Eesti Maaülikool
Estonian University of Life Sciences

GENETIC DIVERSITY, ORIGIN, AND NEW HOSTS OF THE INVASIVE PATHOGEN *LECANOSTICTA ACICOLA* IN NORTHERN EUROPE

INVASIIVSE PATOGEENI *LECANOSTICTA ACICOLA* GENEETILINE MITMEKESISUS, PÄRITOLU JA UUED PEREMEESTAIMED PÕHJA-EUROOPAS

MARILI VESTER

A Thesis
for applying for the degree of Doctor of Philosophy
in Forestry

Väitekiri
filosoofiadoktori kraadi taotlemiseks
metsanduse erialal

Tartu 2022

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Doctoral Theses of the Estonian University of Life Sciences

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According to verdict No 6-14/21-6 of November 11, 2022, the Defence Board of PhD thesis in Forestry of the Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor of Philosophy in Forestry.

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Defence of the thesis:
Estonian University of Life Sciences, room 2A1, Kreutzwaldi 5, Tartu, on January 10th, 2023, at 10:00.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following four original publications (**I-IV**). The papers are referred to in the text by their Roman numerals.

I Adamson, K., **Laas, M.,** Drenkhan, R., Hanso, M. 2018. Quarantine pathogen *Lecanosticta acicola*, observed at its jump from an exotic host to the native Scots pine in Estonia. Baltic Forestry. 24 (1), 36−41.

II **Laas, M.,** Adamson, K., Drenkhan, R. 2019. A look into the genetic diversity of *Lecanosticta acicola* in northern Europe. Fungal Biology. 123 (10), 773−782.

III Oskay, F., **Laas, M.,** Mullett, M., Lehtijärvi, A., Doğmuş-Lehtijärvi, H. T., Woodward, S., Drenkhan, R. 2020. First report of *Lecanosticta acicola* on pine and non-pine hosts in Turkey. Forest Pathology. 50 (6), e12654.

IV **Laas, M.,** Adamson, K., Adamčíková, K., Akiba, M., Barnes, I., Beenken, L., Braganca, H., Bulgakov, T., Capretti, P., Cech, T., Cleary, M., Enderle, R., Gheraldini, L., Jankovský, L., Janoušek, J., Markovskaja, S., Matsiakh, I., Meyer, J., Mullett, M., Oskay, F., Piškur, B., Raitelaitytė, K., Sadiković, D., Drenkhan, R. 2022. Diversity, migration routes and worldwide population genetic structure of *Lecanosticta acicola*, the causal agent of Brown spot needle blight. Molecular Plant Pathology. 23 (11), 1620-1639.

The contribution of the authors to the papers were as follows:

Paper	Original idea and study design	Data collection	Data analysis	Manuscript preparation
I	KA, MH, RD	KA, **MV**	KA, **MV**	KA, MH, **MV**, RD
II	KA, **MV**, RD	KA, **MV**, RD	KA, **MV**	KA, **MV**, RD
III	FO, RD	FO, **MV**	FO, **MV**	AL, FO, HTDL, MM, **MV**, SW
IV	IB, MM, **MV**, RD, TC	All	KA, KR, **MV**	IB, JJ, KA, MC, MM, **MV**, RD

All – all authors of the paper, AL – Asko Lehtijärvi, FO – Funda Oskay, IB – Irene Barnes, HTDL – Hatice Tuğba Doğmuş-Lehtijärvi, JJ – Josef Janoušek, KA – Kalev Adamson, KR – Kristina Raitelaitytė, MC – Michelle Cleary, MH – Märt Hanso, MM – Martin Mullett, **MV – Marili Vester**, RD– Rein Drenkhan, SW – Steve Woodward, TC – Thomas Cech.

ABBREVIATIONS

ABC	Approximate Bayesian computation
AMOVA	analysis of molecular variance
A_R	allelic richness
BSNB	Brown Spot Needle Blight
cc	clone-corrected dataset
h	mean haploid genetic diversity
I_A	index of association
ITS	the internal transcribed spacer
K	number of population clusters
MLH	multilocus haplotype
N	sample size
Na	mean number of different alleles
na	not analysed
non-cc	non-clone-corrected dataset
PA_R	private allelic richness
PCR	polymerase chain reaction
$\bar{r}_d$	standardized index of association
SE	standard error
SSPP	species-specific priming PCR
TBA	Tallinn Botanic Garden
uh	mean unbiased diversity

1. INTRODUCTION

During the last century there has been a rise in the rate of new disease reports from European forests, largely due to global plant trade, climate change, and a failure to enforce proper quarantine measures (Hanso and Drenkhan, 2013; Santini et al., 2013; Drenkhan et al., 2014, 2020; Ramsfield et al., 2016). In the forests of the Northern Hemisphere, the genus *Pinus* is one of the most widely distributed genera and for European forestry, pine stands are of great value, representing the majority of growing stock and having high ecological and economical importance (Richardson and Rundel, 2000), explaining the high interest in pine diseases in northern Europe. Pines in Europe suffer from several diseases, the causal agents of which, such as *Dothistroma septosporum*, *D. pini*, *Diplodia sapinea*, *Fusarium circinatum* and *Lecanosticta acicola* (analysed here) have received a lot of attention during recent decades as emerging pests which cause premature needle loss or shoot dieback (Drenkhan and Hanso, 2009; Drenkhan et al., 2020; Adamson et al., 2021; Mullett et al., 2021).

This current thesis focuses on *L. acicola*, an ascomycete causing Brown Spot Needle Blight (BSNB) – a foliar disease affecting pine species causing premature needle shedding. Since the last century the global range of this pathogen, originating from America, has increased significantly and over the last 15 years the disease agent has become an essential emerging invasive pathogen in Europe with an increased number of outbreaks and new country records coming from distant regions (van der Nest et al., 2019a).

The long-time monitoring of forest diseases in Estonia has followed the distribution changes of several pathogens, including *L. acicola* – since the first record of it for northern Europe in Estonia (Drenkhan and Hanso, 2009; Adamson et al., 2015). Now, *L. acicola* is present in all the Baltic states and southern Sweden where all first records have originated from non-native pine species (Drenkhan and Hanso, 2009; Markovskaja et al., 2011; EPPO, 2012a; Mullett et al., 2018; Cleary et al., 2019). In northern Europe there is only one native pine species - Scots pine (*Pinus sylvestris* L.), and naturally, BSNB has been considered as a potential threat to the northern European pine stands.

The results of population analyses suggest that there have been several separate introduction events of the pathogen into central and southern Europe from genetically distant populations in America (Janoušek et al., 2016; Sadiković et al., 2019) and the pathogen presumably reached Europe and spread within the region largely due to anthropogenic activity, i.e. on infected plant material. The populations in northern Europe are considered to be recently established and thereby represent new regions for the pathogen, compared to those in central and southern parts of Europe. Consequently, it has been unclear if the pathogen has reached northern Europe due to the natural gradual range expansion from central Europe or the outbreaks originate from an independent introduction event from established populations in Europe or even directly from North America.

Additionally, there is not much information about the genetic diversity and reproductive mode of *L. acicola* in the new recently colonized areas, which would be important to assess the risk of developing new and potentially more virulent pathogen genotypes and to establish control strategies.

With invasive pathogens it is unsure what will be the extent of damage. *Lecanosticta acicola*, and overall, the genus *Lecanosticta*, has been predominantly considered a pathogen of pine species (van der Nest et al., 2019a). However, the very similar pathogen *Dothistroma septosporum* is known to infect not only *Pinus* spp. but also several species of non-*Pinus* hosts (e.g., *Cedrus*, *Abies*, *Larix*, *Picea*, *Pseudotsuga*) (Drenkhan et al., 2016). Therefore, it cannot be ignored that under favourable conditions *L. acicola* may also infect other species in the Pinaceae family.

This thesis is a synthesis of four research papers, which discuss distribution, new host records, population diversity and migration history of the invasive needle pathogen *L. acicola* with a focus on northern Europe. In this thesis northern Europe is considered to include populations of the Baltic states, northern Poland and Sweden. Two of the papers (**I** and **III**) study the threat to host species by the pathogen. Papers **II** and **IV** study population genetics and migration pathways of *L. acicola*.

2. REVIEW OF THE LITERATURE

2.1. *Lecanosticta acicola*, the causal agent of Brown Spot Needle Blight

Lecanosticta acicola (Thümen) A. Sydow is the first described and most well-known species in the genus *Lecanosticta* which contains, altogether, nine species (van der Nest et al., 2019b). Needles infected with *L. acicola* develop yellowish lesions which develop into darker brown spots with a lighter yellow border. Eventually infected needles turn brown and drop prematurely (Tainter and Baker, 1996). BSNB is a chronic disease – repeated infection appears from older needle classes to younger ones, which can lead to severe defoliation over several years, often resulting in the tree having only the current year's needles (Tainter and Baker, 1996). Severe needle loss leads to reduced growth and possible death of the tree (Cordell et al., 1990; Wyka et al., 2017).

Initial disease symptoms are easy to miss or to confuse with some other needle diseases, e.g., Dothistroma Needle Blight or other species of *Lecanosticta* (van der Nest et al., 2019a). Moreover, due to the latent phase visual observation of the foliage is not an effective method to detect the disease and molecular methods should be preferred (Aglietti et al., 2021).

Although in a number of countries *L. acicola* is still listed as a quarantine pathogen (EPPO, 2022), in the European Union the species is treated as a Regulated Non-Quarantine Pest, meaning that it is not mandatory to eradicate infected trees, but it is not allowed to sell infected or symptomatic *Pinus* plants for ornamental purposes or as forest reproductive material (European Commission, 2019).

2.2. Global distribution of *Lecanosticta acicola* and host range

Lecanosticta acicola was first described in the state of South Carolina, USA in 1876 (de Thümen, 1878) and since then there have been an abundant number of publications describing damage from the pathogen from all over the USA (van der Nest et al., 2019a). In North America, the disease is also present in south-eastern Canada and Mexico (van der Nest et al.,

2019a), while in South America there are reports only from plantations in Colombia (Gibson, 1980; Evans, 1984).

The first report of *L. acicola* in Europe dates to 1942 when the pathogen was described in Spain (Martínez, 1942). In 1975 the disease agent was found in Croatia (Milatović, 1976) and subsequently, in the 1990s in other southern and even central parts of Europe (Chandelier et al., 1994; Lévy and Lafaurie, 1994; Pehl, 1995; Holdenrieder and Sieber, 1995; Cech, 1997; La Porta and Capretti, 2000). The pathogen is also present in East Asia (Li et al., 1986; Suto and Ougi, 1998; Seo et al., 2012). Despite extensive *Pinus* plantations, *L. acicola* has not been reported in Australasia nor in Africa.

The pathogen has been most problematic in *Pinus* plantations where in some cases clear cuts have been done due to the extensive damage (Huang et al., 1995; Lévy, 1996; Markovskaja et al., 2011). High mortality has been reported in forest nurseries and young plantations in southern regions of the USA, especially on *P. palustris* (Siggers, 1944; Cordell et al., 1990). In the northern states of the USA, *L. acicola* has caused damage in Christmas tree plantations where the trees affected with defoliation and discoloured needles have become unmarketable (Phelps et al., 1978). Plantations of non-native pines in China (Huang et al., 1995) and Colombia have also suffered from the disease (Gibson, 1980; Evans, 1984). In Europe, *L. acicola* has caused the most damage in south-western Europe where, during recent years, the disease intensity seems to be rising and the pathogen is currently of considerable concern in plantations of *P. radiata* and *P. nigra* (Ortíz de Urbina et al., 2017; Mesanza et al., 2021c).

During the current century the distribution of *L. acicola* has grown significantly. The pathogen has been reported from distant regions of Europe, and both in Europe and North America a gradual northward spread has been witnessed (van der Nest et al., 2019a). In northern Europe, the first report of *L. acicola* was in 2008, from *P. ponderosa* in the Tallinn Botanic Garden (TBA), Estonia (Drenkhan and Hanso, 2009). Thereafter, similarly in Latvia presence of the disease was confirmed in the botanical garden of Salaspils and in Alnarp arboretum in Sweden (EPPO, 2012a; Mullett et al., 2018; Cleary et al., 2019) where, so far, those have remained the only reported findings of the pathogen. To date, there are no records from Finland or Norway. In Lithuania the first

report originates from the Curonian Spit region, where large areas are planted with *P. mugo* to prevent erosion of sandy soils (Markovskaja et al., 2011). In 2017 the disease was found to be present in a similar coastal habitat in northern Poland (Raitelaitytė et al., 2020). In eastern Europe new reports originate from Belarus, Bulgaria and Romania (EPPO, 2018; Stamenova et al., 2018; Georgieva, 2020; Golovchenko et al., 2020) and the pathogen has reached the British Isles where it has been currently detected only from Ireland (Mullett et al., 2018).

All species of pines (*Pinus* spp.) are potential hosts for *L. acicola*. In total 53 taxa are already listed as susceptible, though the level of susceptibility differs greatly between species (van der Nest et al., 2019a). Additionally, in the case of *P. sylvestris*, susceptibility seems to vary depending on whether it grows within or out of its native range, e.g., *P. sylvestris* is damaged more seriously in North America than in its native range in Europe (Skilling and Nicholls, 1974; Cech and Krehan, 2008; Jurc and Jurc, 2010; EPPO, 2015). At the same time, *P. mugo* and its subspecies have proven to be especially susceptible to the pathogen with high disease severity in its native range in the mountain valleys of central and southern Europe (Holdenrieder and Sieber, 1995; Jankovský et al., 2009; Jurc and Jurc, 2010; EPPO, 2015; Adamčíková et al., 2021) and also in regions where it is planted as a non-native species (Markovskaja et al., 2011; Adamson et al., 2015; Raitelaitytė et al., 2020).

2.3. Use of genetic analyses

For population genetic analyses eleven microsatellite markers and mating type primers have been developed (Janoušek et al., 2014), and the full genome sequence of the species is available on GenBank (NCBI).

During recent years, the populations of *L. acicola* have been investigated on a global scale and locally in country-specific studies (Janoušek et al., 2016; Sadiković et al., 2019; Adamčíková et al., 2021). These studies have revealed that the pathogen is of North American origin, and based on the Translation Elongation 1-α gene region (TEF1), microsatellite and RAPD markers, together with observations of cultural morphology it comprises three lineages which are present in different regions (Huang et al., 1995; Janoušek et al., 2016; van der Nest et al., 2019a). The "northern lineage" occurs in northern USA and Canada, the "southern lineage" in the southern USA and the third lineage is present only in Mexico.

The southern and northern lineages have been introduced into Europe, possibly on several separate occasions. The southern lineage spreads in south-western Europe. The northern lineage appears to dominate in central and southern Europe, and probably also in northern Europe (Huang et al., 1995; Janoušek et al., 2016; van der Nest et al., 2019a).

The populations in the Mississippi region of USA and in Mexico have shown the highest diversity (Janoušek et al., 2016) while the populations in northern USA, Canada and Europe have lower genetic diversity (Janoušek et al., 2016; Sadiković et al., 2019; Adamčíková et al., 2021). Due to the pathogens' rapid expansion during recent years, the previous population studies have not covered new areas where the disease is present now e.g., northern Europe and western Asia.

2.4. Reproduction mode and dispersal

Lecanosticta acicola is a heterothallic ascomycete fungus (Janoušek et al., 2014). It holds two mating type idiomorphs – *MAT1-1-1* and *MAT1-2* that both need to be present for sexual reproduction to occur. The study by Janoušek et al. (2016) indicated that both mating types of the pathogen are present in Europe, and *L. acicola* probably reproduces sexually in Austria and Germany. The sexual state of the pathogen has been rarely reported and found only from the south-eastern states of USA, Colombia, and most recently from Spain (van der Nest et al., 2019a; Mesanza et al., 2021b), proving that the pathogen indeed reproduces sexually in Europe as well, increasing the danger of developing new genetic lines with unpredictable virulence (McDonald and Linde, 2002).

Lecanosticta acicola needs favourable light, temperature and humidity conditions to develop fruiting bodies, for sporulation and spore germination (Kais, 1975; Tainter and Baker, 1996; Mesanza et al., 2021a). When reproducing asexually the fungus spreads via conidia that are dispersed over short distances by rain splash and dew (Siggers, 1939; Skilling and Nicholls, 1974). The conidia are rarely dispersed more that 1.5 m away from the source (Skilling and Nicholls, 1974), although Wyka et al. (2018) found that small number of conidia could be naturally disseminated even 60 m from the infected tree. The ascospores, produced during the sexual state, are airborne and capable of long-distance dispersal (Kais, 1971).

3. AIMS OF THE STUDY

The hypotheses of the study were:

1. Native *Pinus sylvestris* is susceptible to *L. acicola* in northern Europe (**I**).
2. The genetic diversity of *L. acicola* is similar on different hosts within a single stand (**II**).
3. *Lecanosticta acicola* has been spread into northern Europe by several separate introduction events from already established populations in central Europe (**II**, **IV**).
4. Anthropogenic activity has influenced the spread and formation of population diversity of *L. acicola* in northern Europe (**II**, **IV**).
5. *Lecanosticta acicola* is not a *Pinus* specific pathogen (**III**).

The specific aims of this study were:

1. to study the host range of *L. acicola* and its potential threat to the host species (**I**, **III**);
2. to study the genetic diversity of *L. acicola* within a single stand on different host species (**II**);
3. to study the distribution and possible introduction source(s) of *L. acicola* in northern Europe (**II**, **IV**);
4. to describe the genetic diversity, population structure and reproductive mode of *L. acicola* (**II**, **IV**).

4. MATERIAL AND METHODS

4.1. Study sites and sample collection

During October 2016 needle samples were collected from a group of mixed *P. mugo* and *P. sylvestris* trees in Kõrveküla village, Tartu County, Estonia (N 58.432259, E 26.787881). In total, samples were obtained from 28 *P. mugo* and 43 *P. sylvestris* symptomatic trees at the site (**I**).

During the period of 2010-2017, samples were collected from visibly symptomatic pines across Estonia. In total, six pine taxa were sampled: *P. mugo*, *P. mugo* var. *pumilio*, *P. sylvestris*, *P.* x *rhaetica*, *P. ponderosa* and *P. uncinata*. Samples obtained from the mixed stand of *P. mugo* and *P. sylvestris* observed in Paper **I** were also included in Paper **II**.

Needle samples were collected from a selection of Pinaceae taxa displaying symptoms of BSNB in the Atatürk Arboretum, Istanbul, Turkey (N 41.175010, E 28.984569). In total 37 trees from 28 taxa were sampled during 2017 and 2018 (**III**).

For Paper **IV** needle samples with typical symptoms of BSNB were collected from a variety of *Pinus* taxa and *Cedrus libani*. Samples were obtained from one or several locations per country from a total of 27 countries in North and South America, Europe and Asia. Samples obtained in Paper **I**, Paper **II** and Paper **III** were also included in Paper **IV**.

4.2. Disease severity assessment

In the mixed stand of *P. mugo* and *P. sylvestris* in Kõrveküla, Estonia, for every tree the infection level and year class of symptomatic needles was assessed as shown in Bulman et al. (2004). From the present crown volume, the proportion of diseased needles was assessed in 5% increments. For all trees the distance from the closest symptomatic tree was measured (**I**).

