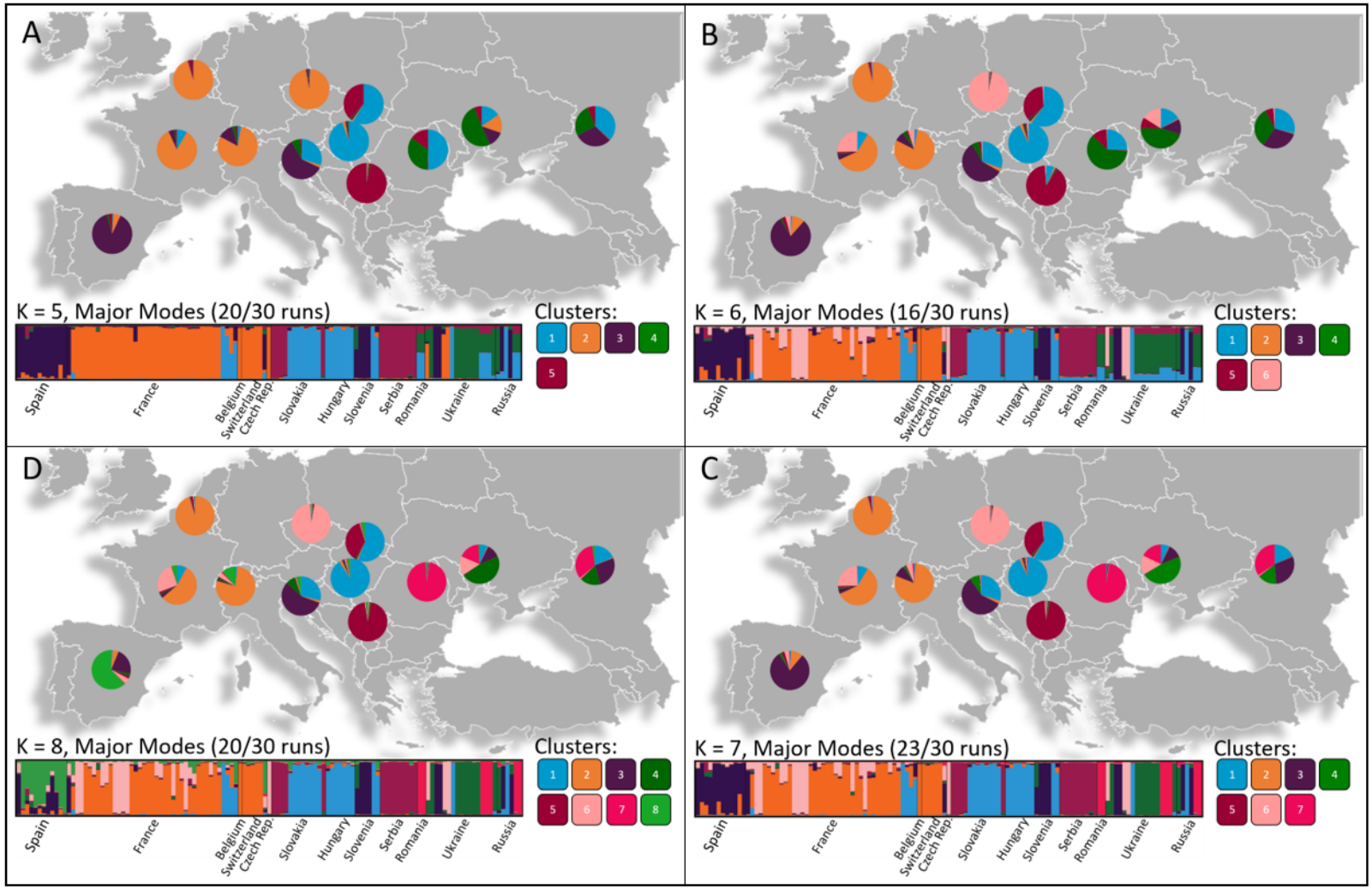

Figure 6. Geographical patterns of population divisions observed using STRUCTURE analyses based on the most likely K values determined by the Puecemaille methods with A) K=5, B) K=6, C) K=7, D) K=8. Clusters are represented as pie charts in each respective country. Four main genetic groups are spread throughout Western, Central and Eastern Europe with several smaller scattered genetic groups residing among the populations.

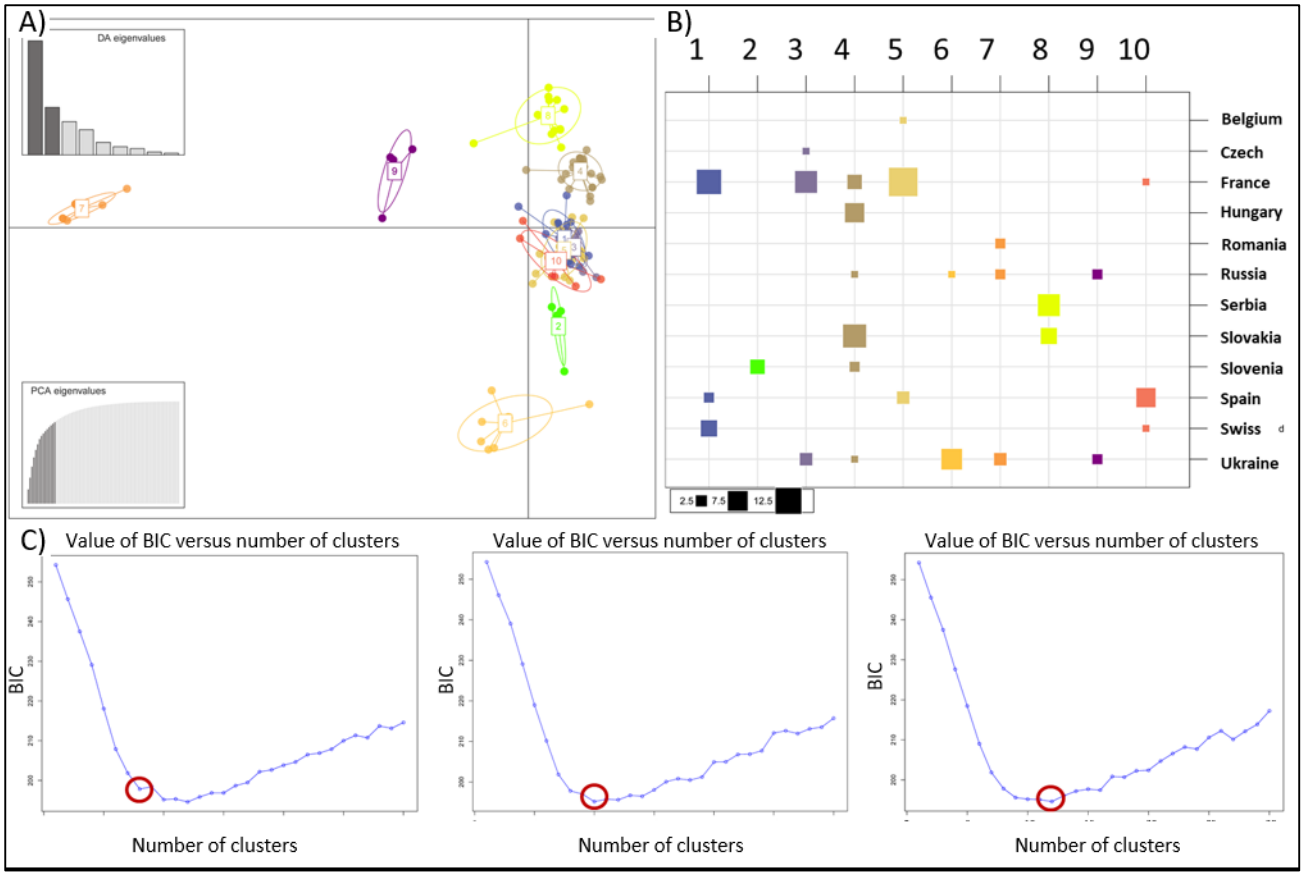

Figure 7. Population structure of the European *D. pini* collection of isolates. (a) Scatterplot of the discriminant analysis of principal components (DAPC) on European *D. pini* multilocus haplotypes. The number and colours represent the 10 groups delineated by the K-means method. Individual multilocus haplotypes are represented by dots and clusters as ellipses. At the top left the eigenvalues of the first nine axes are represented. (b) The composition of the DAPC clusters. The columns correspond to the inferred clusters and the rows correspond to the countries where the populations were sampled. The size of the squares is proportional to the number of individuals comprising each cluster. Cluster one for instance is comprised of individuals isolated from France, Spain and Switzerland with the majority of the individuals in this cluster isolated from France. (c) Multiple '*find.cluster*' runs in adegenet indicated that K resides between 8 and 12. The K=10 scenario was most often proposed as the most optimal scenario.

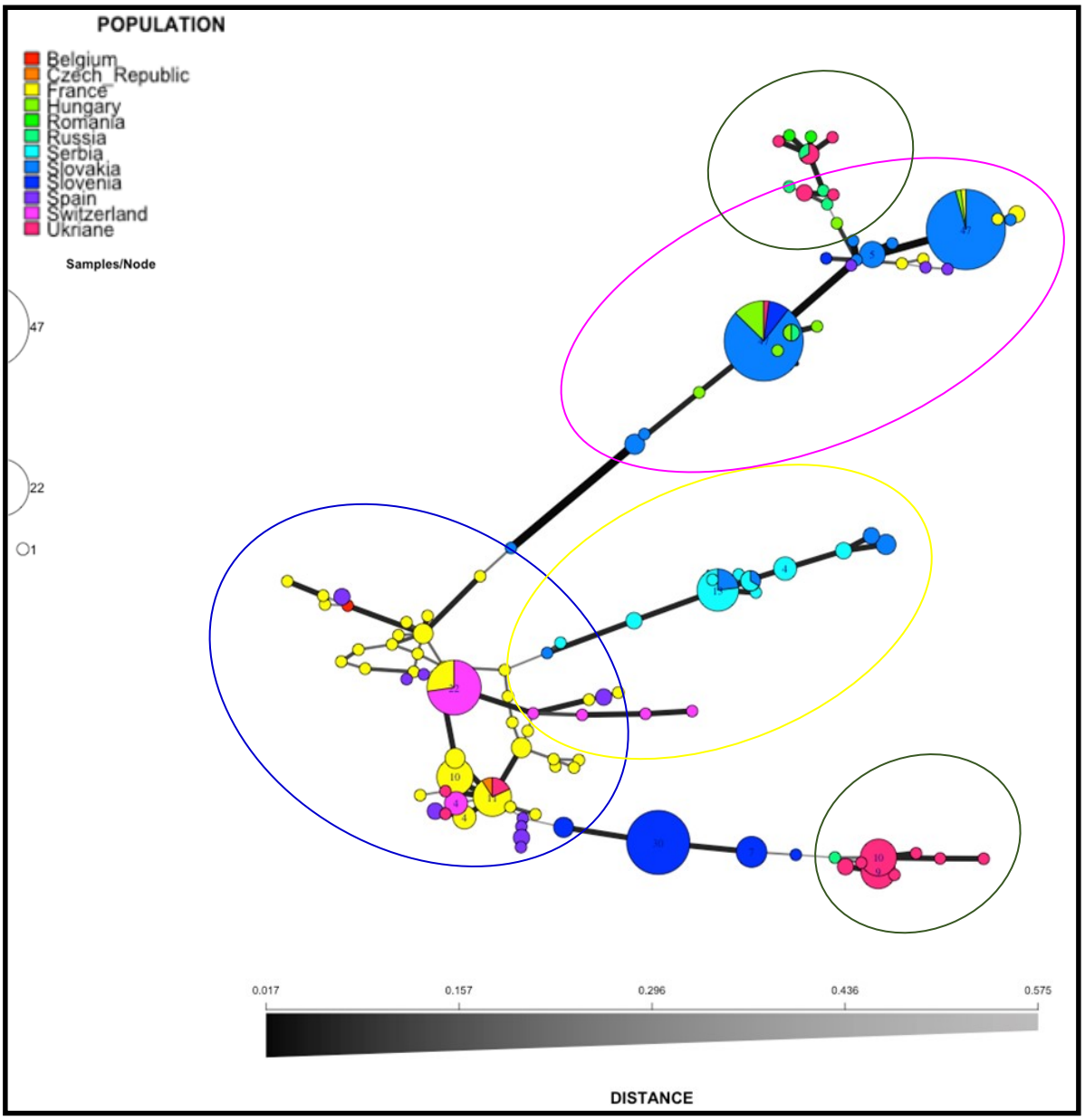

Figure 8. Haplotype network of *D. pini* collected in Europe drawn using Bruvo's genetic distance. Each circle represents a multilocus haplotype. The larger the circle, the more individuals have the same haplotype. The same four major clusters are observed as with the STRUCTURE analysis with the Western Europe individuals (blue oval), Central European clusters (yellow and pink ovals) and Eastern European cluster (dark green) clustering together.

RESEARCH CHAPTER 3

Population structure and diversity of *Dothistroma septosporum* in Cantabria suggests a long term presence of the pathogen

Abstract

Dothistroma septosporum and *D. pini*, the causal agents of Dothistroma needle blight (DNB), are important fungal pathogens of *Pinus* species. DNB was first reported in Spain in 1975 but severe disease outbreaks have only occurred in the Spanish Atlantic region in the past decade. In 2015, a needle blight disease was observed in Cantabria in stands of *P. nigra* subsp. *nigra* and *P. nigra* subsp. *salzmannii* planted alongside each other, and a third site of *Pinus nigra* subsp. *laricio* var. *corsicana* planted seventeen kilometres from the others. The aim of this study was to identify the pathogen present at the different sites on the different hosts, and to compare the population diversity and structure of the three collections using 12 microsatellite and species-specific mating type markers. In total, 120 isolates, all representing *D. septosporum* were obtained. This is the first report of *D. septosporum* on *Pinus nigra* subsp. *laricio* var. *corsicana* and the first report of the pathogen in Cantabria. The consensus between the DAPC and STRUCTURE analyses, as well as pairwise genetic differentiation, showed that there are two genetic clusters in Cantabria. Although both mating types were detected amongst the isolates, sexual recombination was not statistically supported in any of the sites. A high genetic diversity was observed in Cantabria, suggesting that the pathogen is not a recent introduction into the region.

1. Introduction

Dothistroma needle blight (DNB), caused either by *Dothistroma septosporum* (Dorog.) Morelet or *D. pini* Hulbary, is an important disease of *Pinus* in various parts of the world (Drenkhan et al. 2016). The two pathogens can be unequivocally distinguished from each other using DNA-based methods (Barnes et al. 2004). These have shown that *D. pini* is restricted to the Northern Hemisphere while *D. septosporum* has a global distribution and has been the cause of serious losses to plantation forestry in the Southern Hemisphere (Barnes et al. 2004; Barnes et al. 2016; Drenkhan et al. 2016).

Dothistroma needle blight was first reported in Spain in three autonomous communities: Galicia, Asturias and Basque country on *Pinus radiata* in 1974 (Fernández de Ana Magan 1975). The disease was also detected at low levels of incidence in plantations in Palencia in 2007 (Zamora et al. 2008). Although DNB has been known in Spain for at least 50 years, it has not resulted in severe damage until relatively recently, where a field survey conducted from 2009 - 2012 revealed that 13% of *P. radiata* stands in the Basque country were affected by a pine needle blight disease (Ortíz de Urbina et al. 2017). The pathogens responsible for this problem were identified as *Lecanosticta acicola*, the causal agent of Brown spot needle blight (BSNB), and *D. pini* causing DNB (Iturritxa et al. 2015). A second, more intensive study in the same region, revealed the presence of *D. septosporum,* mostly on *Pinus nigra,* but also on single trees of *P. pinaster, P. sylvestris* and *Pseudotsuga menziesii* (Ortíz de Urbina et al. 2017). *Dothistroma septosporum* was also confirmed to be present in Valencia on *P. sylvestris* and *P. nigra* (Mullett et al. 2018), and thus is confirmed in two provinces. The presence of *D. pini,* although not frequent, has only been documented in Aragon (Mullett et al. 2018) and the Basque country (Iturritxa et al. 2015; Ortíz de Urbina et al. 2017).

The host trees affected by DNB in Spain are mostly non-native *P. radiata* and to a lesser extent *P. nigra* planted for commercial forestry purposes (Iturritxa et al. 2015; Ortíz de Urbina et al. 2017). *Dothistroma pini* has been reported from these two species as well as *P. nigra* subsp. *nigra* at low levels of incidence in Spain (Iturritxa et al. 2015; Mullett et al. 2018). *Dothistroma septosporum* was detected on a wider range of hosts including *P. radiata, P. nigra, P. nigra* subsp. *nigra, P. pinaster* and *P. sylvestris* (Mullett et al. 2018; Ortíz de Urbina et al. 2017). It was also recently detected on *P. ponderosa* and *P. brutia* in arboreta surrounded by old *Pinus radiata* plantations (Mesanza et al. 2020). This is consistent with the view that the Mediterranean climate is becoming more suited to the spread of DNB on *Pinus* species (Mullett et al. 2018; Oskay et al. 2020).

Very little is known regarding the origins of *Dothistroma* spp. in Spain. In the case of *D. pini*, high levels of genetic diversity were found for a population from Aragon by van der Nest *et al.* (2021). That study also provided statistical support for sexual recombination, suggesting that the pathogen was not recently introduced into the country and is sexually reproducing. In contrast, a small population of *D. septosporum* collected in Valencia was highly clonal and represented by a single mating type, suggesting that the pathogen was recently introduced (Mullett et al. 2021).

In 2015, a severe outbreak of DNB was reported in Cantabria, a province west of the Basque country. Infected needles were collected from three sites planted to different *P. nigra* sub-species. The aim of this study was to determine the causal agent of the disease and to assess the genetic diversity of the pathogen causing the needle blight. Population genetic analyses were carried out to establish if the disease epidemics were due to a recent introduction, or if the pathogen has been established in the region for a longer time period.

2. Materials and Methods

2.1 Sample collection, fungal isolations and species identification

During 2015, needle blight was observed in three planted *Pinus nigra* stands, aged 28 – 30-years-old, in Cantabria (Fig. 1). Site 29.1 (*Pinus nigra* subsp. *nigra*) and Site 29.2 (*Pinus nigra* subsp. *salzmannii*) were planted adjacent to each other alongside lake San Miguel de Aguayo and Site 25 (*Pinus nigra* subsp. *laricio* var. *corsicana*) was located 17 km North-West from the other two sites (Table 1, Fig. 1). A subset of diseased needles was collected from each of 20 trees at each site and placed in separate paper envelopes. The pine needles were stored at -80°C until further processing.

Conidiomata resembling those of *Dothistroma* were excised from the collected pine needles and three to seven isolations were made per tree sample as described in Barnes *et al.* (2004). After 48 hours, single germinating conidia were plated on Dothistroma Sporulating Media (Mullett and Barnes 2012) with 100 mg streptomycin (Sigma-Aldrich, St Louis, MO) added per litre. The cultures were incubated at 23 °C for 4-6 weeks after which the cultures were processed for DNA extractions. Cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA was extracted from freeze dried fungal mycelia as described by van der Nest *et al.* (2019) using a Zymo Research ZR Fungal/Bacterial DNA MiniPrep™ kit (Irvine, CA) and eluted with 50 µl elution buffer. A final working concentration of 20 ng/µl DNA was prepared for each isolate. The isolates were identified by the amplification and sequencing of the internal transcribed spacer (ITS) 1 and 2 and 5.8S rDNA region with the ITS1 and ITS4 primers (White et al. 1990) using the same protocols and reagents as van der Nest *et al*. (2019). The products were sequenced in both directions using an ABI PRISM 3500xl capillary auto sequencer

(Thermo Fisher Scientific) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) at the sequencing facility of the University of Pretoria.

Consensus fragments for each isolate were created with the forward and reverse ITS sequences using CLC Main workbench version 8.0 (CLC Bio, https://www.qiagenbioinformatics.com/products/clc-main-workbench/). Consensus sequences were initially compared in a BLASTn search against the GenBank database (NCBI; http://www.ncbi.nlm.nih.gov/genbank/) to confirm the species identity of each isolate. The ITS sequences were then aligned and compared against datasets with representatives of all ITS haplotypes that were reported in Barnes *et al.* (2016) and Mullett *et al*. (2018) with the program MEGA 7.0.14 (Kumar et al. 2016).

2.2 Microsatellite amplification and haplotype determination

Twelve labelled microsatellite markers (Barnes et al. 2008) were amplified for the populations collected in this study. A thirteenth monomorphic marker, Doth_A, was included as an internal diagnostic marker that amplifies an allele size of 124 bp for *D. septosporum* and 109 bp for *D. pini* (Barnes et al. 2008; Barnes et al. 2011). PCR reactions were performed in 15 µl reaction volumes. The reactions consisted of 1 µl template DNA (20 ng/µl concentration), 0.15 µl of 100 nM of each of the forward and reverse primers, 0.12 µl Faststart Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN), 200 µM of each dNTP, 0.6 µl of a 2.5mM $MgCl_2$, 1.5 µl 10x PCR reaction buffer mixed containing 2.5mM $MgCl_2$ and the volume was adjusted to 15 µl with sterile SABAX water (Adcock Ingram, Midrand, South Africa). PCR reactions were carried out on an Applied Biosystems® Veriti® 96 well Thermal cycler (Thermo Fisher Scientific, Waltham, MA). The fragments were amplified using the same cycling conditions and primer pair annealing temperatures as described by Barnes *et al.* (2008) with the exceptions of the following annealing temperatures: Doth_E: 50 °C, Doth_DS2: 56 °C, Doth_M:59 °C and DCB2: 56 °C. PCR products (5µl) were stained with GelRed nucleic acid gel stain

(Biotium) (1µl) and separated on 2% SeaKem LE agarose gel (Lonza) for 15 min at 90 V. Successful amplification was visualized under a UV light using a GelDoc EZ Imager (BioRad).

In order to perform the fragment analysis, the PCR products were pooled into two panels with dilution ratios described in Table 2. The panels were prepared for analysis by adding 1 µl of the pooled products to 0.14 µl GENESCAN^{TM}-600 LIZ® (Life Technologies, Applied Biosystems, Warrington, UK) size standard and 12 µl formamide. The prepared reactions were used for fragment analysis on an ABI PRISM 3500xl capillary auto sequencer (Thermo Fisher Scientific). Alleles sizes were scored using GENEMAPPER® Software version 5.0 (Applied Biosystems, Foster City, CA).

The combination of alleles determined for each marker per individual is defined as a multilocus haplotype (MLH). If more than one individual in a population had the same MLH, they were considered to be clones. Two datasets were manually prepared for further analysis: a non-clone-corrected dataset contained all the individuals, and a clone-corrected dataset contained only individuals of each unique MLH per population. Each of the three sites in Cantabria were treated as a separate population.

2.3 Genetic diversity

The number of MLHs, the expected number of MLHs based on rarefaction (Hurlbert 1971), the clonal fraction, the Shannon-Wiener Index (Shannon 2001) and the Stoddart and Taylor's Index (Stoddart and Taylor 1988) were determined for each of the populations with the non-clone-corrected dataset using the R package *poppr* (Kamvar et al. 2014), and the genetic diversity (Nei 1978), was calculated with *poppr* using the clone-corrected dataset. The program ADZE (Szpiech et al. 2008) uses rarefaction to allow for comparisons between populations of different sizes and was used to calculate the allelic richness (A_R) and Private allelic richness (PA_R) for each population with the clone-corrected dataset. Calculations were standardized

corresponding to the population with the smallest population size that was larger than six (Site 29.1, N=9).

2.4 Population structure

The Bayesian clustering program STRUCTURE 2.3.4 (Falush et al. 2003) was used to determine the most likely number of population clusters (K) based on the MLHs for each of the individuals in the non-clone-corrected dataset. Thirty independent runs of K = 1-20 were performed, with a burn-in value of 100 000 and 1 000 000 iterations. An admixture model with correlated allele frequencies was selected and the data was run with no prior differentiations according to sites.

StructureSelector (Li and Liu 2018) was used to estimate the optimal number of clusters. This online program implements the Evanno method (Evanno et al. 2005) as well as an additional four measures (MAXMEAK, MAXMEDK, MEDMEDK and MEDMEAK) that are more accurate to estimate K in populations with uneven population sizes (Puechmaille 2016). CLUMPAK (Kopelman et al. 2015) and DISTRUCT (Rosenberg 2004) was also implemented on the StructureSelector platform and was used to converge all 30 runs of the optimal K and visualize the output.