In Atatürk Arboretum the disease assessment was done in July 2017 and 2018 according to Bulman et al. (2004) in 10% increments (**III**).

4.3. Fungal isolation

Lecanosticta acicola was isolated into pure culture from conidiomata obtained from the symptomatic needles. Isolations were made according to Mullett and Barnes (2012). After rinsing the needles with 96% ethanol, well-developed conidiomata were placed on pine needle agar medium plate (prepared as described in Paper **IV**) and rolled along its surface for separation of conidia. In 7-14 days, germinated single conidia with some mycelia were transferred to a fresh plate. Isolates were grown in the dark at room temperature (21°C). After incubation for three to four weeks, small amount of mycelium from the colony edge was transferred to a 2.0 ml microcentrifuge tube and stored at -20°C until DNA extraction.

4.4. Molecular techniques

4.4.1. DNA extraction and isolate identification

Mycelium was homogenized with a Retsch MM400 homogenizer (Retsch GmbH, Haan, Germany) using metal beads (Ø 2.5 mm). DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) and all details are specified in Paper **IV**. DNA was stored at -20°C until further analyses.

The species of the isolate was confirmed by sequencing of the internal transcribed spacer (ITS) region. ITS-PCR was performed using the fungal-specific PCR primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3'; Gardes and Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; White et al., 1990) as described in Paper **I**. The PCR products were visualized on a 1% agarose gel (SeaKem® LE Agarose, Lonza) under UV light using a Quantum ST4-system (Vilber Lourmat SAS, Marne-la-Vallée, France). The PCR products were sequenced at the Estonian Biocentre in Tartu using the primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White et al., 1990). The sequences were edited using BioEdit 7.2.5. (Hall, 1999) and BLAST searches for the fungal taxa were performed in GenBank (NCBI).

Species-specific priming PCR (SSPP) was performed using *L. acicola* species-specific primers LAtef-F and LAtef-R (Ioos et al., 2010). The conventional PCR reaction was carried out in 20 µl reaction volumes

and cycling conditions as suggested by Ioos et al. (2010). A strain of *L. acicola* (GenBank accession number KJ004507) was used as a reference for SSPP.

4.4.2. Mating type determination

Mating types of the *L. acicola* isolates were determined using two pairs of mating type primers: MdMAT1-1F, MdMAT1-1R and MdMAT1-2F, MdMAT1-2R (Janoušek et al., 2014). PCR reactions were carried out in 20 µl volumes according to Janoušek et al. (2014), with the initial denaturation step at 95°C increased to 12 minutes as described by Adamson et al. (2015).

4.4.3. Haplotype identification

For multilocus haplotyping of *L. acicola* 11 microsatellite marker pairs were used: MD1, MD2, MD4, MD5, MD6, MD7, MD8, MD9, MD10, MD11 and MD12 (Janoušek et al., 2014). The PCR reactions were carried out in 20 µl volumes and amplification conditions were as described in Janoušek et al. (2014, 2016).

For fragment analysis, PCR products were pooled into two panels according to Janoušek et al. (2014) and run on an Applied Biosystems 3130XL genetic analyzer (Applied Biosystems, Carlsbad, USA) with LIZ 500 size standard (Applied Biosystems) at the Estonian Biocentre in Tartu. Alleles were scored using GeneMapper 5.0 (Applied Biosystems).

4.5. Population genetic data analyses

4.5.1. Formation of populations

Isolates of *L. acicola* were divided into three geographical populations according to the location of sampling sites: Tallinn (TLL), central Estonia (CE) and Tartu (TRT) (see Figure 1). Isolates obtained from the mixed stand of *P. mugo* and *P. sylvestris* were considered as two separate subpopulations of the TRT population, indicated as PMUG and PSYL, respectively, with the aim to test the genetic differentiation of *L. acicola* in the same stand on different host species (**II**).

In Paper **IV**, sampling sites in the same country were merged and referred to as populations, except for the USA where samples were divided into two populations – north-eastern USA (N-USA) and south-eastern USA (S-USA).

4.5.2. Genetic diversity

Population genetic data analyses were used in Paper **II** and Paper **IV**. Isolates with identical alleles at all observed microsatellite loci were considered clones. Two datasets were created: one containing all isolates (non-clone-corrected; non-cc) and the other containing only one of each haplotype per population (clone-corrected; cc).

The non-cc dataset was used to calculate the total number of haplotypes using GenAlEx 6.5 (Peakall and Smouse, 2012). The cc dataset was used to calculate the total number of alleles and unique alleles, mean number of different alleles (Na), mean haploid genetic diversity (h), and mean unbiased diversity (uh) for each population, using GenAlEx 6.5. The cc dataset was used to calculate the allelic richness (A_R, number of distinct alleles in the population) and private allelic richness (PA_R, number of unique alleles in the population) in ADZE 1.0 (Szpiech et al., 2008). Since sample sizes across populations differed, a rarefaction approach was used to standardize population sizes (Szpiech et al., 2008). The clonal fraction was calculated according to Zhan et al. (2003).

4.5.3. Isolation by distance

Mantel tests, conducted in GenAlEx 6.5, were used to test for isolation by distance on the cc dataset using Nei's genetic distance (Nei, 1972, 1978) and geographic distances (**IV**). In total, three different analyses were performed for separate sampling regions. First, isolation by distance was tested among all populations. Next isolation by distance was tested separately for populations in Europe, and then for populations in North America, in order to assess if genetic distance between populations increases with geographical distance.

An analysis of molecular variance (AMOVA) was performed in GenAlEx 6.5 on the cc dataset, to test for significant differentiation between populations (**II**, **IV**).

For visualization of Nei's genetic distances and geographic distances, Principal Coordinates Analysis (PCoA) was carried out in GenAlEx 6.5 using the covariance standardized method (**II**, **IV**).

4.5.4. Population clustering

The program STRUCTURE v. 2.3.4. (Falush et al., 2003) was used to estimate the most likely number of population clusters (K). For STRUCTURE analysis the cc dataset was used. Each of 20 independent runs of K=1-9 (**II**) and K=1-25 (**IV**) were carried out with 10,000 burn-in iterations followed by a run of 100,000. The most likely number of clusters (K) was determined using the ln(Pr(X | K)) method (Pritchard et al., 2000, 2010) (**II**, **IV**) and delta K statistic (Δ K) (Evanno et al., 2005) (**IV**) in CLUMPAK (Kopelman et al., 2015). The final assignment of individuals to clusters was carried out on the optimum K by applying 100,000 burn-in iterations, followed by 1,000,000 runs. For each number of clusters, 20 independent runs were performed (**II**, **IV**).

4.5.5. Phylogenetic analysis

POPTREE v. 2 (Takezaki et al., 2010) was used to assess the phylogenetic relationships among the populations. Neighbor joining method (Saitou and Nei, 1987) was used based on allele frequency of a cc dataset of all 28 populations. To generate confidence at branch points, a bootstrap test with 10,000 replications was run (**IV**).

4.5.6. Modelling of demographic history

To reconstruct the history of divergence among the observed *L. acicola* populations, Approximate Bayesian computation (ABC) was performed on the cc dataset using DIYABC v. 2.1.0 (Cornuet et al., 2014). The STRUCTURE clusters were considered in order to develop scenarios describing the demographic history between the main regions: north-eastern America (N-AME), south-eastern USA (S-USA), northern Europe (N-EUR), central Europe (C-EUR), south-western Europe (SW-EUR), and a merged region of Croatia (HRV) and western Asia (W-ASIA) (see Figure 3). For modelling of demographic history populations of Belarus and Ireland were also merged with northern Europe due to the similarities in STRUCTURE clustering. A stepwise

procedure was used to address questions around historical scenarios (Konečný et al., 2013) (**IV**).

In total, three different analyses with various scenarios were performed. In Analysis 1 the relationship among the three main clusters in America and Europe was investigated, Analysis 2 elucidated the origin of northern European populations and Analysis 3 investigated the origin of the cluster dominant in Croatia and western Asia and the origin of the central European populations (**IV**). A detailed description of the scenarios tested is given in Paper **IV**.

Initially, the demographic priors of the tested scenarios were set with a broad range. After 100,000 preliminary runs, the prior checking option was used according to the DIYABC manual and prior distributions adjusted step-by-step. The generalized stepwise model was followed for the microsatellite loci and the default values for the mutation model parameters were used (Cornuet et al., 2014). Ten microsatellite markers (MD1, MD2, MD4, MD5, MD7, MD8, MD9, MD10, MD11, and MD12) were used in the ABC analyses (**IV**).

For each simulation the commonly used genetic summary statistics were used (i.e., mean number of alleles for one sample and between two samples, mean size variance, mean genetic diversity between two samples, F_{st} between two samples, mean index of classification between two samples, and $(d\mu)^2$ distance between two samples). The obtained times of events are in generations. Other statistics available in DIYABC were later used in model checking. One million datasets were simulated for each scenario. The posterior probability of each scenario was estimated by polychotomous logistic regression on 1% of the simulated datasets closest to the observed dataset, transformed by linear discriminant analysis (Cornuet et al., 2014). Posterior distributions of parameters, model checking using the posterior based error and summary statistics not used in model selection, and confidence in scenario choice using 1,000 pseudo-observed test data sets were calculated using the options in DIYABC v. 2.1.0 (**IV**).

4.5.7. Mode of reproduction

To evaluate the possibility of sexual recombination in the populations, both the non-cc and cc datasets were used to test for haploid linkage

disequilibrium using the index of association (I_A) in GenAlEx 6.5 (**II**). In Paper **IV** the I_A and the standardized index of association ($\bar{r}_d$) were calculated in the R package *poppr* (Kamvar et al., 2014; R Core Team, 2017). Both analyses involved comparing the values for the observed data set with the values for 1,000 artificially recombining data sets. In order to assess if the populations deviate significantly from the null hypothesis of a 1:1 ratio of mating types, the exact binomial test was used as described in Barnes et al. (2014) (**II**, **IV**).

5. RESULTS

5.1. New country records, new hosts and disease severity of *Lecanosticta acicola*

In the fall of 2016 Scots pines growing in a small group mixed with *P. mugo* in Kõrveküla, Tartu County, Estonia, were noticed to have symptoms of BSNB. Isolates obtained from both host species were identified by sequencing and species-specific primers and proved to be *L. acicola*. None of the *P. sylvestris* trees growing further than 2.5 meters from infected *P. mugo* were symptomatic and some *P. sylvestris* trees were found to be asymptomatic despite growing close to severely infected trees. *Pinus mugo* trees were more heavily infected than *P. sylvestris* trees. On infected trees, 17% of the needles of *P. sylvestris* and 63% of *P. mugo* needles were symptomatic. On both hosts, in most cases the youngest needle class was also found to be infected (**I**).

Additional locations where infected non-native pines were growing near Scots pine were monitored, resulting in world's first report of *Pinus* x *rhaetica* being infected with *L. acicola* in the TBA (**I**).

Lecanosticta acicola is continuing to spread further into western Asia. The pathogen was isolated from needle samples collected in 2015 from a *P. nigra* plantation in Georgia and it represents the first confirmed record of *L. acicola* in Georgia using molecular methods and the earliest known *L. acicola* sample from western Asia (**IV**).

In the spring of 2017 and summer of 2018, during a survey of needle diseases of the Pinaceae in Atatürk Arboretum in Istanbul, Turkey, *Cedrus libani* was found to have symptoms of BSNB. An isolate obtained from conidia on the diseased needles was identified by sequencing and showed 100% similarity with *L. acicola* strains deposited in GenBank. This is therefore the first molecularly confirmed record of *L. acicola* infecting a host other than *Pinus* spp. (**III**). In the same arboretum *P. sylvestris*, *P. nigra* subsp. *nigra*, *P. nigra* subsp. *laricio*, *P. nigra* subsp. *pallasiana*, and two endemic varieties of *P. nigra* subsp. *pallasiana* (var. *fastigiata* and var. *pallasiana* f. *şeneriana*) were found to be infected with *L. acicola,* the last two being new hosts for the world (**III**).

Disease severity in the Atatürk Arboretum was at least 10% higher during the second year of assessment. The symptomatic crown proportion was lowest on *C. libani* (10%-20%) and highest on *P. nigra* subsp. *pallasiana* (20%-100%) and the endemic varieties of *P. nigra* subsp. *pallasiana* (80%-100%). On *P. sylvestris* the disease severity reached up to 80% of crown being affected (**III**).

5.2. Distribution, diversity, and population structure of *Lecanosticta acicola* in Estonia

For the population genetic study in total 104 *L. acicola* isolates were obtained from 25 sites in Estonia (Figure 1, **II**). The species of all isolates were confirmed by species-specific primers.

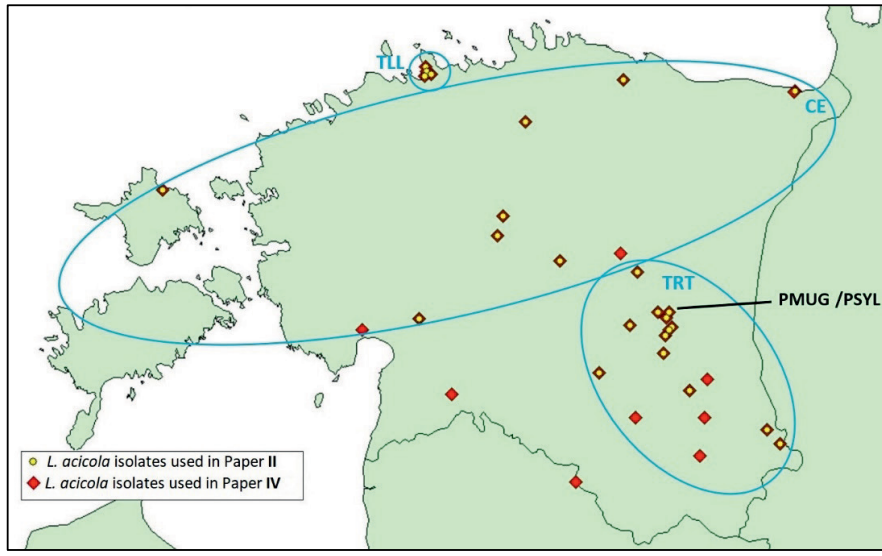

Figure 1. Sampling sites of *Lecanosticta acicola* isolates from Estonia, used in the population studies (**II**, **IV**). The composed populations used in Paper **II** are indicated in blue. PMUG/PSYL indicates the location of a mixed stand of *Pinus mugo* and *P. sylvestris*. Abbreviations: CE – central Estonia, PMUG – *P. mugo* in the mixed stand, PSYL – *P. sylvestris* in the mixed stand, TLL – Tallinn, TRT – Tartu.

From the 104 isolates across 11 microsatellite markers 43 different alleles were detected and 50 unique multilocus haplotypes (MLH) were identified. Ten haplotypes appeared more than once, and four haplotypes occurred at more than one sampling site with the largest distance between two isolates of same haplotype being more than 130

km (**II**). The most common haplotype (MLH 31) was found from 40 trees at three different sampling sites, and it also dominated in the mixed stand of *P. mugo* and *P. sylvestris* on both hosts. Samples collected from TBA, the first location in Estonia and in all northern Europe where *L. acicola* was detected, revealed four different haplotypes, none of those were found from other sampling sites in Estonia (**II**).

According to AMOVA, the molecular variation between geographical populations increases with distance. Populations TLL and TRT were found to be significantly different from each other ($p=0.002$). Significant differences were not found between populations TLL and CE ($p=0.061$) nor between TRT and CE ($p=0.349$). For the following analyses the populations CE and TRT were merged into a single population EST, and TLL, as the initial point of infection in Estonia, was considered as a separate population. The first pathogen population in Estonia (TLL) turned out to be genetically different from all other merged populations in Estonia (EST) ($p=0.001$). Additionally, isolates from the first years after the pathogen's discovery in Estonia (2010-2015) turned out to be significantly different from the isolates collected during later years (2016-2017) ($p=0.007$) (**II**).

According to several diversity indices population EST is more diverse than population TLL, however it covers a significantly larger area of Estonia and, based on the mean allelic richness (A_R) and mean private allelic richness (PA_R) that are calculated based on standardized population sizes, TLL is more diverse (Table 1).

Table 1. Diversity statistics of *Lecanosticta acicola* populations (indicated in bold) and subpopulations based on 11 microsatellite markers in Estonia.

Population code	N[a]	No. of haplotypes	Clonal fraction	Mean haploid genetic diversity h (SE[b])	Total no. of alleles	Unique alleles	Mean allelic richness A_R (SE[b])	Mean private allelic richness PA_R (SE[b])	Mean number of different alleles Na (SE[b])	Mean unbiased diversity uh (SE[b])
TLL	16	11	0.313	0.303 (0.084)	27	3	2.200 (0.583)	0.395 (0.242)	2.455 (0.413)	0.339 (0.094)
EST[c]	88	40	0.545	0.455 (0.077)	40	16	2.068 (0.422)	0.263 (0.164)	3.636 (0.975)	0.468 (0.080)
PSYL	29	7	0.759	0.160 (0.036)	24	0	na	na	2.182 (0.352)	0.166 (0.037)
PMUG	18	4	0.778	0.099 (0.030)	17	0	na	na	1.545 (0.157)	0.105 (0.032)

[a] N – Number of isolates.

[b] SE – Standard error.

[c] EST contains subpopulations PSYL and PMUG.

Population codes: EST – Estonia (covers merged CE and TRT populations), PMUG – *Pinus mugo* in the mixed stand, PSYL – *P. sylvestris* in the mixed stand, TLL – Tallinn.

STRUCTURE analysis based on Estonian isolates suggested the occurrence of three different clusters (K=3). None of the populations fell into only one cluster. The orange cluster has only a marginal presence in the Tallinn (TLL) population whilst it is a significant component in the rest of Estonia (Figure 2, **II**).

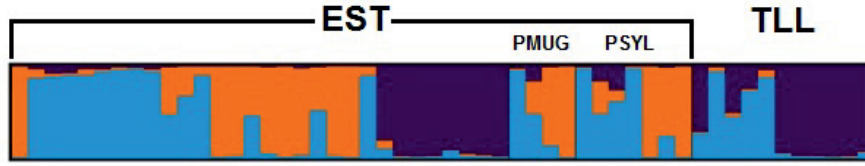

Figure 2. STRUCTURE clustering of *Lecanosticta acicola* isolates in the formed populations (EST and TLL) and subpopulations of the mixed stand (PMUG and PSYL), based on the cc dataset. Abbreviations: EST – Estonia (covers merged CE and TRT populations), PMUG – *Pinus mugo* in the mixed stand, PSYL – *P. sylvestris* in the mixed stand, TLL – Tallinn.

5.2.1. Mixed stand of *Pinus sylvestris* and *Pinus mugo*

In the mixed stand of *P. mugo* and *P. sylvestris* nine different haplotypes were detected – four from *P. mugo* and seven from *P. sylvestris*. Two of the haplotypes (MLH 31 and MLH 14) were found from both hosts. Four of the haplotypes found from *P. sylvestris* were found only from this host species and only from this stand (**II**). It turned out that according to AMOVA there is not a significant difference ($p=0.379$) between isolates obtained from *P. sylvestris* and *P. mugo* in a mixed stand (**II**) and the subpopulations also shared similar genetic STRUCTURE clustering with the light blue and orange clusters dominating (Figure 2). Clonal fraction was high in the subpopulations and overall diversity statistics were lower than in other populations found in Estonia (Table 1). Of the two analysed subpopulations, diversity was higher for the one formed of isolates from *P. sylvestris* (**II**).