Discriminant analysis of principal components (DAPC) was performed with the *adegenet* package in R studio (Jombart and Ahmed 2011) as an additional visualization of the population genetic structure and the clone-corrected dataset was analysed. The optimal number of clusters were determined by assessment of Bayesian information criterion (BIC) using the *find.clusters* function. The *xvalDapc* function was used to retain the optimal number of principal components in the analysis. The *ismn* function in *poppr* was used to draw a minimum spanning network using Bruvo's genetic diversity (Bruvo et al. 2004) in order to compare the multilocus haplotypes determined over the 12 microsatellite loci between the different populations. A

pairwise genetic differentiation (F_{ST}) between the three sampling sites was calculated using GENALEX version 6.5 (Peakall and Smouse 2012) in order to determine if the genetic variation is explained by the population structure observed.

2.5 Mating type determination and random mating

The mating types of the isolates were determined using the species-specific mating type primers designed by Groenewald et al. (2007). The same reagent concentrations, PCR amplification protocols and methods to visualize the amplified products were used as described by Barnes et al. (2014). Isolates for which the mating type fragment size was 820 bp were assigned as MAT1-1-1 and isolates with a fragment size of 480 bp were MAT1-2 individuals.

Three different methods were used to investigate whether sexual recombination is occurring in the populations. An exact binomial test (http://www.biostathandbook.com/exactgof.html), with two-tailed P-values were used to determine if the mating type ratios deviated from a 1:1 ratio (at $P < 0.05$) using the non-clone-corrected dataset. Additionally, the index of association (I_A) (Brown et al. 1980; Smith et al. 1993) and rBarD (Agapow and Burt 2001) were utilized in the R-package *poppr* to test for linkage disequilibrium in the 12 microsatellite loci where the null hypothesis of no linkage due to sexual mating is rejected when $P < 0.05$.

3. Results

3.1 Sample collection, fungal isolations and species identification

A total of 120 isolates were obtained from Cantabria. Forty-nine isolates were from 12 trees at Site 25, 19 isolates were from 11 trees at Site 29.1 and 52 isolates were from 18 trees at Site 29.2 (Table 1). Blast analyses revealed that all 120 isolates obtained were those of *D. septosporum* and all were of a single ITS haplotype (Ds_Hap.1).

3.2 Microsatellite amplification and haplotype determination

All *D. septosporum* isolates produced an allele size of 124 bp when screened with the Doth_A marker which is consistent with *D. septosporum* screened from other countries (Barnes *et al.* 2014). A total of 40 alleles were detected across the 12 microsatellite loci, with DCB2 being monomorphic for allele 156, Doth_F monomorphic for allele 173, and Doth_O monomorphic for allele 200. The number of alleles at each of the nine polymorphic loci ranged from two at Doth_I, to seven alleles at Doth_M (Table 2).

A total of 52 unique MLHs were identified in the 120 isolates analysed (Fig. 2, Appendix 2). Site 29.2 contained four MLH that were present at the other sites: two shared with Site 29.1 (MLH 27 and MLH33, Fig. 2) and two shared with Site 25 (MLH 21 and MLH40, Fig. 2). The collection from Site 29.2 had the greatest number of MLHs (29) and the collection from Site 29.1 had the least (nine). These numbers are, however, biased due to differences in the sample size. In many cases, conidiomata were isolated from the same needle. In 86.2% of these cases, individuals containing different MLHs were isolated from these needles (Appendix 2).

3.3 Genetic diversity

Based on rarefaction, the genotypic richness was the highest in Site 29.2 (eMLG = 14.14) and the collection from Site 29.1 had the lowest genetic diversity (eMLG = 9.0) (Table 3). The clonal fraction ranged from 0.63 in Site 25 to 0.44 in Site 29.2 with 57% clonality overall in the collection (Table 3). Site 29.2 followed by Site 25 displayed the highest level of genetic diversity and richness based on the allelic richness, the Shannon-Wiener index, Stoddard Taylor's index and Simpson index (Table 3). The genotypic evenness in Site 29.1 was the closest to having equal abundance (0.712) followed by Site 29.2 (0.67). The highest gene diversity was observed in Site 29.2 (0.339) followed by Site 29.1 (0.296). The population from Site 25 had the highest frequency of private alleles followed by the population from Site 29.2,

however the number of private alleles was low (0.064 in Site 29.1 – 0.405 in Site 25) in all populations considered (Table 3).

3.4 Population structure

The Evanno ΔK method supported four (K=4) clusters and the four Peuchmaille methods suggested one to three clusters (K = 1 to 3) as the most likely optimal number of clusters in the STRUCTURE analysis (Fig. 3). The structure bar plots for K=2 to K=4 for the major modes are illustrated in Figure 4. The bar plots for K=2 to K=4 together with the geographical distribution of the clusters are represented in Figure 4. The find.clusters function in the adegenet package in R-studio was used to determine that K=2 is the most optimal scenario (Fig. 5). This showed that the majority of individuals at Site 25 clustered together as did those from Site 29.2. In contrast, individuals from Site 29.1 were interspersed between the two clusters. The two major clusters for the three sites sampled were also apparent in the haplotype network drawn using Bruvo's genetic distance (Fig. 6). The F_{ST} calculated between sites was the highest (0.253) between Site 25 and Site 29.2 (Table 4) however all other pairwise comparisons were low (F_{ST} = 0.127 for Sites 29.1 and 29.2; F_{ST} = 0.147 for Sites 25 and 29.1).

3.5 Mating type determination and random mating

Both mating type idiomorphs of *D. septosporum* were detected in isolates from all three sites sampled (Table 5). Although both mating types were detected at these sites, the ratio deviated from 1:1 and random mating was statistically supported only for Site 29.1. The index of association and rbarD, which tests for linkage disequilibrium using microsatellite data, provided no evidence for sexual recombination at any of the sites using the clone-corrected or non-clone-corrected datasets (Table 5).

Although no evidence for sexual recombination was evident from the statistical analyses, isolates of the same MLH having opposite mating types were found at both Site 29.2 and Site

29.1 (Appendix 2). Individuals of opposite mating type were detected amongst isolates collected from the same trees in all of the sites (Appendix 2). Furthermore, in the one case at Site 29.1, and in 28.6% of cases at Site 29.2 where multiple conidiomata had been isolated from the same needle, individuals of opposite mating type were detected amongst isolates from the same needles (Appendix 2).

4. Discussion

This study investigated the population structure and diversity of populations of a *Dothistroma* species collected during a severe outbreak of DNB in 2015 in planted *Pinus nigra* stands in Cantabria. All isolates were shown to represent *D. septosporum.* The results provide a first report of *D. septosporum* in Cantabria and the first report of this pathogen on *Pinus nigra* subsp. *laricio* var. *corsicana.* Of the three host tree species sampled at the affected sites, high susceptibility to *D. septosporum* has previously been reported only on *P. nigra* subsp. *nigra* (Drenkhan et al. 2016). The present study provides evidence that *Pinus nigra* subsp. *laricio* var. *corsicana* and *P. nigra* subsp. *salzmannii* are also highly susceptible to the pathogen.

The genetic diversity of *D. septosporum* found in the collections from Cantabria, and evidence of sexual reproduction at one of the sites sampled, suggests that the pathogen was not recently introduced into the region, but has more likely been present for a long period of time. It is also evident that multiple infections have occurred in the region as evidenced by isolates obtained from the same trees, and often the same needle, having different MLHs and in some cases opposite mating types. The pathogen was likely present in the surrounding area, especially considering that the affected trees were already 25 – 30 years old. The severe outbreak was probably due to a climatic factor that changed in 2015 and prompted high infection levels.

A climate change-driven explanation for the severe disease outbreaks would be consistent with the average precipitation in Cantabria that was above average in 2015

(https://www.worldweatheronline.com/selaya-weather-averages/cantabria/es.aspx). Climate modelling for the Basque country, which is adjacent to Cantabria, suggested that the north of Spain is at high risk for increased infections by *Dothistroma* spp. and its prevalence is influenced by increased precipitation and solar radiation (Iturritxa et al. 2015). This phenomenon is not new as can be seen from increased infection levels due to *D. septosporum* in *P. tecunumanii* stands in Colombia, which have also been linked to unusually high levels of precipitation (Rodas et al. 2016).

Dothistroma septosporum in Cantabria could have originated from neighbouring areas such as the Basque country. Previous surveys (Ortíz de Urbina et al. 2017) have shown that *D. septosporum* infected 10% of the sampled *P. nigra* stands in the Basque country. An increase in infections in the Basque country in pine plantations as well as in arboreta surrounded by infected plantations is indicative that the pathogen is spreading throughout the region (Mesanza et al. 2020). Although extensive efforts have been made in the Basque country to survey the entire region for pine needle pathogens (Iturritxa et al. 2015; Ortíz de Urbina et al. 2017), no population genetics studies have been conducted on populations of *D. septosporum* there. Such information would help inform the understanding of the driving force behind the infections in Cantabria.

In the most recent global population analyses for *D. septosporum*, it was hypothesised that the pathogen is represented by three major population clusters represented by North America, Western Europe and Eastern Europe (Mullett et al. 2021). The population of *D. septosporum* in Eastern Europe and Western Europe differed considerably from each other. A collection of *D. septosporum* from Valencia in Spain was included in the global population study and grouped with the Eastern European population, which also included 17 other European countries (Mullett et al. 2021). In contrast, the *D. septosporum* population collected from France, which is the closest adjacent country to Cantabria, grouped in the Western European

cluster with eight other European countries (Mullett et al. 2021). Severe outbreaks of *D. septosporum* have been reported in France in the past thirty years (Fabre et al. 2012; Lévy 1996; Mullett et al. 2015), even though the pathogen was already present in the country since 1860 (Vuillemin 1896). It is thus conceivable that the French outbreaks could be linked to those more recent in Spain, including Cantabria.

It would be interesting to compare the global population data for *D. septosporum* with the highly diverse populations found in the present study for Cantabria. This would establish whether the pathogen has spread naturally from neighbouring France, or if it is part of the larger Eastern European population. Future studies should include sampling all areas in Spain where *D. septosporum* has been identified in order to better understand potential introductory pathways of the pathogen.

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Table 1. Collection information of isolates collected in Cantabria, Spain for this study.

Site name in Cantabria	**Host**	**GPS coordinates**	**Collectors**	**Date collected**	**Number of isolates/number of trees sampled**
Site 25, near Vega de Pas	*Pinus nigra* subsp. *laricio* var. *corsicana*	43.177788 N, 3.818498 W	Barnes I, Diez JJ, Mullett MS	19 Jul 2015	49/12
Site 29.1, near San Miguel de Aguayo	*Pinus nigra* subsp. *nigra*	43.095454 N, 3.990897 W	Barnes I, Diez JJ, Mullett MS	19 Jul 2015	19/11
Site 29.2, near San Miguel de Aguayo	*Pinus nigra* subsp. *salzmannii*	43.095013 N, 3.995237 W	Barnes I, Diez JJ, Mullett MS	19 Jul 2015	52/18

Table 2. *Dothistroma septosporum* microsatellite PCR annealing temperatures, dilutions for fragment analysis for each microsatellite marker as well as the panel number for fragment analyses.

Marker name	**Optimum annealing temperature (°C)**	**Dilution ratio (µl) for fragment analyses**	**Panel number in fragment analyses**	**Number of alleles per locus/ microsatellite marker[1]**
DCB2	56	0.6/40	1	1
Doth_A	56	1/18	2	1
Doth_DS1	62	1/40	1	6
Doth_DS2	56	1/40	1	3
Doth_E	50	1/18	2	4
Doth_F	57	1.5/40	1	1
Doth_G	60	2/18	2	3
Doth_I	58	1/18	2	2
Doth_J	58	1/18	2	3
Doth_K	60	3/18	2	5
Doth_L	58	3/40	1	4
Doth_M	59	1.2/40	1	7
Doth_O	60	0.8/40	1	1

[1]Determined from 120 isolates analysed from Cantabria.

Table 3. Summary diversity statistics of *Dothistroma septosporum* populations in Cantabria in Spain.