5.2.2. Mating type distribution and reproductive mode of *Lecanosticta acicola* in Estonia

In Estonia both mating types of *L. acicola* are present but appear in unequal proportions ($p<0.05$), with *MAT1-1-1* being more common in the country overall and as well in all formed populations analysed (**II**, **IV**). According to the index of association, random mating takes place only in the TLL population based on the cc dataset ($p=0.063$; **II**).

Both mating types were present in the mixed stand of *P. mugo* and *P. sylvestris* but appeared in an uneven proportion ($p<0.05$) with *MAT1-1-1* dominating on both hosts. However, when the clone-corrected dataset was used, the proportion of mating types was found to be equal on both hosts ($p>0.05$; **II**).

5.3. Global population study of *Lecanosticta acicola*

In total, 650 isolates from 27 countries were obtained for this study. Analyses across 11 microsatellite markers resulted in a total of 172 alleles. The mean number of different alleles (Na) was highest in S-USA, LTU, EST, and SVN (Table 2). The highest number of unique alleles was observed in S-USA (Table 2). In Europe, unique alleles were found in eight of the 21 populations, with the highest numbers in AUT (6) followed by EST, HRV and TUR (3) (Table 2, **IV**).

The additional 23 isolates from eight locations in Estonia used in Paper **IV** increased the number of alleles in Estonia by four to 47 and the number of haplotypes by 12 (**II**, **IV**).

In total, 284 different multilocus haplotypes were found in the collection of 650 *L. acicola* isolates from different countries. All populations with more than four isolates contained clones. The clonal fraction index for Europe (0.600) was higher than for America in total (0.371) (Table 2) but similar to the value of north-eastern America (CAN and N-USA combined, 0.566) (**IV**).

In total, 16 haplotypes were found in more than one country. The most common haplotype (MLH 196) appeared 45 times in four different populations (TUR, BLR, LTU and EST). This is the same haplotype that was found to be the most common one in Estonia in Paper **II**. One haplotype (MLH 225) was found to be present on two continents, in Canada (North America) and Germany (Europe). Several haplotypes were shared between EST and LTU and one haplotype appeared in all three Baltic states. Despite being neighbouring countries, LTU and POL did not share any haplotypes (**IV**).

Overall genetic diversity was highest in S-USA (Table 2). In Europe, the mean unbiased diversity (uh) and the mean haploid genetic diversity (h) were highest in FRA, EST and LTU (**IV**).

Table 2. Diversity statistics of *Lecanosticta acicola* populations based on 11 microsatellite markers.

Region/ Population code	N[a]	No. of haplo-types[b]	No of alleles	Unique alleles	Allelic richness A_R (SE[c])	Private allelic richness PA_R (SE[c])	Mean number of different alleles Na (SE[c])	Mean unbiased diversity uh (SE[c])	Mean haploid genetic diversity h (SE[c])	Clonal fraction
America	**105**	**66**	**125**	**61**	**3.83 (0.34)**	**2.72 (0.35)**	**11.36 (2.03)**	**0.78 (0.05)**	**0.76 (0.05)**	**0.371**
CAN	19	12	31	0	2.25 (0.41)	0.16 (0.13)	2.82 (0.67)	0.41 (0.10)	0.37 (0.09)	0.368
COL[d]	2	1	11	3	na	na	na	na	na	na
MEX[d]	4	4	17	8	na	na	na	na	na	na
N-USA	34	11	21	0	1.73 (0.34)	0.13 (0.12)	1.91 (0.43)	0.25 (0.11)	0.22 (0.10)	0.676
S-USA	46	38	98	36	3.57 (0.47)	1.97 (0.37)	8.91 (1.86)	0.67 (0.10)	0.65 (0.09)	0.174
East Asia	**3**	**2**	**11**	**1**	**na**	**na**	**na**	**na**	**na**	**na**
CHN[d]	1	1	10	0	na	na	na	na	na	na
JPN[d]	2	2	11	0	na	na	na	na	na	na
Europe	**542**	**217**	**103**	**46**	**2.55 (0.43)**	**1.44 (0.46)**	**9.46 (3.86)**	**0.46 (0.10)**	**0.46 (0.10)**	**0.600**
AUT	31	15	30	6	1.90 (0.43)	0.25 (0.25)	2.73 (1.05)	0.26 (0.10)	0.25 (0.10)	0.516
BLR[d]	3	3	17	0	na	na	na	na	na	na
CHE	50	12	22	0	1.77 (0.35)	0.05 (0.03)	2.00 (0.47)	0.26 (0.11)	0.23 (0.10)	0.760
CZE	16	6	19	0	1.73 (0.38)	0.07 (0.06)	1.73 (0.38)	0.24 (0.11)	0.20 (0.09)	0.625
DEU	33	19	33	1	1.92 (0.41)	0.19 (0.13)	3.00 (0.98)	0.27 (0.10)	0.25 (0.09)	0.424
ESP	9	6	15	0	1.36 (0.15)	0.04 (0.04)	1.36 (0.15)	0.18 (0.08)	0.15 (0.06)	0.333
EST	127	62	47	3	2.23 (0.36)	0.17 (0.11)	4.27 (1.29)	0.42 (0.09)	0.41 (0.09)	0.512
FRA	10	6	26	0	2.36 (0.24)	0.03 (0.03)	2.36 (0.24)	0.52 (0.06)	0.43 (0.05)	0.400

Region/ Population code	N[a]	No. of haplo-types[b]	No of alleles	Unique alleles	Allelic richness A_R (SE[c])	Private allelic richness PA_R (SE[c])	Mean number of different alleles Na (SE[c])	Mean unbiased diversity uh (SE[c])	Mean haploid genetic diversity h (SE[c])	Clonal fraction
GEO[d]	1	1	11	0	na	na	na	na	na	na
HRV	24	8	17	3	1.48 (0.32)	0.27 (0.19)	1.55 (0.46)	0.13 (0.09)	0.11 (0.08)	0.667
IRL[d]	3	3	15	0	na	na	na	na	na	na
ITA[d]	4	2	15	0	na	na	na	na	na	na
LTU	106	52	49	1	2.16 (0.39)	0.12 (0.07)	4.55 (1.73)	0.38 (0.10)	0.37 (0.10)	0.509
LTV[d]	1	1	11	0	na	na	na	na	na	na
POL	16	6	16	0	1.46 (0.21)	0.01 (0.01)	1.46 (0.21)	0.20 (0.08)	0.17 (0.07)	0.625
PRT[d]	2	1	11	0	na	na	na	na	na	na
RUS	17	8	18	2	1.46 (0.46)	0.30 (0.30)	1.64 (0.64)	0.09 (0.09)	0.08 (0.08)	0.529
SVK[d]	11	2	10	0	na	na	na	na	na	0.818
SVN	58	16	43	1	2.29 (0.53)	0.33 (0.20)	3.91 (1.29)	0.33 (0.12)	0.31 (0.11)	0.724
SWE[d]	3	1	11	0	na	na	na	na	na	na
TUR	17	6	24	3	2.18 (0.46)	0.44 (0.22)	2.18 (0.46)	0.32 (0.11)	0.26 (0.09)	0.647

[a] Number of isolates

[b] Equivalent to number of isolates in the clone-corrected dataset

[c] SE – standard error

[d] Due to small sample size, (N (cc)<6), these populations were excluded from population genetic analyses

Definition of population codes: AUT – Austria, BLR – Belarus, CAN – Canada, CHE – Switzerland, CHN – China, COL – Colombia, CZE – Czech Republic, DEU – Germany, ESP – Spain, EST – Estonia, FRA – France, GEO – Georgia, HRV – Croatia, IRL – Ireland, ITA – Italy, JPN – Japan, LTU – Lithuania, LTV – Latvia, N-USA – north-eastern USA, MEX – Mexico, POL – Poland, PRT – Portugal, RUS – Russia, S-USA – south-eastern USA, SVK – Slovakia, SVN – Slovenia, SWE – Sweden, TUR – Turkey.

5.3.1. Population structure, genetic differentiation and genetic distance between populations of *Lecanosticta acicola*

The STRUCTURE results were not consistent in the most probable number of clusters that would describe the global *L. acicola* population. The ln(Pr(X | K)) method of choosing the best number of STRUCTURE clusters indicated that seven clusters describe the dataset best, whereas the delta K statistic indicated that two clusters explained the data best (**IV**).

At K=2 one of the clusters (indicated in red) dominates in S-USA, MEX, COL, East Asian populations JPN and CHN but also in south-western European populations FRA, ESP and PRT (Figure 3). The other cluster (indicated in light blue) dominates in N-USA, CAN, western Asia and most of Europe whilst also occurring in the south-western European population FRA (**IV**).

From K=4, in central Europe a single cluster (green) dominates, however, in populations CHE and SVN the structure is more diverse with no single dominating cluster. Populations HRV, RUS and TUR belong primarily to the brown cluster, which also occurs in CAN, N-USA, and MEX (Figure 3, **IV**).

The populations EST, LTV, LTU, POL and BLR shared a roughly similar structure, with the light blue cluster dominating. Isolates from the Curonian Spit region in LTU belonged to the same cluster (green, K=4-K=7) as those in central Europe. In EST the orange cluster also stands out but it is not formed of isolates originating from a certain region or time period (Figure 3, **IV**).

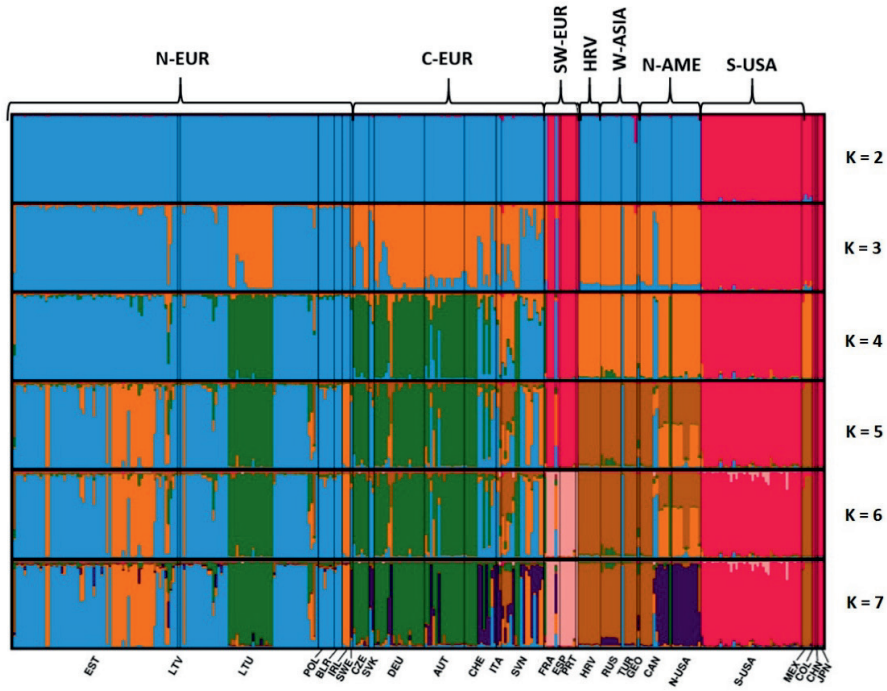

Figure 3. STRUCTURE clustering of the *Lecanosticta acicola* clone-corrected dataset, representing K=2-7. Optimal number of clusters K=2 by delta K and K=7 by ln(Pr(X|K)). Each multilocus haplotype is represented by a vertical line. The proportion of colours in it represents the isolate's estimated membership in each cluster. Population codes are displayed under the figure, above the figure is displayed division into regions as analysed in the migration analyses.

Broadly similar grouping of populations is indicated by the dendrogram, based on Nei's genetic distance (see Paper **IV**).

According to the AMOVA no significant differences were found between population pairs of neighbouring European countries FRA-ESP, AUT-CZE and CZE-DEU ($p>0.05$). All other populations were significantly differentiated from each other ($p<0.05$) (**IV**).

Nei's genetic distance indicated that for most populations in Europe the genetic distance from the populations N-USA and CAN was lower than the average found over all populations. Some of the lowest genetic distances were observed between populations AUT, DEU and CZE but also between EST and LTU. The population S-USA is genetically similar only to ESP and genetically distant from all other populations.

Also, the populations FRA and ESP were genetically distant from most populations in Europe (**IV**).

The Mantel test for isolation by distance among 16 American and European populations revealed significant correlation between geographical distance and Nei's genetic distance ($p=0.030$). Isolation by distance was also supported in Europe ($p=0.010$) but rejected in North America ($p=0.166$, **IV**).

5.3.2. Mating type distribution and reproductive mode

Out of 28 studied populations, both mating type idiomorphs were present in 14 populations (Table 3). The exact binomial test on the mating type ratios indicated that in four populations (ESP, FRA, S-USA and SVN) equal ratios of the mating type idiomorphs ($p>0.05$) were found based on the non-cc dataset, and in eight populations (CHE, DEU, ESP, FRA, N-USA, POL, S-USA and SVN) based on the cc dataset (**IV**). Therefore, in these populations, sexual reproduction is probable.

The index of association indicated that random mating occurred only in ESP and S-USA populations based on the non-cc dataset and additionally in SVN, HRV and CZE populations based on the cc dataset, the last two being unexpected since only one mating type was identified in those populations (Table 3, **IV**).

In all northern European countries, the idiomorph *MAT1-1-1* dominated, except in Sweden where only *MAT1-2* was found (Table 3, **IV**).

Table 3. A summary of the mating type distribution and index of association results.

Population	*MAT1-1-1/ MAT1-2* non-cc[a]	p-value of exact binomial test non-cc[a]	*MAT1-1-1/ MAT1-2* cc[b]	p-value of exact binomial test cc[b]	I_A[c] non-cc[a]	$\bar{r}_d$[d] non-cc[a]	p-value of I_A[c] and $\bar{r}_d$[d] non-cc[a]	I_A[c] cc[b]	$\bar{r}_d$[d] cc[b]	p-value of I_A[c] and $\bar{r}_d$[d] cc[b]
AUT	2/29	<0.001	2/13	0.007	2.582	0.543	0.001	1.901	0.386	0.001
BLR[e]	3/0	na	3/0	na	na	na	na	na	na	na
CAN	17/2	0.001	10/2	0.039	1.708	0.250	0.001	1.053	0.157	0.001
CHE	3/47	<0.001	3/9	0.146	2.025	0.678	0.001	0.558	0.186	0.001
CHN[e]	0/1	na	0/1	na	na	na	na	na	na	na
COL[e]	0/2	na	0/1	na	na	na	na	na	na	na
CZE	0/16	<0.001	0/6	0.031	1.173	0.403	0.001	0.109	0.036	0.509
DEU	24/9	0.014	13/6	0.167	0.987	0.177	0.001	0.921	0.157	0.003
ESP	3/6	0.508	2/4	0.688	-0.173	-0.058	0.848	-0.376	-0.126	1.000
EST	102/24	<0.001	44/17	0.001	1.251	0.163	0.001	0.309	0.039	0.003
FRA	7/2	0.180	4/1	0.375	6.516	0.731	0.001	5.440	0.609	0.001
GEO[e]	1/0	na	1/0	na	na	na	na	na	na	na
HRV	0/24	<0.001	0/8	0.008	0.409	0.410	0.004	0.106	0.106	0.543
IRL[e]	2/1	na	2/1	na	na	na	na	na	na	na
ITA[e]	4/0	na	2/0	na	na	na	na	na	na	na
JPN[e]	0/2	na	0/2	na	na	na	na	na	na	na
LTV[e]	1/0	na	1/0	na	na	na	na	na	na	na
LTU	63/29	0.001	30/13	0.014	0.673	0.104	0.001	0.516	0.078	0.001
MEX[e]	2/2	na	2/2	na	na	na	na	na	na	na
N-USA	31/3	<0.001	8/3	0.227	2.041	0.689	0.001	0.968	0.324	0.001
POL	13/3	0.021	4/2	0.688	1.317	0.439	0.001	1.189	0.396	0.014

Population	*MAT1-1-1/ MAT1-2* non-cc[a]	p-value of exact binomial test non-cc[a]	*MAT1-1-1/ MAT1-2* cc[b]	p-value of exact binomial test cc[b]	I_A[c] non-cc[a]	$\bar{r}_d$[d] non-cc[a]	p-value of I_A[c] and $\bar{r}_d$[d] non-cc[a]	I_A[c] cc[b]	$\bar{r}_d$[d] cc[b]	p-value of I_A[c] and $\bar{r}_d$[d] cc[b]
PRT[e]	1/0	na	1/0	na	na	na	na	na	na	na
RUS	0/17	<0.001	0/8	0.008	na	na	na	na	na	na
S-USA	26/20	0.461	21/17	0.627	0.090	0.010	0.223	-0.068	-0.007	0.809
SVK[e]	11/0	0.001	2/0	na	na	na	na	na	na	na
SVN	26/28	0.892	4/9	0.267	2.001	0.404	0.001	0.139	0.030	0.260
SWE[e]	0/3	na	0/1	na	na	na	na	na	na	na
TUR	17/0	<0.001	6/0	0.031	2.956	0.602	0.001	2.534	0.634	0.001

[a] non-cc – non-clone corrected dataset

[b] cc – clone-corrected dataset

[c] I_A – index of association

[d] $\bar{r}_d$ – standardized index of association

[e] Due to small sample size, (N (cc)<6), these populations were excluded from population genetic analyses

Definition of population codes: AUT – Austria, BLR – Belarus, CAN – Canada, CHE – Switzerland, CHN – China, COL – Colombia, CZE – Czech Republic, DEU – Germany, ESP – Spain, EST – Estonia, FRA – France, GEO – Georgia, HRV – Croatia, IRL – Ireland, ITA – Italy, JPN – Japan, LTU – Lithuania, LTV – Latvia, N-USA – north-eastern USA, MEX – Mexico, POL – Poland, PRT – Portugal, RUS – Russia, S-USA – south-eastern USA, SVK – Slovakia, SVN – Slovenia, SWE – Sweden, TUR – Turkey.

5.3.3. Distribution pathways of *Lecanosticta acicola*

In Analysis 1, which investigated the demographic history between the three main clusters in America and Europe (Figure 3, K=3), the posterior probabilities were highest for Scenario 17 (P=0.376, see Paper **IV**), where populations in south-western Europe originated from an admixture event between north-eastern America and south-eastern USA, while the merged population EUR (containing C-EUR, W-ASIA and HRV) originated from an admixture event between north-eastern America and an unsampled population (Figure 4).

The most supported scenario in Analysis 2 (S2.3) suggested that northern Europe was derived from EUR (P=0.503), a median of 40 and a mode of 32 generations ago with a weak bottleneck occurring, i.e. of meaning short duration and high number of founders.

The scenario with the highest support in Analysis 3 revealed that the populations in Croatia and western Asia originated from N-AME (S3.5, P=0.708). The region of C-EUR originated from an admixture event between N-AME an unsampled population, a median of 115 and a mode of 77 fungal generations ago.

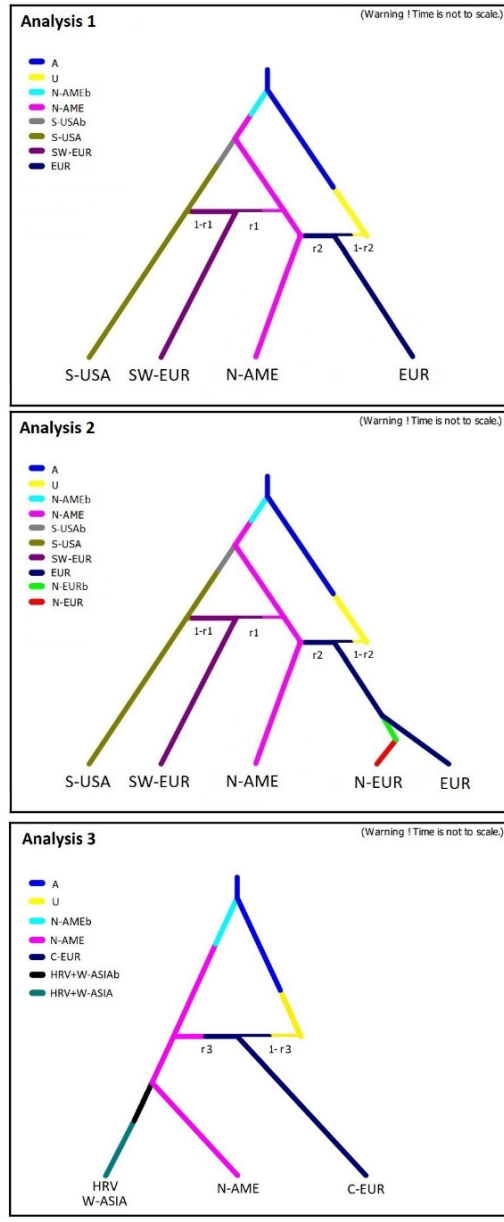

Figure 4. A graphical representation of the combined historical scenarios, most supported by the ABC analyses (**IV**). Abbreviations: A – ancestral population; U – unsampled population; N-AME – north-eastern America (N-USA+CAN); S-USA – south-eastern USA; SW-EUR – south-western Europe; C-EUR – central Europe; N-EUR – northern Europe; HRV – Croatia; W-ASIA – western Asia; EUR – merged population of C-EUR, HRV and W-ASIA; b – bottleneck event; r1, r2, r3 – rate of admixture, thickness of line indicates the contribution from populations (r and r-1).

6. DISCUSSION

6.1. Distribution and introduction source(s) of *Lecanosticta acicola* in northern Europe

In this thesis it is documented that the distribution area of *L. acicola* is expanding in Europe and further into western Asia (**II**, **III**, **IV**). The continuous northward spread of several forest pathogens, including *L. acicola* illustrates the effect of climatic change – previously cold winters that held off pathogens adapted to moderate climates have been replaced with milder temperatures and higher rates of precipitation, which are more suitable for fungi (Hanso and Drenkhan, 2013). This problem is not only observed in Europe – it has been suggested that the outbreak of so-called White Pine Needle Damage in north-eastern USA and Canada, caused by a disease complex including *L. acicola*, has been due to changes in climatic conditions (Broders et al., 2015; Wyka et al., 2017, 2018).

Whereas climate change helps the incremental and gradual natural range expansion of pathogens, long distance jumps are often due to human activity. It was already implied by Adamson et al. (2015) and Adamson (2017) that the chronology of *L. acicola* finds in northern Europe suggests anthropogenic activity. The presence of similar haplotypes over long distances (**II**, **IV**) that the fungus would not be able to overcome naturally in short periods of time (Wyka et al., 2018) suggests that it is unlikely that the pathogen has spread solely via natural dispersal.

The results of this thesis suggest that there have been several separate introduction events of *L. acicola* into northern Europe (**II**, **IV**). According to the analyses, the first populations in Estonia are genetically different from the more recently sampled ones (**II**) suggesting that the first documented disease outbreak in Tallinn has not been the only source for colonization of the whole country. The structure of populations furthermore suggests that there has not only been natural spread of the fungus, but also that human activity has mixed the haplotypes and populations. For example, northern European populations are mainly characterized by a rather homogenous population structure, however, there are haplotypes that stand out as belonging to a different cluster, probably originating from a separate introduction (**IV**). In Lithuania

there is a clearly different subpopulation in the Curonian spit region, represented by a cluster that otherwise dominates in central Europe (**IV**). The same population shares a haplotype with Germany (**IV**). This indicates separate introductions of the Curonian Spit and mainland populations in Lithuania.

The results of the ABC analyses indicated that the populations in northern Europe originate from previously established populations in Europe, and not from separate introduction events directly from North America. However, the support for the scenario suggesting that northern Europe originated from an admixture event between North American and European populations was not much lower (**IV**). The pathogen´s populations in northern Europe most probably originated predominantly from central Europe, as is indicated by shared STRUCTURE clusters and haplotypes. How northern European countries and Turkey ended up sharing the same multilocus haplotype is unknown, but transportation of planting material is the most likely explanation, as it is for the shared haplotype between Canada and Germany (**IV**).

According to the ABC analysis, *L. acicola* reached northern Europe a median of 40 and a mode of 32 generations ago with a high number of founders (**IV**). In DIYABC software generation time is considered to be the time elapsed between the birth of an individual and the birth of its offspring (Cornuet et al., 2014), which in studies of fungal pathogens is generally assumed to be one year (Janoušek et al., 2016; Mullett et al., 2021). The first reported find of *L. acicola* in northern Europe originates from 2008 (Drenkhan and Hanso, 2009) and the oldest sample used in the DIYABC analyses is from 2010. Therefore, there is a difference between the estimated divergence time of the northern European population and the actual time of its first report. In forest pathology it often happens that the pathogens have been present for some time before being noticed, either in a latent phase or spreading on such a small scale that it goes unnoticed (Drenkhan et al., 2013; Adamson et al., 2018). For example, analysing herbaria exemplars of *Fraxinus* spp. from Estonia has shown that *Hymenoscyphus fraxineus*, the causal organism of ash dieback, was present in Estonia at least 19 years before the first documented find (Agan et al., 2022). It cannot be excluded that *L. acicola* may have been present in northern Europe for some time before the first reported finds. Future studies based on herbaria exemplars of *Pinus* spp. may provide more certainty of it.

The high proportion of clones in the European populations, including in northern Europe (**II**, **IV**) confirms that the species is non-native and recently introduced here. Similar results have been observed in the populations of *Diplodia sapinea*, another non-native pine pathogen, in Europe (Brodde et al., 2019; Adamson et al., 2021). In comparison, when *Dothistroma septosporum* populations were studied, all isolates turned out to be different haplotypes (Adamson et al., 2018) and recent studies have indicated that north-eastern Europe may indeed be the native range for this pathogen (Mullett et al., 2021).

Based on the proportion of clones it was concluded that the diversity is rather low in the Estonian population (**II**). However, when the Estonian and Lithuanian populations were included in a global analysis, the genetic diversity in northern Europe was found to be surprisingly high compared to the other populations in Europe and north-eastern America (**IV**). Especially, considering the short duration of time that the pathogen has been known to be present in northern Europe. In both Estonia and Lithuania the number of alleles, clonal fraction and other diversity statistics were approximately similar and were found to be mostly either higher or on a comparable level to what was found in north-eastern USA and Canada (see Table 2), which is presumably the native range of the pathogen. In general, species are expected to have higher diversity in their native areas (McDonald, 1997) but that is not always the only source of high diversity. Higher microsatellite diversity in northern Europe could be explained by several introduction events from genetically different source populations. Part of the effect may be due to the samples from northern USA and Canada being collected during a disease outbreak year (Wyka et al., 2017) which could possibly mean a higher proportion of clones, as has been observed in the *D. sapinea* outbreak in Sweden (Brodde et al., 2019). In addition, in the USA the presence of *L. acicola* has been reported from 28 states (van der Nest et al., 2019a; EPPO, 2022) but in Paper **IV** isolates from only five states were used. Sampling additional populations from North America, from more geographically representative areas would most probably reveal higher genetic diversity of the pathogen in North America.

In most European countries where the pathogen is present both of its mating types are found (**IV**). It is proven that sexual reproduction takes place in south-western Europe (Mezansa et al., 2021b) and the results of this study (**IV**), and of Janoušek et al. (2016), suggest that some level of

sexual reproduction may also take place in central Europe. In the more representative populations studied in the northern European region – Estonia, Lithuania and Poland – both mating types were present and appeared in the same locations, however, the proportion of mating types indicated sexual reproduction was likely only in Poland and in Estonian subpopulations PSYL and PMUG based on a clone-corrected dataset (**II**, **IV**). Genetic analyses did not support the occurrence of random mating in northern Europe, except in the geographically limited Estonian subpopulation TLL (**II**, **IV**). The proportion of clones observed on different trees in one stand in Estonia indicated that on a local scale the pathogen spreads successfully in an asexual state (**II**) and it is probably the dominate reproductive mode in northern Europe.

Interestingly, the results suggest that it is possible to control the disease using fungicides combined with collecting and burning the needle litter. In TBA, after the first detection of the disease, not all infected trees were felled and during the following years fungicides were used along with burning the needle litter to control the disease (Kaur and Hermann, 2021). Genetic analyses did not find the same *L. acicola* haplotypes detected in TBA to be present in other locations in Estonia, not even in Tallinn (**II**, **IV**). Therefore, this method of disease management is worth trying in the case of valuable specimens in restricted areas.

6.2. Host range and the potential threat of *Lecanosticta acicola*

For a long time *L. acicola,* and the entire *Lecanosticta* genus*,* has been considered a pathogen exclusively of pine species. Recent findings from *Cedrus* species in separate locations (**III**, Schenck et al., 2022) indicate that *L. acicola* is not a *Pinus* specific pathogen and other Pinaceae species may prove to be susceptible, especially under high inoculum pressure. Although, for example *Picea abies* has remained uninfected despite proximity to severely infected trees (Beenken et al., 2018). In addition to *C. libani*, the results of this thesis added three new *Pinus* taxa to the host list of *L. acicola*: *P. nigra* subsp. *pallasiana* var. *fastigiata*, *P. nigra* subsp. *pallasiana* var. *pallasiana* f. *seneriana,* and *P.* x *rhaetica* (**I**, **III**).

Native *P. sylvestris* has proven to be susceptible to *L. acicola* in northern Europe (**I**), however, at the moment, the species seems to have low susceptibility to the pathogen. In the mixed stand of *P. mugo* / *P. sylvestris* only *P. sylvestris* trees growing in proximity of infected *P. mugo* trees were

found to be infected. Similar observations originate from two other locations in County of Tartu, Estonia, where young *P. sylvestris* trees growing near infected *P. mugo* trees have been found to be diseased (M. Vester, personal records). In other locations in Europe where *P. sylvestris* has been found to be infected there are often also more susceptible pine species close by affected by *L. acicola*, again suggesting that in its natural habitat in Europe there needs to be an inoculation source close nearby and high inoculum pressure to infect *P. sylvestris* (Cech and Krehan, 2008; Jurc and Jurc, 2010; EPPO, 2012b, 2015; Mullett et al., 2018; Georgieva, 2020; Raitelaitytė et al., 2022; **III**). This is yet another example highlighting the fact that non-native species are a threat to forest stands since they tend to be more susceptible to invasive pathogens, may co-introduce their pathogens, and turn into a source of inoculum (Drenkhan et al., 2014; Adamson et al., 2015).

The infection of dozens of young Scots pines in Estonia took place in a short amount of time. Less than a year before, in the winter of 2015/2016, only infection of *P. mugo* trees was observed in the location and *P. sylvestris* trees seemed visually healthy (**I**). One reason for such a rapid spread of infection could be the extremely suitable weather conditions for the pathogen that year, favourable for production and dissemination of spores. Alternatively, the presence of more virulent haplotypes in the stand, unique haplotypes on *P. sylvestris*, and the host jump (**I**, **II**) could be the result of mutations or sexual recombination creating novel haplotypes. This is supported by the equal proportions of mating types found in the stand indicating probability of sexual reproduction.

From *P. sylvestris* more haplotypes were found and therefore a somewhat larger diversity registered, however, more isolates were also obtained from *P. sylvestris* (**II**), which may affect these results. The results showed that within the limits of one stand, diversity or population structure did not differentiate significantly between the two host species (**II**). The results of this study indicated that there may be strains that are more aggressive and spread better, given the example of one strain being dominant on two hosts in the Estonian stand (**II**). The disease severity and level of defoliation varied greatly between the individual infected trees, even among the trees that were infected with the dominant haplotype in the stand (**II**). This is probably caused by differences in the heritable susceptibility of individual trees – a topic that has been

explored in the case of *P. palustris* in North America (Snyder and Derr, 1972) and *P. elliottii* in China (Ye and Wu, 2011) but not in northern Europe.

There is a distinct lack of relevant information about the potential severity of BSNB on *P. sylvestris* in Europe. It has been noted that northern and southern provenances of *P. sylvestris* have different susceptibility to *L. acicola* with the first being more resistant (Skilling and Nicholls, 1974; Phelps et al., 1978). The observations of the infection levels of *P. sylvestris* in Europe so far seem to confirm it. In the infected *P. sylvestris* stand in Estonia, the disease severity of *P. sylvestris* was on average 17% (**I**) and has not increased significantly during the subsequent years (M. Vester, 2022, personal observation). *Pinus sylvestris* trees growing near infected *P. mugo* stand are reported to be infected with *L. acicola* aswell in Lithuania, although there is no further information about the disease severity (Raitelaitytė et al., 2022). However, in Bulgaria a serious outbreak of *L. acicola* on *P. sylvestris* has been reported with the degree of defoliation reaching up to 100% (Georgieva, 2020). Aswell in Turkey, the infection level of *P. sylvestris* reached 80% of the crown (**III**), unfortunately, nothing is known about the progeny of the infected *P. sylvestris* tree.

The disease severity also varies on the new hosts, *Cedrus* spp. In Atatürk Arboretum in Turkey, disease severity was low on *C. libani* (up to 20%; **III**). However, in south-western France several forest stands of *C. atlantica* have been found to be infected, with defoliation, a measure of disease severity, reported to be from light to severe (Schenck et al., 2022). The more serious damage registered in France could be due to the southern lineage of *L. acicola* (Janoušek et al. 2016; **IV**), which has been reported to be more aggressive than the northern lineage (van der Nest et al., 2019a), but the lineage infecting *Cedrus* plantations in France has not been confirmed. There may also be differences in the susceptibility between *Cedrus* spp. as there is with *Pinus* spp., though in the Atatürk Arboretum in Istanbul *C. atlantica* and *C. deodara* trees were present, in addition to *C. libani*, but were not symptomatic (**III**).

In addition to observing the situation regarding changes in host range and geographic distribution, it would be relevant to pay attention to the actual species of the disease agent. Recently, several new species in the genus *Lecanosticta* have been described, which have so far not been detected outside of Mesoamerica (van der Nest et al., 2019b) but one of

them, *L. pharomachri*, seems to cause serious damage in *Pinus* plantations in Colombia (Theron et al., 2022). This yet again highlights that caution must be applied when planting material is introduced to avoid the spread of new pathogens that have the potential to cause economic damage, and molecular methods should be used to adequately distinguish morphologically similar disease agents (Aglietti et al. 2021).

7. CONCLUSIONS

During the last 15 years, *L. acicola* has become prevalent in some regions of northern Europe and although most of the reports originate from non-native pine species, the host jump to the single native host species *P. sylvestris* (**I**) reflects growth potential of the disease and proves the first hypothesis of this thesis that *P. sylvestris* is susceptible to *L. acicola* in northern Europe under natural conditions.

In the first stand in Estonia where *P. sylvestris* was found to be infected with *L. acicola*, one haplotype was found to dominate on both hosts (**II**). Although more different haplotypes of the pathogen were found from *P. sylvestris* than from *P. mugo*, based on the genetic analyses the two subpopulations were not significantly different from each other, supporting the second hypothesis. Currently the threat to forest stands of *P. sylvestris* in northern Europe seems to be low, but the monitoring should be continued because it has not been long since the host jump in northern Europe.

The northern European populations of this pathogen most likely originate from previously established populations in Europe (**IV**). When geographical distances, shared haplotypes, and population structure are also considered, it can be surmised that the populations of northern Europe predominantly originate from central Europe, most likely through several separate introduction events. The results suggest that the pathogen could have been present in northern Europe for a longer time than previously thought – possibly for more than 30 fungal generations (**IV**).

Although natural spread of the pathogen also clearly occurs, apparently supported by climate change, anthropogenic activity has inevitably affected the spread of *L. acicola*, and, with that, also raised its genetic diversity (**II**, **IV**).

In northern Europe both mating types of the pathogen are present, however, they appear in unequal proportions (**II**, **IV**). Clonal reproduction of *L. acicola* seems to be predominant but the occurrence, in some limited regions, of sexual reproduction cannot be excluded (**II**, **IV**), therefore creating the possibility for the appearance of new haplotypes.

The finding of *L. acicola* from *Cedrus libani* in Turkey (**III**) proves the fifth hypothesis of this thesis - that *L. acicola* is not a *Pinus* specific pathogen and under favourable conditions may also infect other conifer species. Both the continuing expansion of the distribution area and host range shows that *L. acicola* has almost certainly not reached its full potential in Europe nor in Asia. It can be expected that in the future the distribution of *L. acicola* will advance to the north.

In the modern plant protection era it is important not only to avoid new pathogen introductions, but also to avoid introductions of new strains of pathogens originating from genetically different populations, which could raise the genetic diversity of pathogen populations already present. Introduction of new strains may cause the appearance of more virulent pathogen genotypes more suited to specific local climatic conditions.

Some applied recommendations based on this thesis are as follows:

1. Systematic continuous monitoring of invasive pathogens including *L. acicola* will be required to follow changes in the host range and pathogen distribution area. Monitoring should include non-pine hosts growing in proximity of *Pinus* species. Molecular methods should be used to detect early stages of the infection.

2. Quarantine rules need to be updated. Until molecular identification of pathogens is not routinely used, essentially nothing prevents introduction of new plant pathogens, species, or genotypes, with planting material since several disease agents have a latent phase during which they are visually undetectable. Avoiding anthropogenic introduction of new pathogen strains would help prevent a rise in the pathogen's genetic diversity and sudden changes in its virulence.

3. Native species, or established progenies of non-native species, suitable to local climate conditions, should be preferred in forestry and urban areas. Susceptible non-native host species should not be grown near nurseries to avoid infecting nursery stock which would cause unavoidable economic damage if the seedlings would have to be destroyed. Or, even worse, if the infection goes unnoticed and the infected seedlings are used in the planting of new areas, the viability of the forest stand will be compromised and further spread of the pathogen will be likely.

4. Since *L. acicola* is no longer a quarantine species in the European Union, it is not mandatory to remove all infected trees. In arboreta or other valuable greeneries it is possible to use disease management plans to keep spread of the pathogen under strict control by using a combined treatment of fungicides together with collection and burning of the needle litter.