Site	**N[1]**	**MLH[2]**	**eMLH[3]**	**CF[4]**	**A_R[5] ***	**PA_R[6] ***	**H[7]**	**G[8]**	**Lambda[9]**	**E.5[10]**	**H_{exp}[11] ***
Site 25	49	18	9.22 ± (1.55)	0.63	2.174 ± (0.281)	0.405 ± (0.142)	2.23	5.68	0.824	0.565	0.246
Site 29.1	19	9	9.00 ± (0.00)	0.53	1.914 ± (0.236)	0.064 ± (0.044)	1.91	5.08	0.803	0.712	0.296
Site 29.2	52	29	14.14 ± (1.51)	0.44	2.221 ± (0.304)	0.328 ± (0.155)	3.09	15.02	0.933	0.670	0.339
Total	**120**	**52**	**14.22 ± 1.70**	**0.57**			**3.48**	**19.78**	**0.949**	**0.598**	**0.396**

[1]N = Number of isolates.
[2]Number of multilocus haplotypes. Equivalent to samples that have been clone-corrected.
[3]The number of expected MLG at the smallest sample size ≥ 10 (i.e. Site 29.1 = 19) based on rarefaction ± standard error.
[4]CF: Clonal Fraction = 1 – [MLH/N].
[5]Allelic richness ± standard error (Szpiech *et al.,* 2008).
[6]Private allelic richness ± standard error (Szpiech *et al.,* 2008).
[7]H: Shannon-Wiener Index of MLG diversity (Shannon, 2001).
[8]G: Stoddart and Taylor's Index of MLG diversity (Stoddart & Taylor, 1988).
[9]lambda: Simpson's Index (Simpson, 1949). – provides and estimation of the probability that two randomly selected genotypes are different. 0 = no genotypes different. 1 = all genotypes are different.
[10]E.5: Genotypic evenness, (Pielou, 1975; Ludwig & Reynolds, 1988; Grünwald *et al.,* 2003).
[11] H_{exp} = Nei's (1973) gene diversity.
* The clone-corrected dataset was used to determine the Allelic richness, Private allelic richness and Nei's gene diversity. All other calculations were performed using the non-clone-corrected dataset.

Table 4. F_{ST} (Pairwise genetic differentiation) calculated between the populations of *Dothistroma septosporum* grouped by site.

Site	**Site 25**	**Site 29.1**	**Site 29.2**
Site 25	0	0.147	0.253
Site 29.1		0	0.127
Site 29.2			0

Table 5. Mating type ratios and index of association tests for the *Dothistroma septosporum* populations collected in Cantabria in Spain.

Site	**Mating type ratios[a]**					**Linkage disequilibrium – Index of association[b]**					
						Non-clone-corrected data			**Clone-corrected data**		
	MAT1-1	**MAT1-2**	**Could not determine**	**Expected ratio**	**P-value (two tailed test)**	**I_A**	**rbar D**	**P**	**I_A**	**rbarD**	**P**
Site 25	45	4		24.5	< 0.0001	2.402	0.317	0.001	1.975	0.251	0.001
Site 29.1	12	6	1	9	**0.238**	2.031	0.292	0.001	0.580	0.083	0.020
Site 29.2	15	36	1	25.5	0.005	1.253	0.158	0.001	0.506	0.064	0.001
Total	72	46	2								

Significant P-values (P>0.05) is shown in bold.

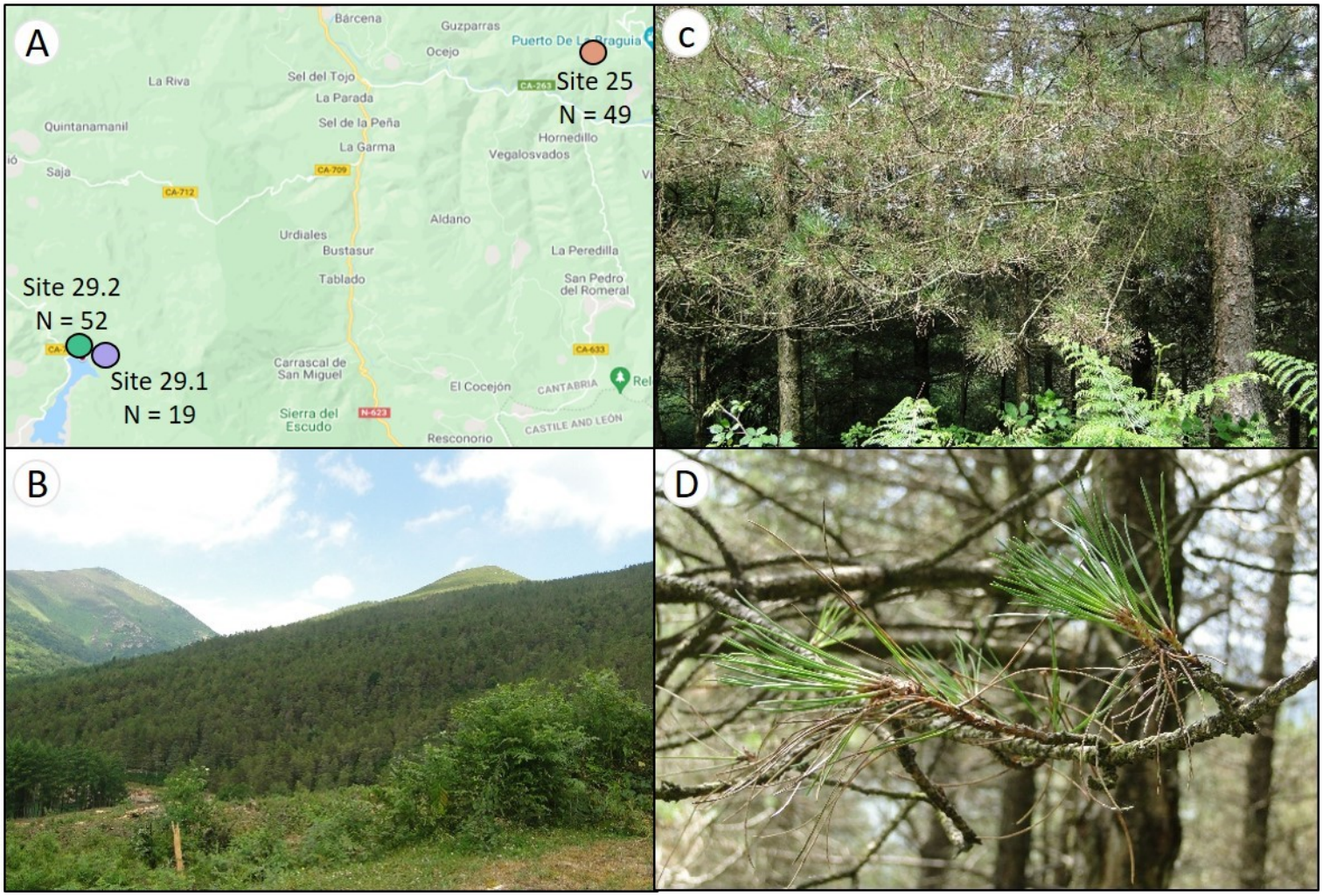

Figure 1. Dothistroma needle blight in Cantabria, Spain A) The three collection sites in Cantabria. B) Outbreaks of DNB were observed in Cantabria on three subspecies of *P. nigra.* and severe needle cast C) was observed, including on a new host for *D. septosporum, P. nigra* subsp. *laricio* var. *corsicana.* D) Severe host susceptibility was observed and only primary needles were left on the infected trees' branches.

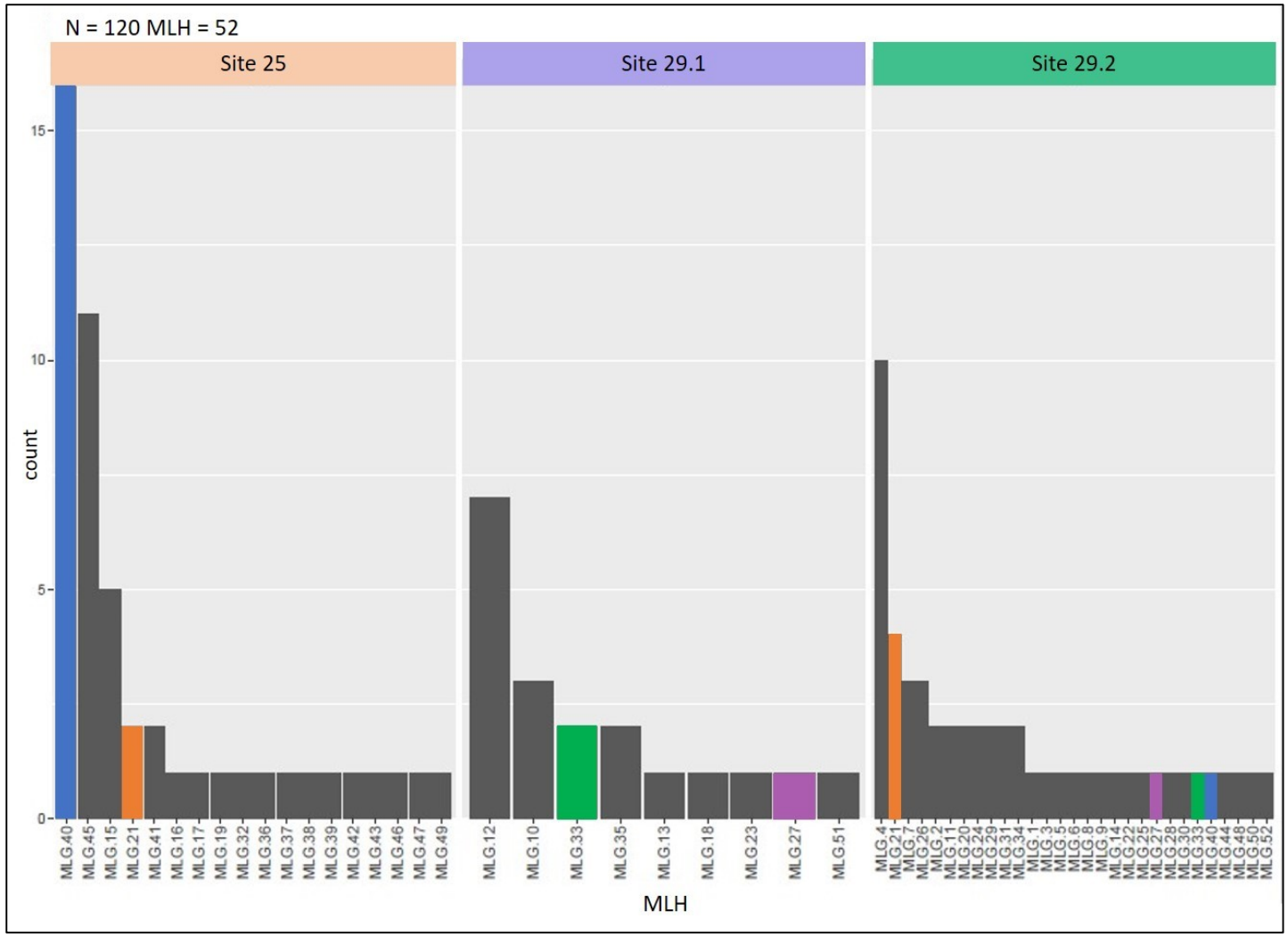

Figure 2. The 52 multilocus haplotypes (MLH) derived from microsatellite data in each site and shared between sites. Four MLHs, indicated by the coloured bars were shared between multiple sites.

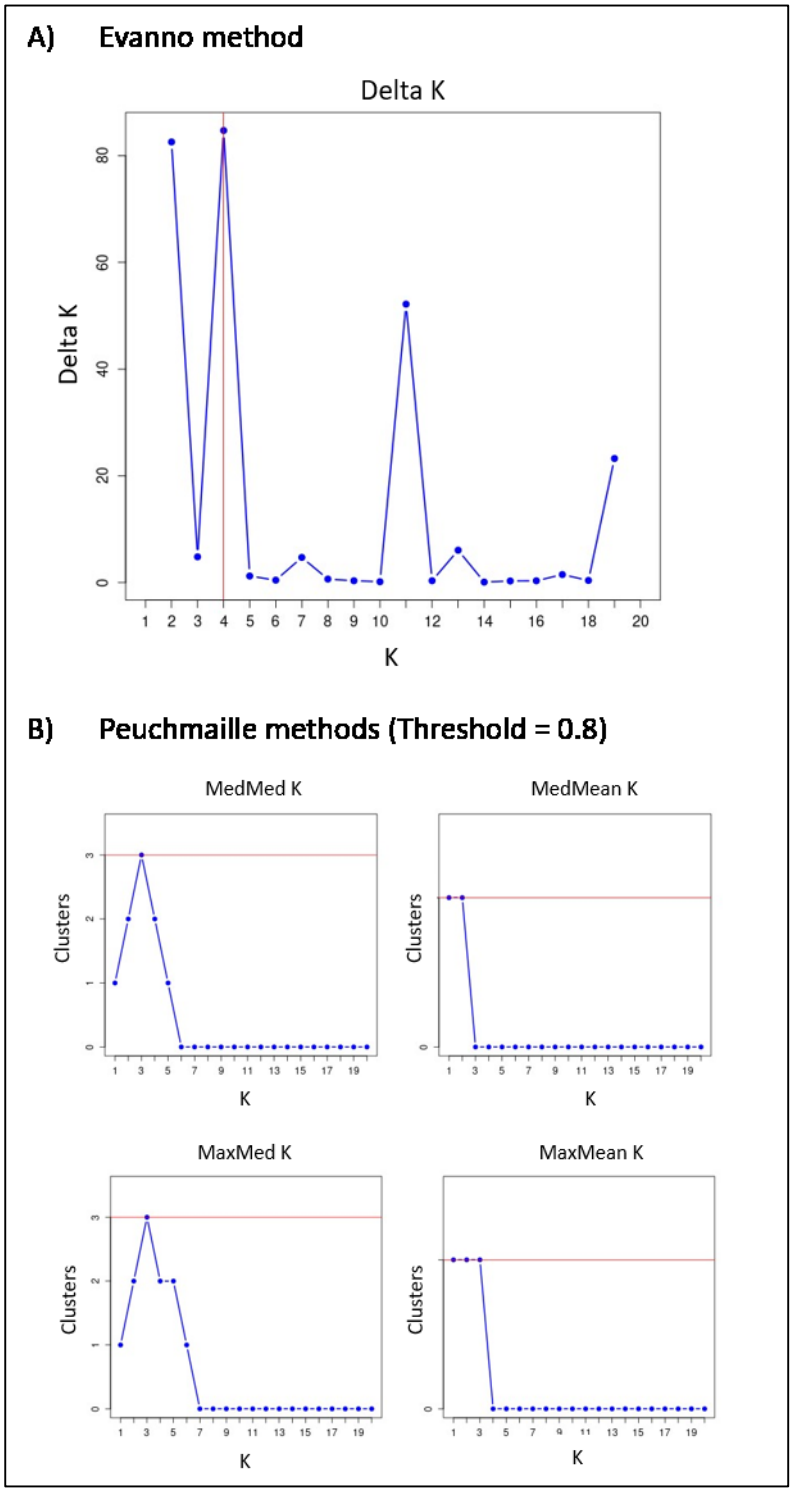

Figure 3. The optimum number of clusters determined using StructureSelector. A) The Evanno method (Delta K) determined K = 4 to be the optimal number of clusters. B) The Puechmaille methods determined that K = 1-3 were optimal however K=3 was selected in all options except for MedMean K when a threshold of 0.8 was set.

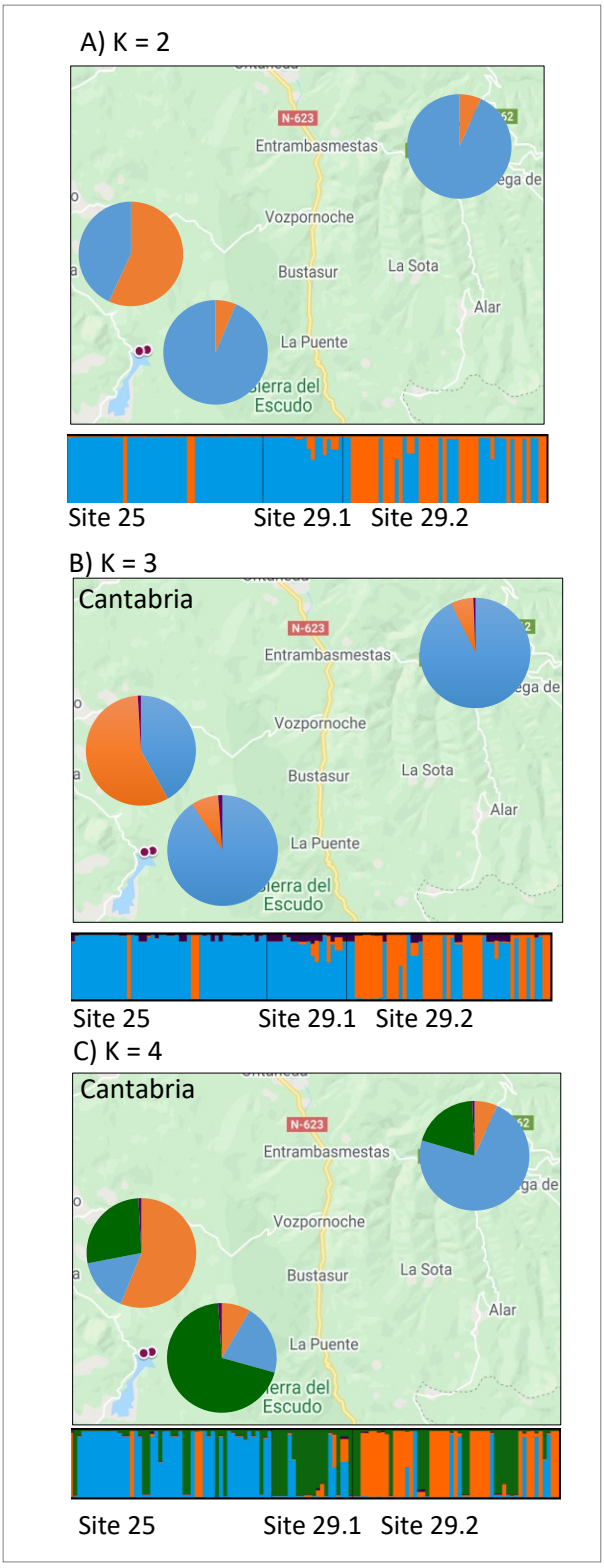

Figure 4. *Dothistroma septosporum* population divisions observed in Cantabria using STRUCTURE analyses based on the most likely K values determined by the Evanno delta K method (K=4) and the Puecemaille methods (K=1-3) with A) K=2 (number of DAPC clusters), B) K=3 and C) K=4. Clusters are represented as pie charts in each sampled site.

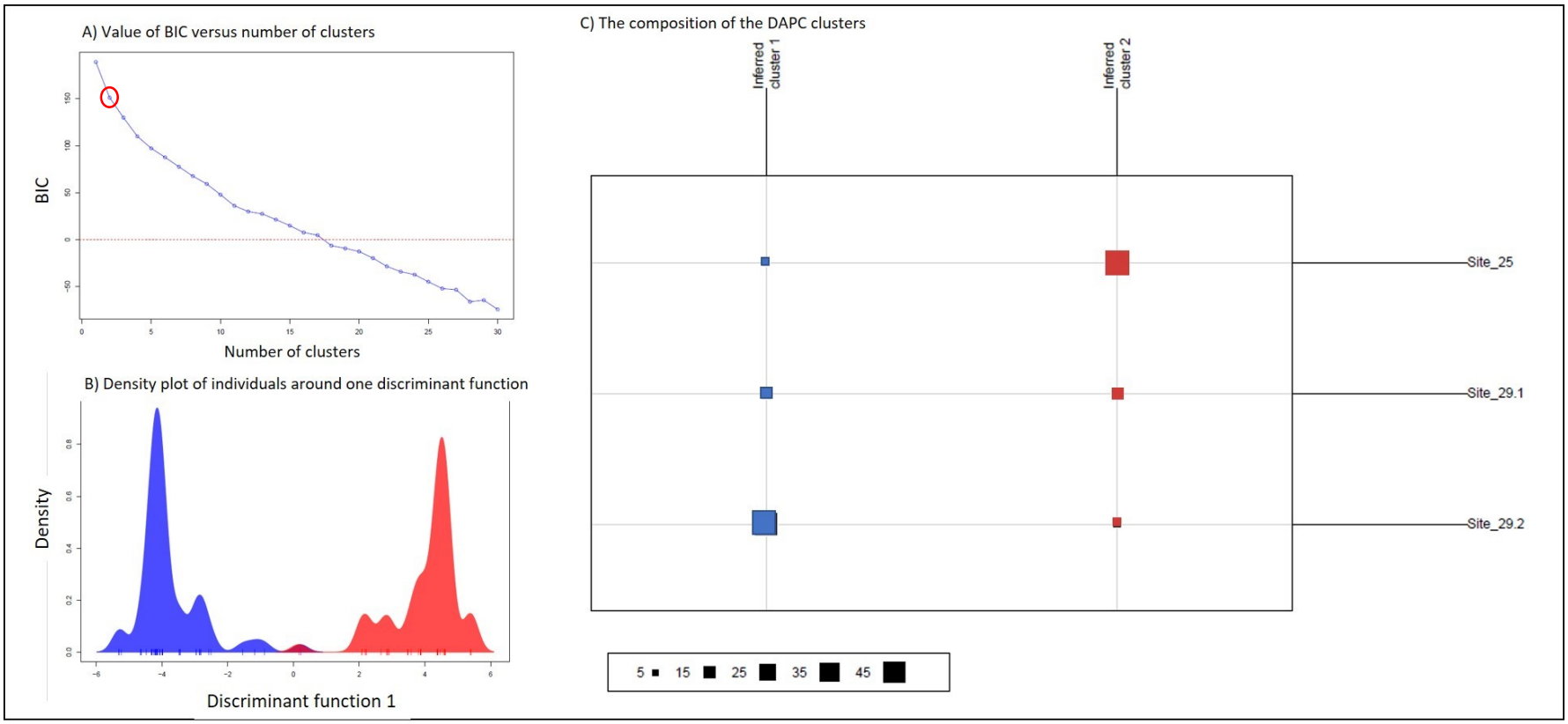

Figure 5. The discriminant analysis of principal components (DAPC) based on the *D. septosporum* multilocus haplotypes obtained from Spain. (A) The value of BIC versus the number of clusters reveals that K=2 is the optimal number of clusters. This is represented (B) in a plot of the densities of individuals on a single discriminant function with different colours for different groups. (C) The composition of the DAPC groups. Each column corresponds to each of the inferred clusters and rows correspond to each location. The size of the squares is proportional to the number of individuals in each cluster. Cluster 1 is mainly composed of individuals from site 25 and Cluster 2 is mainly composed of individuals from site 29.2.