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SUMMARY IN ESTONIAN

INVASIIVSE PATOGEENI *LECANOSTICTA ACICOLA* GENEETILINE MITMEKESISUS, PÄRITOLU JA UUED PEREMEESTAIMED PÕHJA-EUROOPAS

Sissejuhatus

Kottseen *Lecanosticta acicola* (Thümen) A. Sydow on mändidel (*Pinus* spp.) esineva okkahaiguse pruunvöötaud tekitaja. Eelmisel sajandil algas selle Ameerika päritolu patogeeni levikuala laienemine ning tänaseks on *L. acicola* hajusalt levinud kogu Põhja-Ameerikas ja Euroopas, esinedes ka Lõuna-Ameerikas ning Ida-Aasias (van der Nest et al., 2019a).

Lecanosticta acicola esinemine on dokumenteeritud 53-l männi taksonil, kuid kõik liigid perekonnas mänd on patogeenile potentsiaalsed peremeestaimed (van der Nest et al., 2019a). On eeldatud, et soodsates oludes võib *L. acicola* nakatada ka teisi liike männiliste sugukonnas (*Pinaceae*), sarnaselt lähedase liigiga *Dothistroma septosporum* (Drenkhan et al., 2016). Nakatunud okkad varisevad enneaegselt, mis toob kaasa puu juurdekasvu aeglustumise, üldise tervisliku seisundi nõrgenemise ning võib põhjustada ka puude hukkumist (Tainter ja Baker, 1996; Wyka et al., 2017).

Lecanosticta acicola on enim kahju põhjustanud männi istandustes. Ajalooliselt on kahjud olnud suurimad *P. palustris* istandustes USA lõunaosariikides (Cordell et al., 1990), kuid ka USA põhjaosa jõulupuuistandustes on haigus tekitanud majanduslikku kahju, rikkudes puude kaubandusliku välimuse (Phelps et al., 1978). Euroopas on patogeen suurimat kahju tekitanud männi istandustes Prantsusmaal ja Hispaanias (Lévy, 1996; Ortíz de Urbina et al., 2017).

Pruunvöötaudi tekitaja esmakirjeldus Põhja-Euroopas pärineb aastast 2008, kui patogeen leiti Eestis Tallinna Botaanikaaiast (Drenkhan ja Hanso, 2009). Tänaseks on haigustekitaja levinud ka teistes Balti riikides ning Lõuna-Rootsis, kus seene esmaleiud pärinevad eksoot-männiliikidelt (EPPO, 2012a; Markovskaja et al., 2011; Mullett et al., 2018; Cleary et al., 2019). Põhja-Euroopa ainsat looduslikku männiliiki - harilikku mändi (*P. sylvestris* L.) on *L. acicola* tugevalt kahjustanud Põhja-Ameerikas (Skilling

ja Nicholls, 1974). Ka Kesk-Euroopas on registreeritud pruunvöötaud harilikul männil, kuid seni on nakkuskolded olnud lokaalsed (Cech ja Krehan, 2008; EPPO, 2015; Georgieva, 2020). Seega on küsimus, kas *L. acicola* võib nakatada ja kahjustada harilikku mändi ka selle loodusliku areaali Põhja-Euroopa osas ja milliseks võib kujuneda kahjustuste ulatus.

Seenel on kaks paarumistüübi idiomorfi, *MAT1-1-1* ja *MAT1-2*, mille koosesinemine on eelduseks *L. acicola* sugulise arengujärgu tekkele (Janoušek et al. 2014). Suguta arengujärgu eosed ehk koniidid levivad peamiselt vihma, udu ja kastepiiskadega ning läbivad vaid väikesi vahemaid (Skilling ja Nicholls, 1974; Wyka et al., 2018). Sugulise arengujärgu eosed kanduvad edasi õhuvooludega ning võivad läbida ka suuremaid distantse (Kais, 1971). *Lecanosticta acicola* sugulist arengujärku on leitud harva (van der Nest et al., 2019a), Euroopas vaid Hispaaniast (Mesanza et al., 2021b), kuid populatsioonigeneetilised analüüsid viitavad selle esinemise võimalusele ka Austrias ja Saksamaal (Janoušek et al., 2016).

Senised *L. acicola* populatsioonianalüüsid on kinnitanud, et nimetatud patogeeni on Ameerikast Euroopasse introdutseeritud vähemalt kahel erineval korral (Janoušek et al., 2016). Lisaks on Euroopa populatsioonides kirjeldatud üldiselt madalamat mitmekesisust kui seene looduslikus areaalis Ameerikas (Sadiković et al., 2019; Adamčíková et al., 2021). Pruunvöötaudi tekitaja on Põhja-Euroopas avastatud suhteliselt hiljuti, mistõttu haigustekitaja täpne päritolu pole teada, niisamuti ka seene geneetiline mitmekesisus selles regioonis.

Käesolev doktoritöö annab ülevaate uuringute tulemustest pruunvöötaudi tekitaja *L. acicola* leviku kohta, patogeeni uutest peremeestaimedest, seene populatsioonigeneetikast, levikuteedest ja paljunemisviisidest Põhja-Euroopas. Käesoleva töö kontektis on Põhja-Euroopaks loetud Balti riikide, Poola põhjaosa ja Rootsi populatsioonid. *Lecanosticta acicola* levikuteede analüüsis arvestati Põhja-Euroopa regiooni hulka ka Valgevene ja Iirimaa seenetüved (**IV**).

Antud töös püstitati järgmised hüpoteesid:

1. Põhja-Euroopa tingimustes on looduslik harilik mänd (*Pinus sylvestris* L.) vastuvõtlik pruunvöötaudi tekitajale (*Lecanosticta acicola*) (**I**).
2. Sama puistu piires on *L. acicola* geneetiliselt sarnane ka erinevatel peremeestaime liikidel esinedes (**II**).

3. *Lecanosticta acicola* on Põhja-Euroopasse levinud mitme erineva introduktsiooni tulemusel Kesk-Euroopa populatsioonidest (**II**, **IV**).
4. Inimtegevus on mõjutanud *L. acicola* levikut ja populatsioonide mitmekesisust (**II**, **IV**).
5. *Lecanosticta acicola* ei ole vaid perekond männile (*Pinus*) spetsiifiline patogeen (**III**).

Töö põhieesmärgid olid järgmised:

1. Uurida *L. acicola* ulatust ja kahjustusi erinevatel peremeestaime liikidel (**I**, **III**).
2. Uurida *L. acicola* geneetilist mitmekesisust sama puistu piires erinevatel peremeestaime liikidel (**II**).
3. Dokumenteerida *L. acicola* levik ja päritolu Põhja-Euroopas (**II**, **IV**).
4. Uurida *L. acicola* geneetilist mitmekesisust, populatsioonistruktuuri ja paljunemisviise (**II**, **IV**).

Materjal ja metoodika

2016. aasta oktoobris koguti okkaproove Tartumaal (N 58.432259, E 26.787881) segapuistust kokku 43 harilikult männilt ja 28 mägimännilt. Kõigil puudel antud puistus hinnati kahjustatud okaste osakaal Bulman et al. (2004) poolt kirjeldatud metoodika kohaselt ning mõõdeti puu kaugus lähimast sümptomaatilisest puust (**I**).

Aastatel 2010-2017 koguti okkaproove üle Eesti pruunvöötaudi sümptomitega mändidelt, kokku kuuelt männi taksonilt: *P. mugo*, *P. mugo* var. *pumilio*, *P. sylvestris*, *P.* x *rhaetica*, *P. ponderosa* ja *P. uncinata* (**II**).

Aastatel 2017 ja 2018 koguti okkaproove pruunvöötaudi sümptomitega puudelt Atatürk arboreetumis Türgis (N 41.175010, E 28.984569). Kokku koguti proove 37 puult, mis kuulusid 28 erinevasse männiliste (*Pinaceae*) taksonisse. Kõigil puudel hinnati kahjustatud okaste osakaalud Bulman et al. (2004) järgi (**III**).

Lecanosticta acicola globaalse populatsioonianalüüsi koostamiseks koguti pruunvöötaudi sümptomitega okkaproove 27 riigist Põhja- ja Lõuna-Ameerikas, Euroopas ja Aasias (**IV**). Globaalses analüüsis kasutati ka *L. acicola* isolaate, mis olid kogutud ja käsitletud artiklites **I**, **II** ja **III**.

Sümptomaatilistest okkaproovidest, millelt leiti *L. acicola* viljakehi ja koniide isoleeriti seen puhaskultuuri Mullett ja Barnes (2012) kirjeldatud metoodika kohaselt. Isolaatide liik tuvastati molekulaarselt kasutades liigispetsiifilisi praimereid (Ioos et al., 2010) või sekveneeriti ITS regioon Sangeri meetodil (**I**, **II**, **III**, **IV**). Isolaatide paarumistüübid määrati kindlaks PCR meetodil kasutades paarumistüüpide spetsiifilisi praimereid (Janoušek et al., 2014) (**I**, **II**, **IV**).

Patogeeni haplotüüpide määramiseks kasutati 11 mikrosatelliidi markerite paari (Janoušek et al., 2014). Fragmentanalüüsiks koguti PCR produktid kahte paneeli vastavalt protokollile (Janoušek et al., 2014). Fragmentanalüüs viidi läbi Tartu Biokeskuses ning alleelide pikkuste mõõtmiseks kasutati programmi GeneMapper 5.0 (Applied Biosystems, USA) (**II**, **IV**).

Populatsioonigeneetika analüüsid viidi läbi, kasutades programme GenAlEx 6.5 (Peakall ja Smouse, 2012), ADZE v. 1.0 (Szpiech et al., 2008), STRUCTURE v. 2.3.4. (Falush et al., 2003), CLUMPAK (Kopelman et al., 2015), DIYABC v. 2.1.0 (Cornuet et al., 2014) (**II**, **IV**), POPTREE v. 2 (Takezaki et al., 2010), ja statistikaprogrammi R paketti *poppr* (Kamvar et al., 2014; R Core Team, 2017) (**IV**).

Tulemused ja arutelu

2016. aasta sügisel tuvastati Tartumaal Kõrvekülas, hariliku ja mägimänni puude grupis *L. acicola* nakkus esmakordselt Eestis ka hariliku männi isenditelt (**I**). Nakatunud olid vaid need harilikud männid, mis kasvasid kuni 2,5 meetri kaugusel lähimast nakatunud mägimännist. Kahjustatud okaste osakaal osutus puistus suuremaks mägimändidel. Vähem kui aasta varem, 2015/2016 aasta talvel olid nakatunud vaid mägimännid ning harilikel mändidel ei esinenud pruunvöötaudi sümptomeid (**I**).

Hariliku ja mägimänni puude grupis tuvastati kokku üheksa erinevat patogeeni haplotüüpi – neli mägimännilt ja seitse harilikult männilt. Neist kahte haplotüüpi leiti mõlemalt männiliigilt. Haplotüüp nr 31 osutus domineerivaks – seda tuvastati kokku 40 puult kolmes erinevas proovivõtu asukohas Eestis ning see osutus enimlevinud geneetiliseks tüveks mõlemal peremeestaime liigil ülalnimetatud hariliku ja mägimänni puude grupis. Segapuistus eri peremeestaimedelt isoleeritud patogeeni tüved jagasid sarnast populatsioonistruktuuri ega olnud geneetiliselt

oluliselt erinevad (**II**). Uue peremeestaime liigi nakatumine ja unikaalsete haplotüüpide esinemine harilikul männil aga ka virulentsema geneetilise tüve leidumine puistus võib olla patogeeni sugulise paljunemise tulemus.

Populatsioonigeneetilisteks analüüsideks koguti Eestist 104 *L. acicola* isolaati aastatel 2010-2017. Analüüsitud 11 lookuses tuvastati kokku 43 alleeli ning 50 haplotüüpi. Neli haplotüüpi leiti rohkem kui ühest asukohast Eestis ning pikim vahemaa sama klooni kahe leiukoha vahel oli 130 km. See on enam kui *L. acicola* suudaks looduslikult sedavõrd lühikese aja jooksul levida (Wyka et al., 2018). Tallinna Botaanikaaiast leiti kokku neli haplotüüpi, mida Eestis ei esinenud kusagil mujal. Eesti esimene dokumenteeritud *L. acicola* populatsioon Tallinnas osutus geneetiliselt erinevaks ülejäänud Eestit koondavast populatsioonist EST ($p=0.001$), mis viitab populatsioonide erinevale päritolule. Samuti on geneetiliselt erinevad patogeeni tüved dokumenteeritud invasiooni algusaastatest (2010-2015) võrrelduna hilisemate levikuaastatega (2016-2017) ($p=0.007$) (**II**). Eesti *L. acicola* populatsiooni geneetiline analüüs viitab inimtegevuse mõjule patogeeni levimisel ning tõenäoliselt ka patogeeni korduvale introduktsioonile geneetiliselt erinevatest lähtepopulatsioonidest.

Lecanosticta acicola globaalses populatsioonianalüüsis kasutati 650 seene isolaati 27 riigist. Analüüsitud 11 lookuses tuvastati kokku 172 unikaalset alleeli ja isolaatide hulgast 284 haplotüüpi. Leiti kokku 16 haplotüüpi, mida esines enam kui ühes riigis. Neist üks identne patogeeni haplotüüp esines mitmes asukohas Kanadas ja Müncheni botaanikaaias Saksamaal, mis viitab otseselt inimese-poolsele levitamisele kontinentide vahel (**IV**). *Lecanosticta acicola* populatsioonid on kõige mitmekesisemad USA lõunaosas ja Mehhikos, kus tuvastati madalaim klonaalsus ning suurim unikaalsete alleelide osakaal (**IV**). Euroopas on seene geneetiline mitmekesisus suurim Prantsusmaal, kus tuvastati patogeeni kahe erineva geneetilise liini esinemine. Üllatuslikult suur geneetiline mitmekesisus Põhja-Euroopa populatsioonides on tõenäoliselt põhjustatud patogeeni korduvast introduktsioonist, millele viitab ka killustunud populatsioonistruktuur (**IV**).

Tulemused viitavad, et Põhja-Euroopa *L. acicola* populatsioonid pärinevad teistest Euroopa piirkondadest ja mitte eraldi toimunud introduktsioonist Põhja-Ameerikast. Kui arvestada ka populatsioonide geograafilist paiknemist, jagatud haplotüüpe ja sarnast populatsioonistruktuuri

regioonide vahel, siis võib järeldada, et Põhja-Euroopa *L. acicola* populatsioonid pärinevad valdavalt Kesk-Euroopast (**IV**). On teada, et isegi ohtlike invasiivsete patogeenide kohalolu võib jääda peale esmast introduktsiooni veel mõneks ajaks märkamata vaid piiratud kahjustuse esinemise tõttu (Agan et al., 2022). Ka *L. acicola* populatsioonigeneetilised analüüsid viitavad võimalusele, et patogeen esines Põhja-Euroopas juba mõnda aega enne esimest dokumenteeritud leidu – isegi enam kui 30 seene generatsiooni tagasi (**IV**).

Kõigis Põhja-Euroopa riikides, välja arvatud Rootsis, domineerib patogeeni paarumistüüp MAT1-1 ning valdavalt levib seen klonaalselt. Seene suguline paljunemine on tõenäoline Poolas ning mõningates regioonides Eestis, näiteks hariliku ja mägimänni segapuistus ning Põhja-Eesti populatsioonis TLL (**II**, **IV**). Seni ei ole Põhja-Euroopas visuaalselt leitud *L. acicola* sugulist arengujärku.

Lecanosticta acicola levikuala on laienemas ka Lääne-Aasiasse, kus patogeen dokumenteeriti esmakordselt Gruusias ja Türgis (**III**, **IV**). Käesolevas doktoritöös tuvastati *L. acicola* esmakordselt Liibanoni seedrilt (*Cedrus libani*), mis on maailma esimene molekulaarselt kinnitatud leid *L. acicola* esinemisest peremeestaimel, mis ei ole perekonnast mänd (**III**).

Kokkuvõte

Kuigi valdav osa *L. acicola* leide Põhja-Euroopas pärineb eksoot-männiliikidelt on patogeen nakatanud ka harilikku mändi looduslikes tingimustes (**I**). Siiski on pruunvöötaudi tekitaja seni tuvastatud harilikul männil vaid haigusest kahjustatud mägimändide (*P. mugo*) läheduses. Käesoleva töö tulemuste põhjal on seni patogeeni esinemine harilikul männil Põhja-Euroopas tagasihoidlik ja kahjud marginaalsed. Kinnitust leidis, et sama puistu piires ei ole eri peremeestaimeliike nakatanud patogeeni tüved geneetiliselt oluliselt erinevad ning sama geneetiline tüvi võib nakatada ka mitut männiliiki (**II**).

Lecanosticta acicola on Põhja-Euroopasse levinud tõenäoliselt Kesk-Euroopast (**IV**). Patogeeni levikule on kaasa aidanud inimtegevus – nii pikemal distantsil kontinentide ja riikide vahel, kui ka riigisiseselt. Korduv introduktsioon geneetiliselt erinevatest patogeeni populatsioonidest on suurendanud oluliselt ka seene populatsioonide mitmekesisust Põhja-Euroopas (**II**, **IV**). Kiire areaali laienemine ning uute peremeestaimede,

kaasa arvatud männi perekonda mitte kuuluvate liikide nakatamine (**III**), peegeldab selle invasiivse patogeeni potentsiaali muutuda Euroopas senisest ohtlikumaks kahjustajaks.

Doktoritööst tulenevad praktilised soovitused:

1. Oluline on jätkata *L. acicola* seirega, et jälgida seene levikuala muutusi ning hinnata patogeeni ohtlikkust. Lisaks tuleb seirata nakatunud mändide läheduses ka teistesse okaspuude perekondadesse kuuluvaid isendeid. Eelistatult peaks seire olema DNA-põhine, et tuvastada patogeeni nakkus ka latentses arengufaasis.
2. Karantiinireeglid vajavad täiendamist. Kuni imporditavate taimede kontrollis ei kasutata molekulaarseid meetodeid, ei takista sisuliselt mitte miski nakatunud taimede transporti riikide vahel ning koos sellega uute patogeeni liikide ja nende uute geneetiliste tüvede introduktsiooni, kuna nakkus pole alati visuaalselt nähtav. Oluline on vältida juba saabunud, s.o. invasiivsete patogeenide populatsioonide mitmekesisuse suurendamist, mida võib põhjustada patogeeni korduv introduktsioon geneetiliselt erinevatest lähtepopulatsioonidest.
3. Metsa uuendamisel ja haljastuses tuleb eelistada kodumaiseid või eksootilisi haiguskindluse suhtes kontrollitud puude järglasi. Patogeenile vastuvõtlikke peremeestaime liike ei tohiks kasvatada taimlate läheduses, et vältida nakkuse ülekandumist istutusmaterjalile, mis tooks kaasa majandusliku kahju, kui taimed tuleb hävitada. Taimlas nakkuse märkamata jäämine põhjustaks probleeme metsakultuuri elujõulisuses ning levitaks haigustekitajat.
4. Kuna *L. acicola* pole Euroopa Liidus enam karantiinne patogeen, vaid on reguleeritud mitte-karantiinne liik, siis pole õiguslikku alust nõuda nakatunud puude hävitamist väljaspool puukoole ja taimlaid. Arboreetumites või teistes puude kollektsioonides on väärtuslike isendite puhul võimalik haiguse levikut kontrollida kombineerides tõrjet fungitsiididega ning okkavarise kogumist ja põletamist.