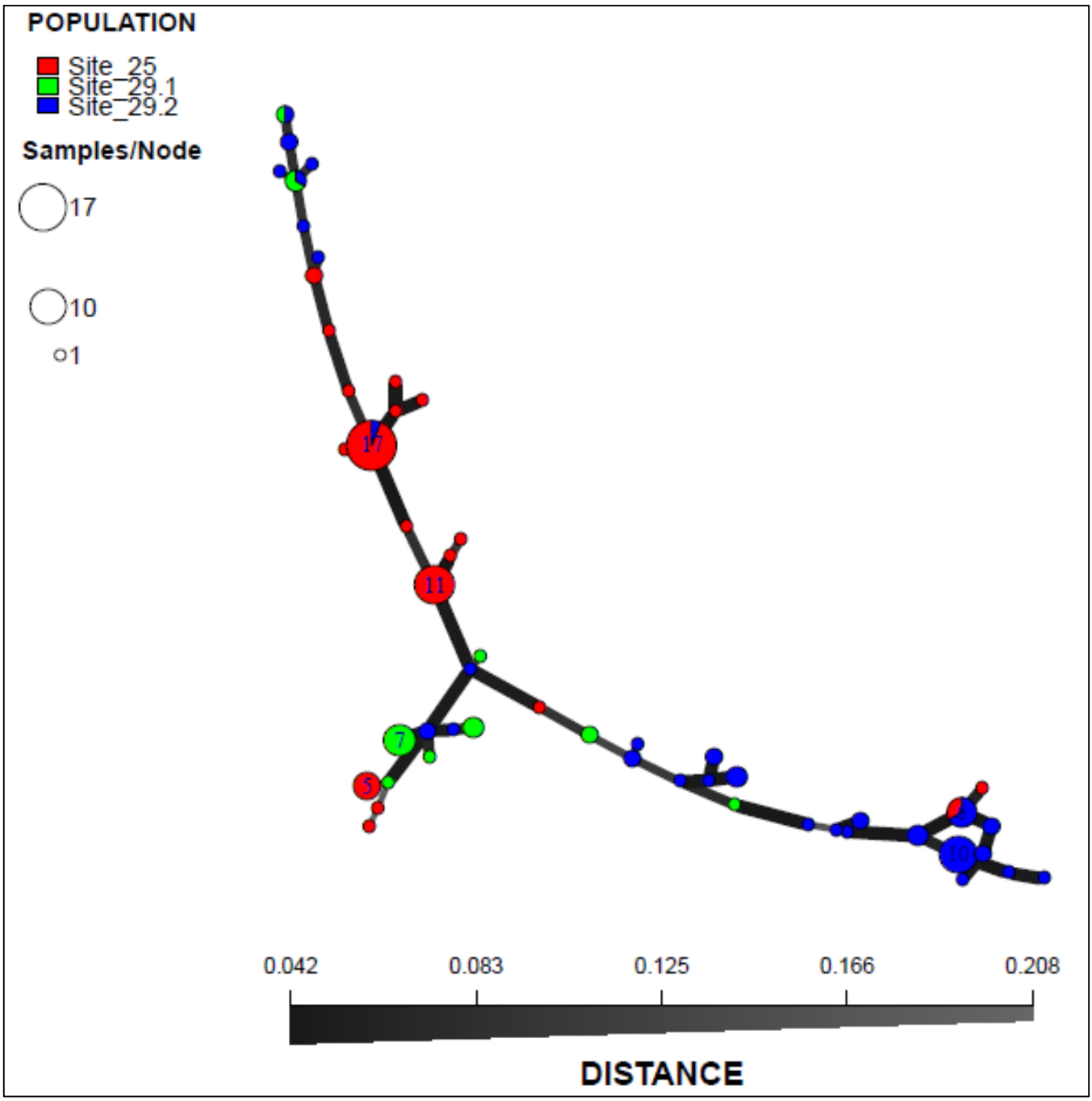

Figure 6. Haplotype network of *D. septosporum* collected in Cantabria drawn using Bruvo's genetic distance. Each circle represents a multilocus haplotype and thicker lines between the circles indicates less genetic distance between the MLHs. The larger the circle, the more individuals have the same haplotype. One to three clusters are observed with the isolates collected from Cantabria with the most likely interpretation that there are two clusters, with majority of individuals from Site 25 (red) genetically distant from the individuals from Site 29.2 with site 29.1 interspersed throughout.

APPENDIX 1

Allele sizes for 345 individuals of *Dothistroma pini* in Europe based on 17 microsatellite markers

Table 2. Allele sizes for 345 individuals of *Dothistroma pini* in Europe based on 17 microsatellite markers

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
BEL_2	Belgium	-	*Pinus sp.*	4	2	109	111	169	264	250	307	352	390	204	197	219	234	270	319	353	353	398	397
43896	Czech Republic	-	*Pinus jeffreyi*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
38542	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	45	111	169	264	250	301	352	364	204	197	219	234	267	322	359	350	386	403
41492	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	2	1	57	111	169	264	250	301	352	387	204	203	217	234	267	319	353	350	386	406
38756	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
38757	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
41483	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
41487	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	60	111	169	264	250	301	352	390	204	197	217	234	270	319	353	350	398	403
38538	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	61	111	169	264	250	301	352	390	204	203	219	234	270	322	353	350	398	397
41488	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	63	111	169	264	250	301	352	390	206	197	219	234	270	319	359	353	389	397
38537	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	65	111	169	264	250	301	352	390	206	203	217	234	267	322	353	350	401	406
38541	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	73	111	169	264	250	301	352	390	206	203	219	234	267	319	353	350	386	403
41485	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	2	1	77	111	169	264	250	301	352	396	204	197	219	234	270	319	353	350	398	397
38539	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	79	111	169	264	250	301	352	396	206	203	217	234	267	319	353	350	386	406
41480	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
41489	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
41481	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	4	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
41484	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	84	111	169	264	250	307	352	384	206	197	217	234	270	319	353	350	386	403
41491	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	4	1	86	111	169	264	250	307	352	387	204	197	217	234	270	319	353	350	386	403
41475	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	87	111	169	264	250	307	352	390	204	197	219	234	267	319	353	350	398	397
41490	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	4	1	88	111	169	264	250	307	352	390	204	197	219	234	270	319	353	350	398	397
38540	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	90	111	169	264	250	307	352	390	206	203	219	234	270	319	353	350	386	406
38534	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	91	111	169	264	250	307	352	390	206	209	217	234	267	319	353	350	386	403
41478	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	94	111	169	264	250	307	352	396	204	203	219	234	267	319	353	350	389	406
41486	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	96	111	169	264	250	307	352	399	204	197	217	234	270	319	353	350	398	406
41479	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	2	1	102	111	169	289	250	307	352	390	206	197	217	234	270	319	353	350	401	397
38536	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*		1	103	111	169	289	250	307	352	390	206	203	217	234	267	319	353	350	398	406
41493	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	2	104	111	169	289	250	307	352	396	204	197	217	234	270	319	353	350	401	397
38758	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	105	111	169	289	250	307	352	396	206	197	217	234	267	322	353	350	401	385
41482	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	1	106	111	169	295	250	307	352	387	204	197	217	234	267	319	353	350	428	409
41476	France	La Ferté-Imbault	*Pinus nigra* subsp. *laricio*	1	2	107	111	169	295	250	307	352	396	206	197	217	234	267	319	353	350	401	394
FR_1.3_N3	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
43903	France	La Bouyale	*Pinus nigra* subsp. *laricio*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
43911	France	La Bouyale	*Pinus nigra* subsp. *laricio*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
FR_1.5_N1	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	1	79	111	169	264	250	301	352	396	206	203	217	234	267	319	353	350	386	406
43907	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
FR_1.12_N1	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
FR_1.13_N1	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
FR_1.8_N1	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	2	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
41474	France	La Bouyale	*Pinus nigra* subsp. *laricio*	1	2	97	111	169	264	250	307	352	399	204	203	217	234	267	319	353	350	386	406
43904	France	Nueng-sur-Beuvron	*Pinus nigra* subsp. *laricio*	4	1	62	111	169	264	250	301	352	390	206	197	217	234	270	322	353	350	386	406
43901	France	Nueng-sur-Beuvron	*Pinus nigra* subsp. *laricio*	4	1	99	111	169	264	250	307	352	402	204	197	219	234	267	319	353	350	389	406
43897	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
FR_3.1_N3	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	79	111	169	264	250	301	352	396	206	203	217	234	267	319	353	350	386	406
41506	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	80	111	169	264	250	301	352	399	204	203	217	234	267	319	353	350	386	406
43910	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	82	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	385
FR_3.4_N3	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	82	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	385
FR_3.5_N3	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	85	111	169	264	250	307	352	384	206	197	219	234	270	319	353	350	380	409
41495	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	88	111	169	264	250	307	352	390	204	197	219	234	270	319	353	350	398	397
41505	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	93	111	169	264	250	307	352	396	204	197	219	234	270	322	356	353	398	403
FR_3.5_N1	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	95	111	169	264	250	307	352	396	206	197	219	234	270	319	353	350	398	409
41499	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	98	111	169	264	250	307	352	402	204	197	217	234	270	322	353	350	386	406

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
41494	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	100	111	169	264	250	307	352	405	204	197	219	234	270	322	356	353	398	403
43908	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	1	101	111	169	264	250	307	352	393	204	197	219	234	270	319	368	350	398	397
41498	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	108	111	169	295	250	319	352	364	206	197	217	234	267	325	353	350	428	409
41507	France	Souesmes	*Pinus nigra* subsp. *laricio*	1	2	108	111	169	295	250	319	352	364	206	197	217	234	267	325	353	350	428	409
41501	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	2	44	111	169	249	250	301	360	344	204	203	217	234	267	319	353	350	380	397
41497	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
41503	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
43899	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
43900	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
43902	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
43905	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
FR_2.5_N2	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
FR_2.6_N1	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
FR_2.7_N2	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
FR_2.9_N3	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	78	111	169	264	250	301	352	387	204	209	217	234	267	319	353	350	386	406
43906	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	80	111	169	264	250	301	352	399	204	203	217	234	267	319	353	350	386	406
41477	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	2	80	111	169	264	250	301	352	399	204	203	217	234	267	319	353	350	386	406
FR_2.13_N2	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	82	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	385
FR_2.7_N3	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	82	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	385
FR_2.4_N2	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
41496	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	4	2	88	111	169	264	250	307	352	390	204	197	219	234	270	319	353	350	398	397
41504	France	Villefranche-sur-Cher	*Pinus nigra* subsp. *laricio*	1	2	89	111	169	264	250	307	352	390	204	197	219	234	270	319	353	350	401	397
26412	Hungary	Diszel	*Pinus nigra*	1	1	46	111	169	295	250	307	352	364	204	197	217	222	267	319	353	350	440	394
26407	Hungary	Diszel	*Pinus nigra*	1	1	47	111	169	295	250	307	352	364	204	203	217	234	267	319	353	350	425	394
26422	Hungary	Diszel	*Pinus nigra*	1	1	48	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	403
26395	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26398	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26436	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26437	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26441	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26442	Hungary	Diszel	*Pinus nigra*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
26424	Hungary	Diszel	*Pinus nigra*	1	1	53	111	169	295	250	307	352	364	204	197	219	234	267	319	353	350	425	394
26405	Hungary	Diszel	*Pinus nigra*	1	1	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
26417	Hungary	Diszel	*Pinus nigra*	1	1	58	111	169	295	250	307	352	364	204	203	217	234	267	319	353	350	443	394
46789	Romania	Botasani	*Pinus nigra*	2	1	10	111	185	284	250	292	340	358	212	197	217	222	273	322	389	350	404	394
46790	Romania	Botasani	*Pinus nigra*	2	1	12	111	185	284	250	292	340	358	212	197	217	222	267	322	389	350	419	394
29368	Russia	Donskoye forest	*Pinus nigra*	2	1	15	111	185	249	250	331	340	361	206	203	217	234	267	319	353	350	410	400
29369	Russia	Donskoye forest	*Pinus pallasiana*	2	1	37	111	169	249	250	310	340	364	206	197	217	222	267	319	353	350	410	394
29370	Russia	Donskoye forest	*Pinus mugo*	2	1	47	111	169	295	250	307	352	364	204	203	217	234	267	319	353	350	425	394
29367	Russia	Gorodishchenskoye forestry	*Pinus pallasiana*	2	1	13	111	169	229	256	322	352	344	204	203	217	222	270	319	359	350	389	394
29366	Russia	Gorodishchenskoye forestry	*Pinus pallasiana*	2	1	71	111	185	284	250	292	340	358	212	197	217	222	267	322	353	350	404	394
24852	Russia	Rostov oblast	*Pinus pallasiana*	2	1	11	111	185	284	250	292	340	358	212	197	217	222	267	322	389	350	404	394
48239	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	28	111	169	219	250	298	352	393	204	197	217	234	267	322	353	353	386	397
47085	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	30	111	169	219	250	298	352	396	206	197	217	234	267	322	353	353	386	397
47093	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	31	111	169	219	250	298	352	399	206	197	217	234	270	322	353	353	386	397
47086	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	33	111	169	219	250	298	352	461	204	197	217	234	267	322	353	353	386	400
47088	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
47089	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
47155	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
47157	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
48238	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
48718	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
47156	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
47087	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	35	111	169	219	250	298	352	396	204	197	217	234	267	319	353	353	386	397
47091	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	35	111	169	219	250	298	352	396	204	197	217	234	267	319	353	353	386	397
47092	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	35	111	169	219	250	298	352	396	204	197	217	234	267	319	353	353	386	397
48240	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	35	111	169	219	250	298	352	396	204	197	217	234	267	319	353	353	386	397
47084	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	36	111	169	219	250	325	352	396	204	197	217	234	267	319	353	353	386	397
47090	Serbia	Deliblatski pesak, Susara	*Pinus nigra*	2	1	36	111	169	219	250	325	352	396	204	197	217	234	267	319	353	353	386	397
T4N1	Serbia	Suboticka Pescara	*Pinus nigra*	2	2	27	111	169	219	250	298	352	396	204	197	217	234	270	322	353	353	386	397
T4N2	Serbia	Suboticka Pescara	*Pinus nigra*	2	2	27	111	169	219	250	298	352	396	204	197	217	234	270	322	353	353	386	397
48819	Serbia	Suboticka Pescara	*Pinus nigra*	4	2	32	111	169	219	250	298	352	399	204	197	217	234	267	322	353	353	386	397
48820	Serbia	Suboticka Pescara	*Pinus nigra*	4	2	32	111	169	219	250	298	352	399	204	197	217	234	267	322	353	353	386	397
48821	Serbia	Suboticka Pescara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
48822	Serbia	Suboticka Pescara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
T2N2	Serbia	Suboticka Pescara	*Pinus nigra*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
D539	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	2	2	1	111	185	219	250	325	352	396	204	197	217	234	267	319	353	353	386	397
D545	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	2	2	1	111	185	219	250	325	352	396	204	197	217	234	267	319	353	353	386	397
48237	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
D542	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
48724	Slovakia	Arborétum Mlyňany	*Pinus schwerinii*	1	2	51	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	376
51017	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D443	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	55	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	431	397
47094	Slovakia	Arborétum Mlyňany	*Pinus jeffreyi*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47095	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47096	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47097	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47098	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47099	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47100	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47101	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
47102	Slovakia	Arborétum Mlyňany	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48230	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48231	Slovakia	Arborétum Mlyňany	*Pinus jeffreyi*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48232	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48233	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48234	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48235	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48236	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48721	Slovakia	Arborétum Mlyňany	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48722	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
48723	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D413	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D438	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D440	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D451	Slovakia	Arborétum Mlyňany	*Pinus coulteri*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D456	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D459	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D520	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D544	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D546	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D548	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D571	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D572	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D573	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D623	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
M540	Slovakia	Arborétum Mlyňany	*Pinus ponderosa*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D609	Slovakia	Banská Belá	*Pinus nigra*	2	1	26	111	169	219	250	325	352	396	204	197	217	234	267	319	353	353	407	397
D610	Slovakia	Banská Belá	*Pinus nigra*	2	1	26	111	169	219	250	325	352	396	204	197	217	234	267	319	353	353	407	397
D624	Slovakia	Banská Belá	*Pinus nigra*	2	1	26	111	169	219	250	325	352	396	204	197	217	234	267	319	353	353	407	397
D245	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
48228	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D156	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D157	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D316	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D750	Slovakia	Gabčíkovo	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D747	Slovakia	Jahodná	*Pinus nigra*	1	2	49	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	368	394
48720	Slovakia	Jahodná	*Pinus nigra*	1	2	50	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	419	394
48229	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
49955	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D110	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D158	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D678	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D733	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D734	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D735	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D738	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D739	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D740	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D742	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D743	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D749	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D751	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D752	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D758	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D759	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D760	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D761	Slovakia	Jahodná	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
51011	Slovakia	Jahodná	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D681	Slovakia	Jahodná	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D741	Slovakia	Jahodná	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
51012	Slovakia	Košice	*Pinus ponderosa*	1	1	25	111	169	269	250	307	352	364	204	197	217	234	267	319	353	350	407	394
51013	Slovakia	Košice	*Pinus ponderosa*	1	1	25	111	169	269	250	307	352	364	204	197	217	234	267	319	353	350	407	394
51014	Slovakia	Košice	*Pinus ponderosa*	1	1	25	111	169	269	250	307	352	364	204	197	217	234	267	319	353	350	407	394
D682	Slovakia	Košice	*Pinus ponderosa*	1	1	25	111	169	269	250	307	352	364	204	197	217	234	267	319	353	350	407	394
D720	Slovakia	Košice	*Pinus ponderosa*	1	1	25	111	169	269	250	307	352	364	204	197	217	234	267	319	353	350	407	394
D708	Slovakia	Kováčová	*Pinus mugo*	2	negative	32	111	169	219	250	298	352	399	204	197	217	234	267	322	353	353	386	397
D494	Slovakia	Ľubochňa	*Pinus sylvestris*	1	2	54	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	397
D431	Slovakia	Sečovce	*Pinus mugo*	2	2	29	111	169	219	250	298	352	402	204	197	217	234	270	322	353	353	386	397
51015	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
51016	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
51018	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D725	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D748	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D754	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D762	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D768	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D769	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D770	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
D771	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D772	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D773	Slovakia	Trstice	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D767	Slovakia	Trstice	*Pinus nigra*	1	negative	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
D715	Slovakia	Trstice	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D753	Slovakia	Trstice	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D763	Slovakia	Trstice	*Pinus nigra*	1	2	56	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
D744	Slovakia	Zvolen	*Pinus jeffreyi*	4	2	34	111	169	219	250	298	352	396	204	197	217	234	267	322	353	353	386	397
D516	Slovakia	Zvolen	*Pinus jeffreyi*	1	2	54	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	397
D746	Slovakia	Zvolen	*Pinus jeffreyi*	1	2	54	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	397
43428	Slovenia	Dutovlje (Karst)	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43429	Slovenia	Dutovlje (Karst)	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43430	Slovenia	Hruševica (karst)	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43431	Slovenia	Hruševica (karst)	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43402	Slovenia	Panovec	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
43420	Slovenia	Panovec	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
43421	Slovenia	Panovec	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
43423	Slovenia	Panovec	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
43395	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43396	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43397	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43398	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43399	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43400	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43401	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43403	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43405	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43406	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43407	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43409	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43411	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43412	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43413	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43414	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43415	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43416	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43417	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43418	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43419	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43422	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43408	Slovenia	Panovec	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43410	Slovenia	Panovec	*Pinus nigra*	1	2	70	111	169	249	250	331	360	344	204	197	217	240	270	319	353	350	380	406
134690	Slovenia	Panovec	*Pinus nigra*	1	2	70	111	169	249	250	331	360	344	204	197	217	240	270	319	353	350	380	406
134689	Slovenia	Pivka	*Pinus nigra*	1	2	24	111	169	229	250	325	352	344	204	197	217	240	270	319	359	350	389	409
39746	Slovenia	Pivka	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
48823	Slovenia	Pivka	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
43394	Slovenia	Pivka	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
48825	Slovenia	Prebold	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
48826	Slovenia	Prebold	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
48827	Slovenia	Prebold	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
48828	Slovenia	Prebold	*Pinus nigra*	1	2	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
48824	Slovenia	Radenci	*Pinus nigra*	1	2	39	111	169	304	250	307	352	364	204	197	217	234	267	319	353	350	398	394
43425	Slovenia	Ribnica	*Pinus nigra*	1	2	67	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
43424	Slovenia	Ribnica	*Pinus nigra*	1	2	70	111	169	249	250	331	360	344	204	197	217	240	270	319	353	350	380	406
43426	Slovenia	Škocjan	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
43427	Slovenia	Škocjan	*Pinus nigra*	1	2	68	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
51027	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	4	2	16	111	169	249	250	322	360	361	204	203	217	240	267	319	353	350	386	406
D1371.5	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	18	111	169	259	250	322	360	396	204	214	217	240	267	319	353	362	398	394
D1372.5	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	19	111	169	264	250	307	364	344	204	197	217	246	270	322	353	350	398	394
51026	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	2	2	20	111	169	264	250	322	352	344	204	197	217	234	267	319	353	362	437	391
51028	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	21	111	169	259	250	307	451	390	206	197	217	329	267	319	353	350	395	397
D1373.2	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	21	111	169	259	250	307	451	390	206	197	217	329	267	319	353	350	395	397
D1371.4	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	4	2	22	111	169	259	250	322	352	344	204	197	217	234	270	319	353	353	398	397
D1372.2	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	4	2	22	111	169	259	250	322	352	344	204	197	217	234	270	319	353	353	398	397
D1371.2	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	23	111	169	259	250	322	352	361	204	209	217	234	267	319	353	350	386	400
D1371.7	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	23	111	169	259	250	322	352	361	204	209	217	234	267	319	353	350	386	400
D1371.3	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	2	1	72	111	169	264	250	325	352	344	206	197	217	234	267	319	353	350	380	391
D1371.1	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	4	2	74	111	169	310	250	312	360	402	204	197	217	240	267	322	353	350	380	391
D1372.3	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	2	75	111	169	264	250	322	360	361	204	203	217	240	267	322	353	350	386	418
D1372.4	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	2	75	111	169	264	250	322	360	361	204	203	217	240	267	322	353	350	386	418
51025	Spain	Aragon, Puente de Montana	*Pinus nigra* subsp. *nigra*	1	1	76	111	169	320	250	307	364	390	204	197	219	246	267	322	350	350	398	406
FID6.N3	Spain	Boixar	*Pinus nigra/pallasiana*	2	1	92	111	169	341	250	328	400	384	204	197	217	281	267	319	353	362	422	394
46833	Switzerland	Walensee	*Pinus nigra*	1	2	17	111	169	320	250	322	352	358	204	203	217	234	267	319	353	350	386	394
46834	Switzerland	Walensee	*Pinus nigra*	1	2	17	111	169	320	250	322	352	358	204	203	217	234	267	319	353	350	386	394
46835	Switzerland	Walensee	*Pinus nigra*	1	2	17	111	169	320	250	322	352	358	204	203	217	234	267	319	353	350	386	394
46836	Switzerland	Walensee	*Pinus nigra*	1	2	17	111	169	320	250	322	352	358	204	203	217	234	267	319	353	350	386	394
46825	Switzerland	Walensee	*Pinus nigra*	1	2	41	111	169	229	250	307	352	390	206	197	217	234	270	319	353	350	416	403
46820	Switzerland	Walensee	*Pinus nigra*	1	2	42	111	169	229	250	307	352	390	206	197	217	234	270	319	353	350	437	403
46824	Switzerland	Walensee	*Pinus nigra*	1	2	43	111	169	229	250	307	352	393	206	197	217	234	270	319	353	350	416	403
46821	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46822	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46823	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46826	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46827	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46829	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46830	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46831	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46832	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
47103	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
47104	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
47105	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
47106	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
48241	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
48242	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
48243	Switzerland	Walensee	*Pinus nigra*	2	1	59	111	169	289	250	307	352	387	206	197	217	234	270	319	353	350	401	403
46828	Switzerland	Walensee	*Pinus nigra*	2	1	64	111	169	289	250	307	352	390	206	197	217	234	270	319	353	350	401	403
UKH3_N1	Ukraine	Hola prystan	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
42940	Ukraine	Hola prystan	*Pinus pallasiana*	2	2	6	111	169	264	250	301	352	344	204	203	217	234	267	319	353	350	386	406
47078	Ukraine	Hola prystan	*Pinus pallasiana*	2	1	7	111	185	284	247	292	340	358	212	197	217	222	267	322	389	350	404	394
47077	Ukraine	Hola prystan	*Pinus pallasiana*	2	1	11	111	185	284	250	292	340	358	212	197	217	222	267	322	389	350	404	394
47079	Ukraine	Hola prystan	*Pinus pallasiana*	2	1	11	111	185	284	250	292	340	358	212	197	217	222	267	322	389	350	404	394
42938	Ukraine	Hola prystan	*Pinus pallasiana*	2	2	69	111	169	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42939	Ukraine	Hola prystan	*Pinus pallasiana*	2	2	81	111	185	295	256	322	352	396	204	203	217	234	270	319	359	350	389	400
42937	Ukraine	Hola prystan	*Pinus pallasiana*	2	1	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
42936	Ukraine	Hola prystan	*Pinus pallasiana*	2	2	83	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
42942	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	1	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400

Isolate number	Country	Location	Host	ITS HAP	Mating type	Assigned MLH [a]	Doth A	DP_4	DP_1	DP_10	DP_11	DP_5	DP_13	DP_2	DP_6	DP_18	DP_17	DP_15	DP_16	DP_9	DP_8	DP_12	DP_7
42944	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	1	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
23767	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
47076	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
50262	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
42948	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
42943	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42945	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42949	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42950	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42951	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42952	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42953	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
UKT_4_N6	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
UKT3_N4	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	5	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42955	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	1	2	8	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	403
UKT4_N4	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	9	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	403
47075	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	38	111	169	249	250	331	340	361	206	197	217	222	267	319	353	350	410	394
42947	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	38	111	169	249	250	331	340	361	206	197	217	222	267	319	353	350	410	394
23769	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	1	40	111	185	229	256	322	352	396	204	203	217	234	270	319	359	350	389	400
42954	Ukraine	Kherson, Tsjurupinsk	*Pinus pallasiana*	2	2	69	111	169	229	256	322	352	344	204	203	217	234	270	319	353	350	389	400
42941	Ukraine	Kindburg peninsula	*Pinus pallasiana*	2	2	2	111	169	229	250	301	352	396	204	203	217	234	267	319	353	350	386	400
UKK5_N1	Ukraine	Kindburg peninsula	*Pinus pallasiana*	1	1	52	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
47080	Ukraine	Mykolaiv kinburn	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
47083	Ukraine	Mykolaiv kinburn	*Pinus pallasiana*	2	1	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
47081	Ukraine	Mykolaiv kinburn	*Pinus pallasiana*	2	1	4	111	169	249	250	334	340	361	206	197	217	222	267	319	353	350	410	394
47082	Ukraine	Mykolaiv kinburn	*Pinus pallasiana*	2	1	14	111	185	229	256	322	352	344	204	209	217	234	270	319	359	350	389	400
48719	Ukraine	Mykolaiv kinburn	*Pinus pallasiana*	2	1	66	111	185	284	250	292	340	358	212	197	217	222	285	322	389	350	404	394
42946	Ukraine	Nova Zburivka	*Pinus pallasiana*	2	2	3	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400

[a]: Multilocus haplotypes that are shared between sites are colour coded. These colours also correlate with the colours in Figure 3.