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I

ORIGINAL PUBLICATIONS

Adamson, K., **Laas, M.,** Drenkhan, R., Hanso, M. 2018. Quarantine pathogen *Lecanosticta acicola*, observed at its jump from an exotic host to the native Scots pine in Estonia. Baltic Forestry. 24 (1), 36−41.

Quarantine Pathogen *Lecanosticta acicola*, Observed at Its Jump from an Exotic Host to the Native Scots Pine in Estonia

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Abstract

In October 2016, the causal agent of Brown Spot Needle Blight (BSNB) *Lecanosticta acicola* (Thüm.) Syd. was discovered for the first time on young native Scots pines in a small mixed mountain pine/Scots pine stand in central-eastern Estonia. The pathogen was isolated from both pine species and identified by molecular methods. Both ITS sequences were deposited in GenBank. It is the first record of the fungus on Scots pine in northern Europe confirmed by the molecular methods. An intensive monitoring of this EPPO quarantine pest has started at this apparently initial point of infection of BSNB in Estonia and in other mixed exotic/native pine stands. When searching for Scots and other pine species in northern Estonia infected by *L. acicola*, *Pinus* × *rhaetica* Brügger was added to the world host list of *L. acicola*.

Keywords: invasive species, quarantine pest, *Mycosphaerella dearnessii*, *Pinus sylvestris, P. mugo*, *P.* × *rhaetica*.

Introduction

Lecanosticta acicola (Thüm.) Syd. is a foliage pathogen that causes Brown Spot Needle Blight (BSNB) on various pines (*Pinus* spp.). The pathogen was first described in the 19th century by F. Thümen (1878) in South Carolina, USA. By now, in addition to North America, this pathogen has invaded many regions in Europe, Asia, Africa and South America (Lévy and Lafaurie 1994, Patton 1997, Suto and Ougi 1998, CABI/EPPO 2010), infecting more than 30 different pine species (Tainter and Baker 1996, Sinclair and Lyon 2005). As stated by EPPO (2010), potentially all pines can act as hosts of *L. acicola*.

During the early 2010s, several outbreaks of BSNB were reported on Eastern white pine (*P. strobus* L.) needles in the northern USA and eastern Canada (Broders et al. 2015). BSNB has also been reported in southern, central and western Europe, e.g. in Austria, Czech Republic, Italy, Slovenia, and Switzerland (Holdenrieder and Sieber 1995, La Porta and Capretti 2000, Cech and Krehan 2008, Jankovský et al. 2009a, 2009b, Jurc and Jurc 2010; EPPO 2015, 2016).

Still, *Pinus sylvestris,* the single native pine species in the Nordic countries of Europe, has been discovered to be infected by *L. acicola* in North America (Skilling and Nicholls 1974, Huang et al. 1995), also as an exotic for that continent host species. In central Europe, there are only few references regarding the fungus on native *P. sylvestris*: in Austria (Cech and Krehan 2008, EPPO 2012b, 2015) and in Slovenia (EPPO 2008, Jurc and Jurc 2010). However, these reports lack molecular affirmation – that is, there are no deposited sequences of the pathogen species in GenBank – and this infectious agent can easily be confused with the agent of Dothistroma Needle Blight (DNB), *Dothistroma septosporum* (Dorog.) M. Morelet, when identified by morphological characteristics.

Concerning the general occurrence of *L. acicola* in northern Europe, in 2008 this fungus was first detected in Estonia: the symptoms of BSNB were noticed on *Pinus ponderosa* Lawson in Tallinn Botanic Garden (Drenkhan and Hanso 2009). The pathogen was isolated and investigated by molecular methods, and the ITS sequence was deposited in GenBank (Adamson et al. 2015). In 2009, in Lithuania, *L. acicola* was molecularly identified for the first time by Markovskaja et al. (2011) on *P. mugo* and afterward described there by EPPO (2012b) in three *P. sylvestris* and *P. mugo* needle samples. However, the Lithuanian identification of *L. acicola* on *Pinus sylvestris* is still not registered in the GenBank database.

In the following years, several new cases of *L. acicola* (in new areas of infection) were registered in Estonia on *P.*

mugo, P. mugo var. *pumilo* (Haenke) Zenari and *P. uncinata* Ram. (Adamson et al. 2015). In 2012, BSNB symptoms were reported in Latvia on a single *Pinus pumila* tree in the National Botanical Garden of Latvia in Salaspils, where the identity of *L. acicola* was confirmed by PCR-based methods at the ANSES Plant Health Laboratory in France (EPPO 2012a). Unfortunately, as late as in February 2017 no pure cultures or sequenced strains of the fungus from Latvia were available, which could affirm the identification and be used to determine the origin of the fungus in the Baltic countries and northern Europe.

Based on the higher represented data, BSNB and its agent *L. acicola* have deserved serious attention for the possible emergence of an epidemic on native Scots pines. Until 2016, *L acicola* has been found in the northern Baltics only on exotic pine species, these having silvicultural importance mainly as ornamental trees in parks and botanical gardens. However, the pathogen would present a danger to extensive forest stands only if it will be discovered on the native pine in the northern Europe, the Scots pine (*Pinus sylvestris* L.). The aim of this study was to investigate native pines growing in the immediate vicinity of the regions known as being infected by *L. acicola* exotic pine species in Estonia, since these could be at the highest risk.

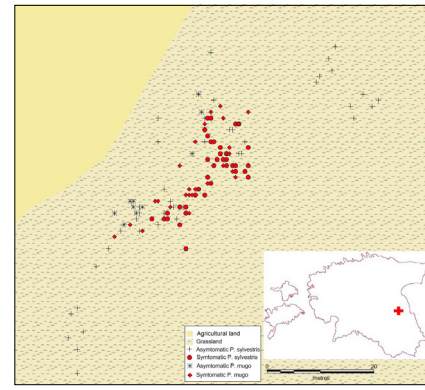

Figure 1. Map of the infected by *L. aciola* young mixed *P. mugo*/*P. sylvestris* stand in Estonia

Materials and Methods

Sampling

In October 2016, several 4-13 years old *P. sylvestris* trees, symptomatic to *L. acicola*, were noticed in a young mixed *P. mugo*/*P. sylvestris* stand (County of Tartu, 58.43237ºN, 26.78829ºE) in Estonia (Figures 1 and 2).

At once, the other six known nidi of *L. acicola* on exotic pine species as well as Scots pine trees growing in their neighbourhood were examined for occurrence of the pathogen.

The nidus in Tartu county, where *L. acicola* was first recorded on Scots pine is a mixed, naturally regenerated mountain pine/Scots pine stand growing in a ca 110 × 30 m area (Figure 1), surrounded by extensive agricultural fields. A next group of solely Scots pine individuals growing, several hundreds of metres away, was also examined.

Every tree in that *L. acicola* nidus (Figure 2) was classified as symptomatic or asymptomatic, and the infection level and year class of symptomatic needles was assessed. The mean height of the *P. mugo* trees was 5 m and 8 m of the elder *P. sylvestris* trees, the last ones being apparently the parents of the younger, ca 4-6 years old, from 0.6 m to 1.2 m high, found as infected *P. sylvestris* trees.

The infection level was assessed as shown in Bulman et al. (2004), where scoring was done in 5% steps of crown volume (of needles) present on the diseased tree. Additionally,

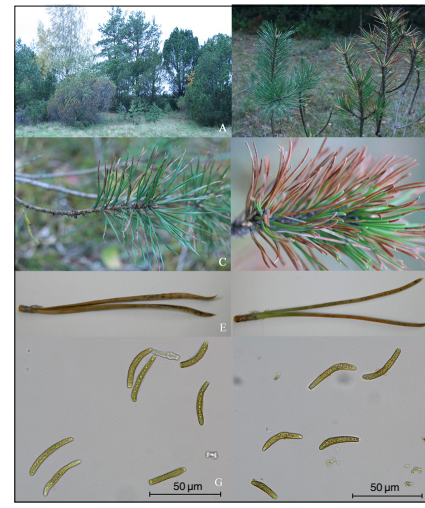

Figure 2. The investigated mixed stand of *P. mugo*/*P. sylvestris,* infected by *L. acicola.* A) the site; B) *P. sylvestris* (left) next to the heavily infected *P. mugo* tree (right); C) *P. sylvestris* shoot with the infected by *L. acicola* needles; D) *P. mugo* shoot with the heavily infected by *L. acicola* needles; E) *P. sylvestris* needle, infected by *L. acicola*; F) *P. mugo* needle, infected by *L. acicola*; G) conidia of *L. acicola* on *P. sylvestris*; H) conidia of *L. acicola* on *P. mugo*

the numbers of fruiting bodies per symptomatic needle pair (5 needle pairs per tree) on 15 randomly-selected trees of both host species were counted. For all trees the distance from the closest symptomatic tree in its area was measured. In total 135 symptomatic and asymptomatic trees were assessed, including samples for phytopathological analyses collected from 43 *P. sylvestris* and 28 *P. mugo* symptomatic trees.

Morphological measurements (length and width) of 30 random fruiting bodies and 30 of conidia for both host species were carried out according to the program NIS Elements 4.12.01 (Nikon, Japan). Spore counting was done as follows: 3 fruiting bodies from separate needles of one tree per species were crashed in water drop for a slide. Conidia were counted from three random areas on the slide under the 60x magnification (which determined the field of vision) under the 10x ocular lens. It means that per both host species 30 fruiting bodies were counted from 30 random places of 10 slides. Average number of conidia was calculated per each 60× magnification area, the result of which was later called as "index of conidia".

Isolation of L. acicola

The needles were first rinsed in 96% ethanol, the conidiomata were scraped from the needle surface under sterile conditions, placed on pine needle agar medium and, to release the conidia, rolled along its surface as described by Mullett and Barnes (2012). The pine needle agar medium (PNA) was used, prepared, according to Drenkhan and Hanso (2009). The plates were incubated at room temperature (21 °C) and the germinated conidia with mycelia were transferred onto fresh PNA plates.

DNA extraction, PCR, and sequencing

For DNA extraction, a small amount of mycelium from pure culture was transferred into 2 ml sterile micro-centrifuge tubes. Until DNA extraction, the samples were stored at -20 °C. Cell disruption was carried out as described by Drenkhan et al. (2014). DNA was extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit (Lithuania), following the manufacturer's protocol.

For the identification of *L. acicola* the species-specific primers LAtef-F (5'-GCAAATTTTCGCCGTTTATC-3') and LAtef-R (5'-TGTGTTCCAAGAGTGCTTGC-3') were used (Ioos et al. 2010). The conventional PCR cycling conditions and reaction mixture were performed in 20 µl reaction volumes, according to Ioos et al. (2010), with some modifications (see Drenkhan et al. 2014). PCR products were visualized on 1 % agarose gel (SeaKem® LE Agarose, Lonza) under UV light. The results were visualised using the Quantum ST4-system (VilberLourmat SAS, Marne-la-Vallée, France).

The identity of *L. acicola* was confirmed in pure cultures by sequencing the internal transcribed spacer (ITS) region. ITS-PCR was performed using the fungal-specific PCR primers ITS1-F (5' -CTTGGTCATTTAGAGGAAGTAA-3', Gardes and Bruns 1993) and ITS4 (5' -TCCTCCGCTTATTGATATGC-3' , White et al. 1990). PCR products were sequenced using the primer ITS5 (5' -GGAAGTAAAAGTCGTAACAAGG-3' , White et al. 1990) at the Estonian Biocentre in Tartu. The sequences were edited with the BioEdit version 7.2.5. BLAST searches for the fungal taxa were performed at GenBank (NCBI).

Identification of mating types

For establishing the occurrence and distribution of mating types of the fungus in symptomatic needles, the following mating type primers of *L. acicola* were used: Md MAT1-1F (5'-CGCATTCGCACATCCCTTTGT-3'), Md MAT1-1R (5'-ATGACGCCGATGAGTGGTGCG-3') and Md MAT1-2F (5'-GCATTCCTGATCTACCGTCT-3'), Md MAT1-2R (5'-TTCTTCTCGGATGGCTTGCG-3') (Janoušek et al. 2014). The PCR cycling conditions and reaction mixture were performed in 20-µl reaction volumes according to Janoušek et al. (2014), with some few modifications (see Adamson et al. 2015).

Results

For the first time, BSNB was diagnosed on *P. mugo* in that young mixed pine stand in Tartu county in winter 2015/2016. No infected *P. sylvestris* trees among and around the heavily infected *P. mugo* tree groups could be found.

When visiting the same area in October 2016, when finding of first infected by *L. acicola* Scots pines was approved, needle samples were collected from 43 symptomatic *P. sylvestris* and 28 *P. mugo* trees. In the laboratory, 33 and 25 of those samples, respectively, were found to be carrying conidiomata with ripe conidia of *L. acicola*. Instantly it was also noticed, that *Dothistroma septosporum,* though commonly found in many areas nowadays, was not represented on those infected by BSNB trees. Since this observation marked a landmark event in the colonization process of Estonia by the quarantine pathogen *L. acicola*, from the conidiomata on *P. sylvestris* needles in total 28 pure cultures of *L. acicola* were isolated. Four *L. acicola* isolates were sequenced and deposited in GenBank (Table 1). On both host species, the MAT1-1 was found dominating, with 89.3% on *P. sylvestis* and 94.4% on *P. mugo.*

Not all *P. sylvestris* trees were infected. Some individuals were visually evaluated as healthy despite growing between heavily infected trees of *P. mugo* and/or *P. sylvestris*. All the infected *P. sylvestris* trees, however, were observed growing only near to the infected *P. mugo* trees. Farther away than 2.5 m from the closest infected *P. mugo* tree, all *P. sylvestris* trees were visually classified as uninfected.

Table 1. Deposition of sequences and samples of *L. acicola* from *P. sylvestris*, *P. mugo* and *P.* × *rhaetica* in GenBank and in mycological herbaria

Host	Sampling date	GenBank accession no.	Mating type [a)]	Herbarium [b)] deposition no.
P. sylvestris	20.10.2016	KY576855	MAT1-1	TAAM206890
P. sylvestris	20.10.2016	KY576854	MAT1-2	TAAM206893
P. mugo	20.10.2016	KY576853	MAT1-1	TAAM206982
P. mugo	20.10.2016	KY576852	MAT1-2	TAAM206891
P. x rhaetica	17.08.2016	KY620271	MAT1-1	TAAM200894

a) Mating type priming PCR (as in Janoušek et al. 2014)
b) TAAM, Mycological herbarium of the Estonian University of Life Sciences

On average, 17% of the needles of *P. sylvestris* and 63% of *P. mugo* needles on infected (i.e. symptomatic) sampled trees were infected by *L. acicola*. On 61% of the symptomatic *P. sylvestris* trees (N=43) the youngest (2016. year-class) needles were also found to be infected, while on *P. mugo* trees (N=28) this value was 86%. An average of 5.1 fruiting bodies per infected needle pair were found in *P. sylvestris* and 4.1 in *P. mugo*. The dimensions of fruiting bodies (N= 30) per each infected host species varied and were 100.9 (405.6)-802.4 x 88.5-(134.4)-186.1 µm and 186.1-(468.2)-845.4 x 111.9-(157.0)-213.9 µm, on *P. sylvestris* and *P. mugo,* respectively. Dimensions of conidia varied on *P. sylvestris* between 21.4 (40.1)-51.7 x 3.8- (5.2)-6.3 µm and in *P. mugo* 27.1-(39.6)-54.1 x 4.2-(5.3)-6.7 µm. Index of conidia on *P. sylvestris* was 1.1 and on *P. mugo* 0.9. As visible, no significant differences in spore dimensions could be found.

Immediately after the discovery of *L. acicola* on *P. sylvestris* in Tartu county, the Scots pine trees growing in or around other Estonian nidi of *L. acicola* on exotic pine species were examined for occurrence of the pathogen. First, it was established that only in Tallinn Botanic Garden some 40-year-old Scots pine trees were growing close nearby (an infected *P. ponderosa* tree stem was located to the nearest Scots pine tree at the distance of 24 m, and at the distance of 13 m between the closest tree crown borders). These Scots pines turned to be asymptomatic, but *L. acicola* was identified on a *P.* × *rhaetica*, also growing in the immediate vicinity of the infected *P. ponderosa* tree.

It is the first recorded observation of *L. acicola* on *P. x rhaetica* anywhere. In other known nidi of *L. acicola* on *P. mugo* in Estonia, the nearest Scots pines were found growing at least several hundreds of metres away from the infected mountain pines.

Discussion

Abundant infection of young native Scots pine (*Pinus sylvestris)* trees by *Lecanosticta acicola* in a limited area of Estonia, documented by identification of the pathogen by isolation and ITS sequence, is apparently the first observation in the northern Baltics, and indeed for all northern Europe, of the EPPO quarantine disease BSNB. In the winter of 2015/2016, no infected *P. sylvestris* trees were found in this mixed mountain pine/Scots pine stand, which eight months later turned out to be seriously infected by BSNB. Therefore, it appears that we may have observed a host jump at the very beginning of the process, which could either fade in the future or develop into a serious epidemic.

Considering the large economic and environmental role of Scots pine throughout Europe, it was fully understandable that in most of the papers describing the first national observations of BSNB (e.g. in Switzerland by Holdenrieder and Sieber 1995, in Italy by La Porta and Capretti 2000, in the Czech Republic by Jankovský et al. 2009a, 2009b; in Estonia by Adamson et al. 2015) the question of infection of Scots pine by BSNB was seriously considered. All these investigations found no such infection.

Cases of BSNB on Scots pine in central Europe described during the past decade, though still lacking information in GenBank databases, have apparently still not developed into serious epidemics, although this possibility has been considered (e.g. Cech and Krehan 2008). However, the behaviour of the Dothistroma Needle Blight (its agent *Dothistroma septosporum*) that preceded the BSNB in Estonia should not be forgotten. After being restricted for a few years to exotic pine species, DNB started, step by step, to infect and damage the native Scots pine widely. Therefore, as a minimum an improved monitoring of BSNB on Scots pines in Estonia should occur, first in nearby infected exotic pine species and then, if needed, more widely in managed forest stands. Climate warming and selective pressure on generative variability of the pathogen may make northern Europe an epicentre of an epidemic of BSNB on native Scots pine.

Individual Scots pine plants may differ in their heritable susceptibility or resistance, and this may be particularly the case in this naturally regenerated young mixed stand of *P. mugo* and *P. sylvestris* in Tartu county. It may explain the large variation when discussing the level of infection, which in our investigation ranged from 0 to 90%. Also, the pathogen should have experienced a similar natural selection pressure at the very beginning of the colonization of *P. sylvestris* as of a new host species, which is more adapted to the Estonian environment than is this alien pathogen itself.

We observed *L. acicola* also on the youngest, that is, on the first-year needles of Scots pine. Angst and Engesser (2009) have described the same in Switzerland and Jurc and Jurc (2010) in Slovenia. When observing the development of the epidemic of DNB and its agent

D. septosporum in Estonia, we documented this pathogen on the first (current) year needles of *P. sylvestris* only several years after the first discovery of the fungus on exotic pine species and a few years after the initial discovery of it on older needles of Scots pine (Drenkhan and Hanso 2009, Drenkhan et al. 2016). This comparison clearly indicates a higher virulence of *L. acicola* versus *D. septosporum*.

D. septosporum, now generally occurring on Scots pines in Estonia, was missing on infected by *L. acicola* trees. Similar observations are known from Austria (Brandstetter and Cech 2003). Jankovský et al. (2009a) also could not observe both pathogen species on the same trees. However, in Lithuania, the both pathogens have been found on the same trees and needles of *P. mugo* (Markovskaja et al. 2016). Also, Ortíz de Urbina et al. (2017) have reported in Spain such an association of both pathogens on the same trees of *P. sylvestris* and, as well, of *P. radiata*.

Mating type MAT1-2 of *L. acicola* arrived in Estonia much later and apparently through an introduction separate from that of MAT1-1 (Adamson et al. 2015), and this possibly can explain why it is currently so rare in exotic pine plantations, as discussed above. Recent genetic analyses have generally suggested separate introductions of *L. acicola* into Europe (Janoušek et al. 2016), and apparently the same also may have happened in Estonia.

Artificial inoculations with *L. acicola* carried out in Japan seasonally in June resulted in heavy infection, while inoculations performed in September resulted only in slight infection (Suto and Ougi 1998). Further research should establish if similar seasonal variations are essential to be considered at creation of the effective control measures for northern Europe.

At present, it is hard to forecast whether-or-not the host jump of *L. acicola* in the natural conditions of northern Europe, discussed in this paper, will bring about any further changes in the genetic background of the pathogen, including possible alterations in virulence. Therefore, genetic investigations—including population genetic studies—should be continuously conducted.

In the mixed *P. mugo/P. sylvestris* stand, that we studied, farther than 2.5 m from the closest infected *P. mugo,* no infected *P. sylvestris* trees could be found but it was only the first year of the process under observation. Concerning applied results of this investigation, we recommend, however, that planting of exotic pine species in the immediate vicinity of native Scots pines should be avoided.

It is also the first recorded observation of *L. acicola* on *P. x rhaetica* anywhere. Taxonomic position of *P.* × *rhaetica*, close to the both infected pine species under investigation in the nidus in Tartu county, certainly awake need for a more thorough investigation of the taxonomy of the found as infected *P. sylvestris* young trees. Observable today vegetative organs of these classified as Scots pine young trees were typical for *P. sylvestris*. If some still not documented in the Estonian nature putative hybrids of *P. sylvestris*/*P. mugo* are actually exist, then a question rises about their possible role in the transmission of this pathogen from the exotic mountain pine to the native Scots pines, forming extensive forest stands.

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A look into the genetic diversity of *Lecanosticta acicola* in northern Europe

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ABSTRACT

For northern Europe *Lecanosticta acicola* is an emerging pine needle pathogen. This study gives a first look into the population genetics of the pathogen in Estonia, the first population documented in that region. The main aim of this study was to investigate the genetic diversity and population structure of the pathogen in this new region for the fungus. For this purpose, 104 isolates from 2010 to 2017 were analysed with 11 microsatellite and mating type markers. The stand where the pathogen's jump from an exotic host to the native Scots pine was recorded was also involved in this analysis. The analysis revealed low genetic diversity and a high number of clones that indicated *L. acicola* is an invasive species in northern Europe. Results suggest that several separate introductions have taken place and anthropogenic activity has apparently affected the spread of the pathogen. Clonal reproduction is dominating and although sexual reproduction is possible, it probably takes place infrequently.

1. Introduction

During this young century, several new forest pathogens have already reached northern Europe, including Estonia (Drenkhan and Hanso, 2009). Generally, nonindigenous pathogens harm the environment and cause major economic loss. For instance, in the United States, it has been estimated that non-native forest pathogens are causing damage totalling 2.1 billion dollars each year (Pimentel et al., 2000) and in Great Britain the annual cost of nonindigenous pathogens to forestry reaches over 1.3 million pounds (Williams et al., 2010).

Lecanosticta acicola (Thümen) A. Sydow. is a pathogen that causes a foliar disease named Brown Spot Needle Blight (BSNB) on many *Pinus* species, where damage is expressed as premature needle shedding that results in growth reduction and even possible death of infected trees (EPPO, 2008). The pathogen was first described in 1876 in South Carolina, USA (Thümen, 1878), but several studies and genetic analyses indicate that it is actually native to Central America (Evans, 1984; Janoušek et al., 2016), from where it has spread to North America, Europe and Asia (Huang et al., 1995; Janoušek et al., 2016; Suto and Ougi, 1998). *L. acicola* has proven to be a very adaptable pathogen since it is now present in different climate regions around the world and infects more than 30 pine species (Janoušek et al., 2016; Sinclair and Lyon, 2005; Tainter and Baker, 1996). The pathogen also belongs to the A2 list of quarantine pathogens posted by the European and Mediterranean Plant Protection Organization (EPPO).

In southern and central regions of Europe the pathogen has been observed for decades (Jankovský et al., 2009; La Porta and Capretti, 2000; Lévy and Lafaurie, 1994; Pehl, 1995), but in northern Europe it was found for the first time in 2008 in Estonia on non-native *Pinus ponderosa* (Drenkhan and Hanso, 2009). During the following years, *L. acicola* expanded its range in Estonia, but was still found infecting only non-native pine species (Adamson et al., 2015), until in 2016 it was detected for the first time in Estonia and northern Europe on native *Pinus sylvestris* (Adamson et al., 2018a). In addition, the Estonian population of the pathogen is apparently the northernmost in the world currently registered. In northern Europe, the pathogen has also been documented in Lithuania (Markovskaja et al., 2011), Latvia (EPPO, 2012a; Mullett et al., 2018) and most recently in southern Sweden (Cleary et al., 2019), but there is still no documented information about its occurrence in neighbouring Finland.

Lecanostica acicola has two mating type idiomorphs – *MAT1-1* and *MAT1-2* (Janoušek et al., 2014). During the six years after its first discovery in Estonia, only *MAT1-1* was found to be present.

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Thereafter, in 2014, the second idiomorph *MAT1-2* was detected here (Adamson et al., 2015). Still, although the presence of both mating types has been documented in Estonia, the sexual state of the pathogen has not been recorded visually (Adamson et al., 2018a). When reproducing asexually, the pathogen spreads via conidia that are able to spread only short distances, reaching mostly the same or a neighbouring tree (Skilling and Nicholls, 1974; Tainter and Baker, 1996), but with small numbers of conidia sometimes registered even 60 m away (Wyka et al., 2018). As generally known, the sexual recombination may increase the genetic diversity of the pathogen and develop strains that are genetically suitable to a new environment and therefore more viable (Gandon et al., 1996; McDonald and Linde, 2002; Milgroom, 1996). Also, sexual recombination would produce ascospores that are airborne and able to spread long distances (Kais, 1971).

Recently there has been a growing interest about BSNB in northern Europe because this area represents a new environment for *L. acicola*. Our observations have witnessed that the pathogen is expanding here fast and has already succeeded to infect the lone native pine species *P. sylvestris* (Adamson et al., 2018a). However, so far population studies of *L. acicola* haven't been carried out in northern Europe. In the previous papers about BSNB in Estonia it has been hypothesised that, after the first arrival in northern Estonia, the pathogen probably has spread here from north to south and, also, that the possibility of several separate introductions of genetically different strains should not be neglected (Adamson et al., 2015, 2018a). Although as a result of yearly monitoring and regular sample collection the spread of *L. acicola* is well documented in Estonia, there has still been a distinct lack of knowledge concerning the genetic diversity and population structure that would support conclusions about the history of introduction, possible distribution pathways and the viability of the pathogen's population in the new Nordic environment.

The objectives of this study were: i) to document the genetic diversity and population structure of *L. acicola* in Estonia, ii) to study the genetic diversity of *L. acicola* in a mixed stand of different host species – a non-native (*Pinus mugo*) and native (*P. sylvestris*), iii) to determine the frequency of mating types and evaluate the possibility of sexual reproduction or random mating occurring in the population.

2. Materials and methods

2.1. Sample collection, fungal isolation, DNA extraction and molecular identification

During the period of 2010–2017, samples of pine needles with symptoms of BSNB were collected from random sampling sites across Estonia, from visibly symptomatic trees only (Table 1, Fig. 1). Samples were collected from 6 different pine species (incl. one variety): *Pinus mugo*, *P. mugo* var. *pumilio*, *P. sylvestris*, *P.* x *rhaetica*, *P. ponderosa* and *P. uncinata*. In addition, samples were collected from the mixed stand of exotic *P. mugo* and native *P. sylvestris*, which was the first site for Estonia and northern Europe, where *L. acicola* was identified on native *P. sylvestris* (Adamson et al., 2018a). Only one isolate per sampled tree was used in further analyses. In total, 104 isolates of *L. acicola* were used in the analyses.

In order to obtain single individuals, isolations to pure cultures were made from single germinated conidia per conidiomata on an infected needle per tree according to Mullett and Barnes (2012). Isolates were grown at room temperature (21 °C) on pine needle agar media that was prepared as described by Drenkhan et al. (2013). DNA was extracted from pure cultures that had distinctive morphological features, characteristic to *L. acicola* (Pehl et al., 2015). In sterile conditions mycelium from the colony edge was transferred into 2.0 ml micro centrifuge tubes that were stored at −20 °C until DNA extraction. For mycelium homogenization a Retsch MM400 homogenizer (Retsch GmbH, Haan, Germany) was used with sterile metal beads (Ø 2.5 mm). DNA was extracted using a Thermo Scientific GeneJET Genomic DNA Purification Kit (Lithuania) according to the manufacturer's instructions.

The species were confirmed by PCR with species-specific primers LAtef-F and LAtef-R in 20 µl reaction volumes according to Ioos et al. (2010). PCR reactions were carried out using a TProfessional Thermocycler (Biometra, Göttingen, Germany). PCR products were visualized on 1 % agarose gel (SeaKem® LE Agarose, Lonza) under UV light using a Quantum ST4-system (VilberLourmat SAS, Marne-la-Vallée, France). Positive amplification in gel electrophoresis confirmed the presence of *L. acicola*.

2.2. Genetic analyses

2.2.1. Haplotype identification

For multilocus haplotyping, 11 microsatellite markers were used: MD1, MD2, MD4, MD5, MD6, MD7, MD8, MD9, MD10, MD11 and MD12 (Janoušek et al., 2014). The PCR mix was prepared and reaction carried out as described in Janoušek et al. (2014, 2016). For fragment analysis, PCR products were pooled into two panels according to Janoušek et al. (2014) and run on an Applied Biosystems 3130XL (Applied Biosystems) genetic analyser at the Estonian Biocentre in Tartu. Alleles were scored using GeneMapper 5.0 (Applied Biosystems, Carlsbad, USA).

Isolates with identical multilocus haplotypes were considered clones. Two datasets were created: one containing all isolates (non-clone-corrected (non-cc)) and the other containing only one of each haplotype per population (clone-corrected (cc)).

2.2.2. Mating type determination

Mating types of the isolates were determined using mating type primers developed by Janoušek et al. (2014). PCR reactions were carried out in 20 µl volumes as described in the protocol presented by Janoušek et al. (2014), with changes in the initial denaturation step of 95 °C for 12 minutes according to Adamson et al. (2015). PCR products were visualized with gel electrophoresis as described before. The expected size of the PCR products was 560 bp for *MAT1-1* and 288 bp for *MAT1-2*. Two strains of *L. acicola* (from USA and Canada, respectively) were used for reference.

2.3. Statistical analyses

2.3.1. Formation of populations

Isolates of *L. acicola* were divided into three geographical populations according to the location of sampling site: Tallinn (TLL), Central Estonia (CE) and Tartu (TRT) (Fig. 1). The Tallinn population includes isolates from the Tallinn Botanic Garden, the first site where *L. acicola* was found in Estonia (Drenkhan and Hanso, 2009), and therefore it was investigated as a separate population, as possibly the primary source in the pathogen's colonization of the country. In GenAlEx 6.5 (Peakall and Smouse, 2012) an analysis of molecular variance (AMOVA) was performed to test for the significance of differentiation between the formatted populations. The clone-corrected dataset was used for that purpose. Geographical populations that were not significantly different from each other according to AMOVA ($p > 0.05$), were merged for further analyses.

Isolates obtained from the mixed stand of *P. mugo* and *P. sylvestris*, which is situated less than 10 km from Tartu, were considered as a part of the TRT population. This stand concluded two separate sub-populations for both host species, indicated as PMUG and PSYL, respectively, which were grouped in order to test

Table 1
Data of *Lecanosticta acicola* isolates used in the study.

Location no.	Sampling site	Sampling date	Geographical coordinates		Host species	No. of isolates	Geographical population	Population according AMOVA[a]
			N	E				
1.	TBG[b]	13.05.2010	59.46907	24.88347	*Pinus ponderosa*	1	TLL[c]	TLL[c]
		17.11.2011				1		
		19.08.2013				1		
		20.09.2013				1		
		15.08.2011			*P. mugo* var. *pumilio*	1		
		15.09.2011				1		
		15.08.2011			*P. uncinata*	1		
		17.08.2016			*P. mugo*	1		
		17.08.2016			*P.* x *rhaetica*	1		
2.	Viimsi	05.11.2016	59.49961	24.83463	*P. mugo*	1		
		27.11.2016				2		
3.	Merivälja street	27.11.2016	59.48062	24.84102	*P. mugo*	3		
4.	Pirita	05.11.2016	59.46222	24.82599	*P. mugo*	1		
5.	Tori	04.07.2013	58.46614	24.78436	*P. mugo*	1	CE[c]	EST[c]
		29.10.2015			*P. mugo*	1		
6.	Kärdla	04.08.2014	58.99827	22.74815	*P. mugo*	4		
7.	Adavere	17.08.2016	58.70577	25.90078	*P. mugo*	1		
8.	Aegviidu	18.08.2016	59.27575	25.62464	*P. mugo*	1		
9.	Sillamäe	20.08.2016	59.39961	27.76655	*P. mugo*	2		
10.	Türi	27.09.2016	58.81225	25.40803	*P. mugo*	3		
11.	Lääne-Virumaa	27.09.2016	59.44669	26.40755	*P. mugo*	2		
12.	Väätsa	09.10.2016	58.88993	25.45269	*P. mugo*	1		
13.	Kaarepere	03.10.2016	58.66109	26.51598	*P. mugo*	1	TRT[c]	
14.	Vasula	20.06.2012	58.47143	26.74399	*P. mugo*	1		
15.	Kärevere	15.10.2014	58.43980	26.45623	*P. mugo*	1		
		20.01.2015				2		
		28.10.2015				2		
16.	Vedu	21.07.2015	58.49552	26.76925	*P. mugo*	1		
17.	Vastse-Kuuste	03.11.2015	58.16658	26.93347	*P. mugo*	1		
18.	Värska	03.11.2015	57.94327	27.65031	*P. mugo*	1		
		10.11.2016				5		
19.	Mikitamäe	03.11.2015	58.00079	27.54155	*P. mugo*	1		
		10.11.2016			*P. mugo*	1		
20.	Rannu	20.06.2016	58.24006	26.21542	*P. mugo*	2		
21.	Lähte	20.10.2016	58.49297	26.68247	*P. mugo*	2		
22.	Kõrveküla	20.10.2016	58.42120	26.77127	*P. mugo*	1		
23.	Tartu	20.10.2016	58.39783	26.73672	*P. mugo*	1		
		05.01.2017				1		
24.	Ülenurme	23.12.2016	58.32101	26.72005	*P. mugo*	1		
25.	Kõrveküla stand	25.09.2016	58.43237	26.78829	*P. sylvestris*	1	PSYL[d]	
		20.10.2016				28		
		20.10.2016			*P. mugo*	18	PMUG[d]	

[a] Based on the results from the AMOVA, the isolates of Central Estonia (CE) and Tartu (TRT) were merged into a single population EST.
[b] TBG – Tallinn Botanic Garden.
[c] *Lecanosticta acicola* population codes: TLL – Tallinn, EST – all of Estonia (except Tallinn), CE – Central Estonia, TRT – Tartu.
[d] PMUG/PSYL – sub-populations of *Lecanosticta acicola* isolated in the mixed stand from *P. mugo* (PMUG) and *P. sylvestris* (PSYL).

L. acicola genetic differentiation on different hosts in a single stand.

Additionally, genetic difference was tested between the isolates originating from different time periods of this apparently early colonization of the country. For that reason, the isolates (based on clone-corrected dataset) were divided into two groups according to the sampling year: 2010–2015 (N = 19) and 2016–2017 (N = 32), respectively.

2.3.2. Calculations of genetic diversity

The non-cc dataset was used to calculate for each population the total number of haplotypes and alleles, unique alleles, mean number of different alleles (Na), mean haploid genetic diversity (h), and mean unbiased diversity (uh), using GenAlEx 6.5 (Peakall and Smouse, 2012). With ADZE 1.0 the allelic richness (A_R, number of distinct alleles in the population) and private allelic richness (PA_R, number of unique alleles in the population) were calculated. Since sample sizes across populations were different, rarefaction approach was used with standardized population sizes (Szpiech et al., 2008).

The clonal fraction was calculated for each population according to Zhan et al. (2003). For visualization of Nei's genetic distances (Nei, 1972, 1978), Principal Coordinates Analysis (PCoA) was carried out in GenAlEx 6.5, based on the cc dataset.

2.3.3. Population clustering

The program STRUCTURE 2.3.4 (Falush et al., 2003). was used to estimate the most likely number of population clusters, assign isolates into genetically different groups and thereby determine structure within populations, without any prior data on geographic location or host provided. For the STRUCTURE analysis the cc dataset was used. The most likely number of clusters (K) was determined using the ln(Pr(X|K)) method (Pritchard et al., 2000, 2009) in CLUMPAK (Kopelman et al., 2015).

2.3.4. Random mating

To evaluate the possibility of sexual recombination in the populations, the exact binomial test was used as described in Barnes et al. (2014), to see whether the populations deviated from the null hypothesis of the 1:1 ratio of mating types. In addition, the

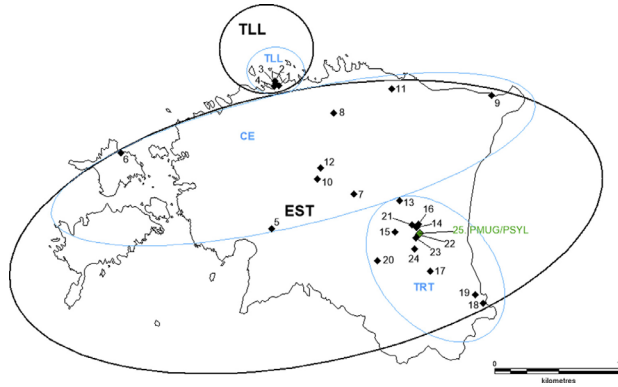

Fig. 1. Map of sampling sites (diamonds) in Estonia, where *Lecanosticta acicola* isolates were obtained and the composed populations of *L. acicola*. The blue lines indicate the geographically-separate populations, and the black lines indicate the populations that were merged according to the results of AMOVA and were used in the further analyses. The mixed stand (no. 25) of *P. mugo* and *P. sylvestris* is marked with green. Numbers of the sampling sites correspond to Table 1. Abbreviations: TLL – Tallinn, EST – all of Estonia, TRT – Tartu, CE – Central Estonia (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

index of linkage disequilibrium (I_A) was calculated in GenAlEx (Peakall and Smouse, 2012) to test if random mating takes place. The mating type ratios and linkage disequilibrium index (I_A) was calculated based on both the non-cc and cc datasets.