APPENDIX 2

Sites, hosts, multilocus haplotypes and mating types of *Dothistroma septosporum* isolates collected in Cantabria

Appendix 2. Sites, hosts, multilocus haplotypes and mating types of *Dothistroma septosporum* isolates collected in Cantabria.

CMW	Sample File[a]	Site	GPS	Host	MLH[b]	Mating type[c]	Doth_A	DCB2	DS1	Doth_F	Doth_O	Doth_M	Doth_L	Doth_DS2	Doth_E	Doth_G	Doth_J	Doth_I	Doth_K
-	SP25.1.N2A	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	43	2	124	156	142	173	200	255	334	383	210	184	184	303	null
-	SP25.1.N7	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
-	SP25.2.N10	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N2S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N3S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N4	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N7S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N7S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N7S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	39	1	124	156	142	173	200	241		383	258	186	184	305	null
-	SP25.2.N8	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.2.N9	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1 (feint band)	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.3.N1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	42	1	124	156	142	173	200	241	321	383	270	186	184	305	null
-	SP25.3.N7	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
-	SP25.4.N7	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
-	SP25.4.N8	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	21	2	124	156	150	173	200	228	334	383	210	182	188	303	363
-	SP25.5.N4S1A	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.11.N2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
-	SP25.13.N1S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	15	1	124	156	161	173	200	221	321	383	210	186	184	303	361
49607	SP25.13.N1S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	15	1	124	156	161	173	200	221	321	383	210	186	184	303	361
-	SP25.13.N3S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	37	1	124	156	142	173	200	241	321	383	258	186	188	305	null
49608	SP25.13.N3S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP25.13.N3S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	16	1	124	156	142	173	200	241	321	383	210	184	184	303	null
49385	SP25.15.N2S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	38	1	124	156	142	173	200	241	321	383	258		184	305	null
49649	SP25.15.N4S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
49386	SP25.15.N4S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49650	SP25.15.N5S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49651	SP25.15.N5S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	41	1	124	156	142	173	200	241	321	383	258		184	305	363
-	SP25.16.N1S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	15	1	124	156	161	173	200	221	321	383	210	186	184	303	361
-	SP25.16.N1S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	15	1	124	156	161	173	200	221	321	383	210	186	184	303	361
49387	SP25.16.N1S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
49609	SP25.16.N2S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	46	2	124	156	150	173	200	228	317	383	210	182	188	303	363
49652	SP25.16.N2S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	21	2	124	156	150	173	200	228	334	383	210	182	188	303	363
49389	SP25.16.N3S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
49390	SP25.16.N3S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49391	SP25.16.N4S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
49392	SP25.16.N4S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	47	1		156	161	173	200	248	404	387	252	184	184	303	357
49393	SP25.18.N1S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49653	SP25.18.N2S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	49	1	124	156	161	173	200	255	404	383	210	186	184	305	357
49654	SP25.18.N2S4	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	36	1	124	156	142	173	200	241	321	383	258	186	184	305	363
49655	SP25.18.N3S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	32	1	124	156	142	173	200	241	321	383	258	186	184	305	361
49394	SP25.18.N3S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49395	SP25.19.N4S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49396	SP25.19.N4S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	19	1	124	156	142	173	200	241	321	383	252	186	184	305	null
49950	SP25.19.N5S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	41	1	124	156	142	173	200	241	321	383	258		184	305	363
49656	SP25.19.N5S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	45	1	124	156	142	173	200	241	321	383	210	186	184	303	null
49951	SP25.19.N6	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null

CMW	Sample File[a]	Site	GPS	Host	MLH[b]	Mating type[c]	Doth_A	DCB2	DS1	Doth_F	Doth_O	Doth_M	Doth_L	Doth_DS2	Doth_E	Doth_G	Doth_J	Doth_I	Doth_K
-	SP25.20.N2S1	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	15	1	124	156	161	173	200	221	321	383	210	186	184	303	361
49397	SP25.20.N2S2	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	17	1	124	156	142	173	200	241	321	383	210		184	303	null
49398	SP25.20.N2S3	Site 25	43.177788N, 3.818498W	*Pinus nigra* subsp. *laricio* var. *corsicana*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
49954	SP291.1.N1S1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.1.N3S2	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.1.N5S1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.1.N6	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1 and 2	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.1.N7	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	33		124	156	142	173	200	241	334	383	258	182	195	305	null
-	SP291.2.N1A	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	27	1	124	156	142	173	200	248	334	383	258	184	195	303	363
-	SP291.4.N1B	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.4.N2	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.4.N4	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	10	2 (feint band)	124	156	158	173	200	248	334	383	210	182	184	303	null
-	SP291.5.N1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	10	1	124	156	158	173	200	248	334	383	210	182	184	303	null
-	SP291.6.N7S1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	12	1	124	156	158	173	200	248	321	383	210	186	184	303	null
-	SP291.7.N6S1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	51	1	124	156		173	200	248	321	383	210	182	188	303	null
-	SP291.11.N4	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	18	2	124	156	150	173	200	248	334	383	210	182	184	303	357
-	SP291.14.N5S1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	23	1 (feint band)	124	156	158	173	200	248	321	383	210	186	184	303	
-	SP291.14.N5S3	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	33	2	124	156	142	173	200	241	334	383	258	182	195	305	null
50354	SP291.16.N1	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	13	2	124	156	150	173	200	241	334	383	210	186	188	305	null
50355	SP291.16.N2	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	10	1	124	156	158	173	200	248	334	383	210	182	184	303	null
-	SP291.17.N2	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	35	2	124	156	142	173	200	241	334	383	210	182	188	305	null
50357	SP291.19.N2	Site 29.1	43.095454N, 3.990897W	*Pinus nigra* subsp. *nigra*	35	2	124	156	142	173	200	241	334	383	210	182	188	305	null
-	SP292.1.N2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	50	1	124	156	158	173	200	248		383	210	186	184	303	null
-	SP292.2.N4S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	28	2	124	156	152	173	200	248	334	383	210	182	184	305	357
-	SP292.2.N5	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	52	1	124	156		173	200	248	321	383	210	186	184	303	null
-	SP292.3.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	2	2	124	156	142	173	200	241	334	383	258	182	195	303	363
-	SP292.3.N4S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
-	SP292.4.N5S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	27	1	124	156	142	173	200	248	334	383	258	184	195	303	363
-	SP292.7.N5	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	3	2	124	156	142	173	200	241		383	258	182	195	303	363
-	SP292.8.N2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
-	SP292.9.N5	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
50547	SP292.10.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	7	2	124	156	142	173	200	248	334	387	210	184	184	305	357
-	SP292.10.N1S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	7	2	124	156	142	173	200	248	334	387	210	184	184	305	357
-	SP292.10.N3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	31	2	124	156	154	173	200	235	334	383	210	182	188	305	363
-	SP292.10.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	31	2	124	156	154	173	200	235	334	383	210	182	188	305	363
50548	SP292.10.N4S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	21	2	124	156	150	173	200	228	334	383	210	182	188	303	363
50549	SP292.11.N1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
50550	SP292.11.N2S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	48	2	124	156	154	173	200	235	334	381	210	182	188	305	363
-	SP292.11.N2S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	25	2	124	156	152	173	200	235	334	383	210	182	188	303	363
-	SP292.11.N3S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	22	2	124	156	152	173	200	214	334	383	210	184	188	303	363
-	SP292.11.N3S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	8	2	124	156	142	173	200	248	334	387	210	182	184	305	357
50551	SP292.12.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	24	2	124	156	150	173	200	228	334	381	210	182	188	303	363
50552	SP292.13.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	24	2	124	156	150	173	200	228	334	381	210	182	188	303	363
50553	SP292.13.N1S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
-	SP292.13.N2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	14	2	124	156	150	173	200	241	334	383	258	182	188	305	null
50554	SP292.13.N4	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	26	2	124	156	152	173	200	228	334	383	210	182	188	303	363
-	SP292.14.N4	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	40	1	124	156	142	173	200	241	321	383	258	186	184	305	null
-	SP292.15.N1S2A	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	11	2	124	156	142	173	200	248	334	383	210	182	188	303	359

CMW	Sample File[a]	Site	GPS	Host	MLH[b]	Mating type[c]	Doth_A	DCB2	DS1	Doth_F	Doth_O	Doth_M	Doth_L	Doth_DS2	Doth_E	Doth_G	Doth_J	Doth_I	Doth_K
49595	SP292.15.N1S2B	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	11	2	124	156	142	173	200	248	334	383	210	182	188	303	359
-	SP292.15.N3S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	7	2	124	156	142	173	200	248	334	387	210	184	184	305	357
-	SP292.15.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
49596	SP292.16.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	21	2	124	156	150	173	200	228	334	383	210	182	188	303	363
-	SP292.16.N1S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	26	2	124	156	152	173	200	228	334	383	210	182	188	303	363
-	SP292.16.N1S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
-	SP292.16.N3S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	21	2	124	156	150	173	200	228	334	383	210	182	188	303	363
49382	SP292.16.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	33	2	124	156	142	173	200	241	334	383	258	182	195	305	null
49597	SP292.16.N4S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	2	124	156	152	173	200	214	334	383	210	182	188	303	363
49383	SP292.17.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	2	2	124	156	142	173	200	241	334	383	258	182	195	303	363
49598	SP292.17.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	34	2	124	156	158	173	200	248	321	383	210	186	188	303	null
-	SP292.17.N4S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	34	2	124	156	158	173	200	248	321	383	210	186	188	303	null
49599	SP292.17.N4S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	29		124	156	150	173		228	334	383	210	182	188	303	363
-	SP292.18.N2S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	26	2	124	156	152	173	200	228	334	383	210	182	188	303	363
49600	SP292.18.N2S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363
49601	SP292.18.N3S2A	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	29	2	124	156	150	173		228	334	383	210	182	188	303	363
-	SP292.18.N4S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	21	2 (feint band)	124	156	150	173	200	228	334	383	210	182	188	303	363
49602	SP292.19.N2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	1	1	124	156	142	173	200	241	321	383	210	182	184	305	363
-	SP292.19.N5S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	20	2	124	156	142	173	200	248		383	210	182	184	305	357
-	SP292.19.N5S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	20	2	124	156	142	173	200	248		383	210	182	184	305	357
49603	SP292.20.N1S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	6	2	124	156	142	173	200	248	334	383	252	182	184	303	359
49604	SP292.20.N1S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	5	2	124	156	142	173	200	248	334	383	210	182	184	305	357
49605	SP292.20.N1S3	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	30	2	124	156	152	173		214	334	383	210	182	188	303	363
49384	SP292.20.N2S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	9	2	124	156	142	173		241	334	383	258	182	195	303	363
49648	SP292.20.N3S1	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	44	1	124	156	152	173		228	334	383	210	182	188	303	363
49606	SP292.20.N3S2	Site 29.2	43.095013N, 3.995237W	*Pinus nigra* subsp. *salzmannii*	4	1	124	156	152	173	200	214	334	383	210	182	188	303	363

[a]: Isolates are numbered according to the site number (SP25 = Site 25, SP291 = Site 29.1, SP292 = Site 29.2) followed by the tree number. "N" indicates a needle that was selected for isolation for the tree sample and if isolations were made from more than one conidiomata on the needle, this is indicated by "S".

[b]: MLHs that are shared between sites are colour coded. These colours also correlate with the colours in Figure 2.

[c]: Individuals that share the same MLH but have different mating types are indicated in Red. All missing data is indicated in yellow.