3. Results

3.1. Isolates and haplotype identification

In total, 104 isolates of *L. acicola* were obtained for the analyses from 25 sampling sites and 6 different pine species (incl. one variety) in Estonia (Table 1, Fig. 1). Twenty nine of those isolates originated from *P. sylvestris* and 18 from *P. mugo* trees in the mixed *P. mugo* and *P. sylvestris* stand described in Adamson et al. (2018a). The species of all *L. acicola* isolates were confirmed by species-specific primers.

Across the 11 analysed microsatellite loci, a total of 43 different alleles were detected in the 104 isolates. One of the analysed loci (MD1) was monomorphic in all the isolates and the number of alleles in the rest of the loci ranged from 2 alleles at loci MD2, MD4, MD5, MD9, MD11 and MD12 to 12 alleles at locus MD8.

Based on the microsatellite analyses, altogether 50 unique multilocus haplotypes were identified in the collection of the isolates. Ten haplotypes appeared more than once and four haplotypes occurred at more than one sampling site (Fig. 2). The longest distance that was found between two individuals of the same clone was 134 km (haplotype no. 48). The most common haplotype (no. 31) was found from 40 trees at three different sampling sites. It was the dominant haplotype in the mixed stand of *P. mugo* and *P. sylvestris*, where it was found from 15 *P. mugo* and 22 *P. sylvestris* trees. At two other sites the haplotype no. 31 was isolated only from *P. mugo*, the furthest located 75 km from the mixed stand (Fig. 2). Also, haplotypes no. 10 and no. 21 were found from several locations, therewith at all sites only on *P. mugo*. The distance between two locations was 75 km for haplotype no. 21 and 59 km for haplotype no. 10.

Samples collected from the first place in Estonia where *L. acicola* was found (Tallinn Botanic Garden), revealed four different haplotypes, but none of those appeared in other locations in Estonia, not even in Tallinn.

3.2. Molecular variation and formation of populations

According to AMOVA, the molecular variation between geographical populations increases with distance. No significant differences were found between populations TLL and CE ($p = 0.061$) nor between TRT and CE ($p = 0.349$) (Table 2). It shows that populations TRT and CE are closer to each other than neighbouring populations TLL and CE. However, TLL and TRT were found to be distinct from each other ($p = 0.002$). Since TLL, as the presumed initial point of infection in Estonia, was intentionally considered as a separate population – to compare it with the populations of the rest of the country – for further analyses geographical populations TRT and CE were merged into the single population EST, which turned out to be genetically different from TLL ($p = 0.001$). The following analyses, considering the genetic diversity of *L. acicola* in Estonia, were based on the two remaining populations TLL and EST.

Isolates of *L. acicola* from the period 2010–2015 ($N = 19$) were genetically different ($p = 0.007$ by AMOVA) from the isolates of the period 2016–2017 ($N = 32$) (Table 2).

3.3. Genetic diversity

Both populations that were compared in the analyses (TLL and EST) contained clones, but the clonal fraction was higher in EST (Table 3). Genetic diversity of the Estonian *L. acicola* population turned out to be low ($h = 0.30–0.46$).

According to several diversity indices the population EST is more diverse than TLL. The mean unbiased diversity value (uh) is higher in EST, as is the genetic diversity (h). EST has a higher number of alleles, mean number of different alleles, and a higher value of unique alleles compared to TLL. However, there is not any

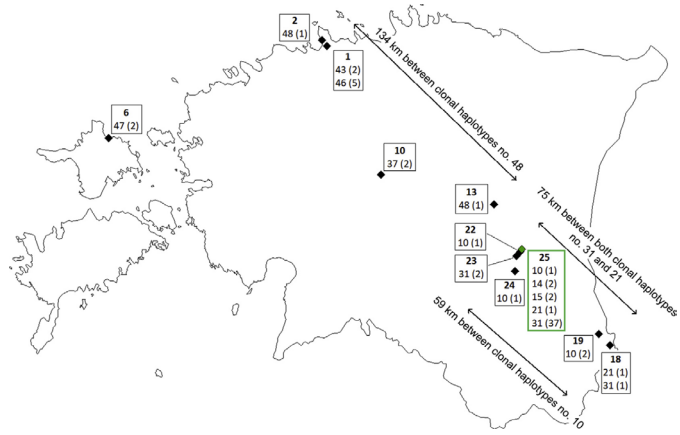

Fig. 2. The sampling sites (diamonds) of *Lecanosticta acicola* haplotypes that were identified several times and their quantity at the sites. In the boxes are the number of definite sampling site (in bold), the numbers of haplotypes and their quantity (in brackets). Numbers of the sampling sites correspond to Table 1. Arrows indicate distances between the locations of the same haplotypes that were identified in several sampling sites. The mixed stand of *Pinus mugo* and *P. sylvestris* is indicated in green (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

Table 2
Molecular variance between grouped populations and sub-populations according to AMOVA.

Population 1 code	Population 2 code	Population 1 N[a] cc[b]	Population 2 N[a] cc[b]	P-value
TLL	TRT	11	26	0.002
TLL	CE	11	14	0.061
TRT	CE	26	14	0.349
TLL	EST	11	40	0.001
PSYL	PMUG	7	4	0.379
2010–2015	2016–2017	19	32	0.007

[a] N – number of isolates.
[b] cc – clone-corrected dataset.

Table 3
Diversity statistics of *Lecanosticta acicola* populations (bold) and sub-populations based on 11 microsatellite markers.

Population code	N[a]	No. of haplotypes	Clonal fraction	Mean haploid genetic diversity h (SE)[b]	Total no. of alleles	Unique alleles	Mean allelic richness A_R (SE)[b]	Mean private allelic richness PA_R (SE)[b]	Mean number of different alleles Na (SE)[b]	Mean unbiased diversity uh (SE)[b]
TLL	16	11	0.313	0.303 (0.084)	27	3	2.200 (0.583)	0.395 (0.242)	2.455 (0.413)	0.339 (0.094)
EST[c]	88	40	0.545	0.455 (0.077)	40	16	2.068 (0.422)	0.263 (0.164)	3.636 (0.975)	0.468 (0.080)
PSYL	29	7	0.759	0.160 (0.036)	24	0	–	–	2.182 (0.352)	0.166 (0.037)
PMUG	18	4	0.778	0.099 (0.030)	17	0	–	–	1.545 (0.157)	0.105 (0.032)

[a] N – Number of isolates.
[b] SE – Standard error.
[c] EST contains sub-populations PSYL and PMUG.

significant differences in allelic richness and private allelic richness that are calculated based on standardized population sizes (Table 3).

3.4. Mating type distribution and haploid linkage disequilibrium

The mating type idiomorphs were successfully identified for 99 of 104 isolates. Both mating type idiomorphs are present in Estonia, but appear in unequal ratios ($p < 0.05$), with *MAT1-1* being more common in grouped populations TLL and EST (Table 4).

The index of association considered random mating being possible only in TLL, based on the clone-corrected dataset ($p = 0.063$). Random mating was rejected in EST, based on both the cc and non-cc datasets, and in TLL based on the non-cc data (Table 4).

3.5. Isolation by distance and population structure

STRUCTURE analysis suggested occurrence of most likely three different clusters ($\Delta K = 3$). None of the populations or sub-populations fell into only one cluster; instead, they were divided

Table 4
Distribution of mating types and linkage disequilibrium statistics of *Lecanosticta acicola* in the populations (indicated in bold) and sub-populations.

Population code	*MAT1-1* non-cc[a]	*MAT1-2* non-cc[a]	P-value of exact binomial test non-cc[a]	*MAT1-1* cc[b]	*MAT1-2* cc[b]	P-value of exact binomial test cc[b]	I_A[c] non-cc[b]	P-value of I_A[c] non-cc[a]	I_A[c] cc[b]	P-value of I_A[c] cc[b]
TLL	14	1	0.001	9	1	0.021	2.801	0.003	2.741	0.063
EST[d]	72	12	0.000	25	11	0.029	3.761	0.001	2.242	0.001
PSYL	26	3	0.000	5	2	0.453	–	–	–	–
PMUG	17	1	0.000	3	1	0.652	–	–	–	–

[a] non-cc – non clone-corrected data.
[b] cc – clone-corrected data.
[c] I_A – Index of association.
[d] EST contains sub-populations PSYL and PMUG.

among at least two clusters (Fig. 3). Isolates from EST were divided among all three clusters, with blue dominating, but orange and purple clusters represented at almost equal proportions, demonstrating that EST is genetically more diverse than TLL and more diverse than sub-populations in the mixed stand of *P. mugo* (PMUG) and *P. sylvestris* (PSYL). Isolates of TLL fell mostly into the blue or purple cluster. Note that the orange cluster has a high proportion in EST, but is significantly less represented in TLL.

The results of PCoA correspond with STRUCTURE analysis (Fig. 4). Isolates from EST are separated in the chart and there is a great variety that is in accordance with the three clusters suggested by STRUCTURE analysis. Isolates from TLL are more concentrated and show less variation. It is notable that several isolates originating from Tartu and Värska area (with a distance of more than 50 km between them) are genetically close (see group of isolates I, Fig. 4). Also, isolates from Tallinn (North Estonia) are very close to isolates from Kärdla (Hiiumaa island, West Estonia), Lääne-Virumaa (north-eastern Estonia) and Kärevere (central-eastern Estonia) (see group of isolates II, Fig. 4). In addition, group III (Fig. 4) shows also that the Central Estonian isolate is genetically close to that of central-eastern and south-eastern Estonia.

3.6. *The mixed stand of* P. mugo *and* P. sylvestris

In the mixed stand the sub-populations PMUG and PSYL were determined according to the host species, respectively *P. mugo* and *P. sylvestris*. However, statistically only an insignificant differentiation between these populations was found according to AMOVA analyses ($p = 0.379$, Table 2). Also, the visual STRUCTURE analysis shows that the sub-populations share a similar structure, with isolates divided mostly into the blue and orange clusters (Fig. 3).

In this stand nine different multilocus haplotypes were detected, four of which were obtained from *P. mugo* and seven from *P. sylvestris* (Fig. 5). Two of the haplotypes (14 and 31) were found from both host species. Although both mating types were represented, *MAT1-1* was found dominating in both sub-populations as in the rest of the country (Table 4). According to the non-cc dataset, mating types appeared in uneven distribution ($p < 0.05$), but according to the clone corrected dataset they occurred at equal ratios ($p > 0.05$). In the sub-populations, genetic diversity and mean unbiased diversity were low for both host species (Table 3). Also, the genetic diversity was lower in the sub-populations and clonal fraction much higher than in the EST and TLL populations, which is in accordance with the small number of different individuals and indicates the importance of asexual reproduction.

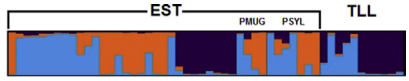

Fig. 3. Structure clustering of *Lecanosticta acicola* populations (EST and TLL) and sub-populations of the mixed stand (PMUG and PSYL), based on the clone corrected dataset. Optimal number of clusters (K = 3) by ln(Pr(X|K)). Abbreviations: EST – all of Estonia (except TLL), PMUG – *Pinus mugo* in the mixed stand, PSYL – *Pinus sylvestris* in the mixed stand, TLL – Tallinn.

4. Discussion

This is the first analysis of the population genetic structure of the pine needle pathogen *L. acicola* in Estonia, northern Europe, which is the first documented population of the fungus in this region. The distribution of BSNB has been monitored here and samples collected for laboratory analyses yearly, since the first record of the pathogen in 2008 (Drenkhan and Hanso, 2009). The isolates used in this study enable us to give an overview of the genetic structure of the pathogen's population for the period 2010–2017. Based on 11 microsatellite and mating type markers, the analysis revealed that the genetic diversity of the pathogen's population in Estonia is low and the proportion of clones is high. Population structure and haplotype dispersal suggest that there have been several separate introductions of the pathogen. Furthermore, with the great distances between the representatives of same clones shown in this study, anthropogenic activity and/or an unknown vector has supported the dispersal of the fungus. However, none of the haplotypes found from the Tallinn Botanic Garden, the first place in Estonia where *L. acicola* was recorded, were found from other locations in Estonia. In addition, mating type idiomorph distribution and a high proportion of clones indicate that in this region the pathogen reproduces mostly asexually, although there is still the possibility that sexual reproduction also can occur, since both mating types are present in the same areas.

4.1. *Genetic diversity and population structure*

From the 104 analysed isolates, 43 unique alleles and 50 haplotypes were detected. Generally, the low genetic diversity and high number of clones that were detected in the studied population are characteristic of non-native species, which results from the introduction of only a limited number of individuals (McDonald, 1997). In Guatemala and Mexico, i.e., in the probable area of origin of *L. acicola*, Janoušek et al. (2016) detected a high level of diversity in the pathogen's population – in comparison to northern America and Europe, where, in contrary, the haplotypic diversity was found to be lower. This confirms the study of Huang et al. (1995), who found that isolates from southern China were close to the isolates from the southern United States, although with lower genetic diversity. Unfortunately, it is difficult to compare directly the results of our study with that of Janoušek et al. (2016) because of different sampling strategies. In the current work, every isolate of *L. acicola* originated from a separate tree. Still, it is rather obvious that in Estonia the population diversity is much lower. For example, the

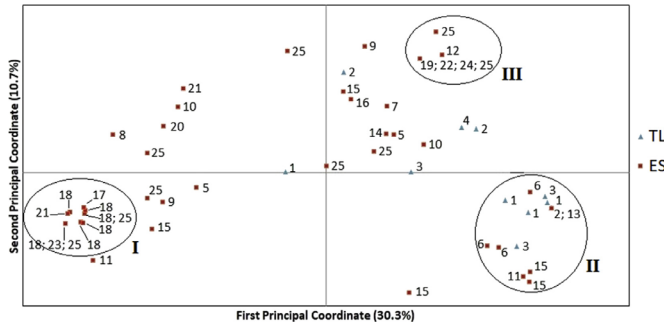

Fig. 4. Results of Principal Coordinates Analysis of the clone corrected dataset. Numbers represent the sampling sites of the isolates and correspond to Table 1. I – Group of isolates from Tartu and Värska area (central-eastern and south-eastern Estonia). II – Group of isolates from Tallinn and Kärdla (north and north-western Estonia). III – Group of isolates from central, central-eastern and south-eastern Estonia. Abbreviations: TLL – Tallinn, EST – all of Estonia (except TLL).

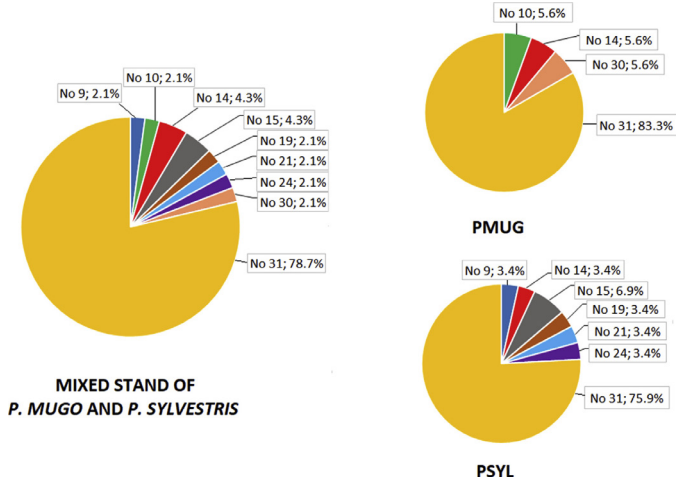

Fig. 5. Distribution of *Lecanosticta acicola* haplotypes in the mixed stand of *Pinus mugo* and *P. sylvestris* and in the sub-populations PMUG and PSYL. The haplotype numbers and their percentages are shown in the boxes.

clonal fraction for the EST population, which included most of Estonia, is 0.545, therefore higher than that detected by Janoušek et al. (2016) for populations of *L. acicola* in North America (0.358) and south-western Europe (0.444), respectively. Janoušek et al. (2016) also found in Mississippi (USA) a high genetic diversity of the pathogen according to the microsatellite analyses, where 40 isolates from one sampling site showed 34 haplotypes, accompanied by the lowest (0.150) clonal fraction in the study. In the current study, in the mixed stand of *P. mugo* and *P. sylvestris* (sub-populations PMUG and PSYL, respectively), although samples were collected from 47 trees, only nine different haplotypes were found (see Fig. 5).

In comparison to *L. acicola*, when isolates of a very similar pine needle pathogen *Dothistroma septosporum* were analysed in Estonia, no shared haplotypes were found between the isolates from different locations of the country (Drenkhan et al., 2013). It indicated that the populations of *D. septosporum* did not originate from a recent introduction and the fungus has resided in northern Europe for a longer time (Adamson et al., 2018b). Since pathogens spread fast through human activity and often stay unnoticed

because of the latent phase, inadequate quarantine rules and lack of investigations, the genetic structure of pathogen populations may also change fast. In addition, multiple introductions and sexual recombination can increase genetic diversity, resulting in the possibility that new fungal strains may become better adapted to the environment or have higher virulence (McDonald and Linde, 2002). However, when isolates from the two time periods 2010–2015 and 2016–2017 were compared, no significant differences in the genetic diversity were detected, indicating that so far the diversity of the pathogen has not notably risen.

Natural distribution of *L. acicola* is limited when the fungus reproduces only clonally. Skilling and Nicholls (1974) revealed that conidia of *L. acicola* rarely spread more than 1.5 m away from the infected tree. This means that in a young plantation the fungus may not reach more than the neighbouring tree. Wyka et al. (2018) also found that the majority of the disseminating conidia spread in close proximity of the source tree, although a few of them were found even 60.6 m away from the source. However, it has been demonstrated that the similar pathogen *D. septosporum* can naturally spread by conidia up to 1400 m (Mullett et al., 2016). In the current study, the distances between the identical clones in sampling sites of *L. acicola* were much longer than conidia would be able to spread naturally. In addition to haplotype 31, two more haplotypes (10 and 21) moved between the Tartu and Värska sampling sites (central-east and south-east Estonia, distance 75 km). Also, clones of haplotype 48 were found from two separate locations with a distance of 134 km between them (see Fig. 2). It is possible that during some years the clones of the pathogen have spread step by step and dispersal by insects or birds must also be considered (Skilling and Nicholls, 1974). However, it is quite probable that anthropogenic activity has influenced the spread of the pathogen, possibly through transportation of infected plant material, which has generally been considered to be an effective way for pathogens to expand their area (Barnes et al., 2014; Drenkhan et al., 2016; Santini et al., 2013). Moreover, even the disease agent itself was probably brought to Estonia inadvertently with infected plants (Adamson et al., 2015). In several cases the impact of tourism has been considered to support the distribution, for example in Austria (EPPO, 2016) and the Czech Republic (Jankovský et al., 2009), where the pathogen was found at protected natural areas visited by tourists.

According to the STRUCTURE analysis the populations TLL and EST have different genetic structures. Notably, the Estonian isolates were divided into three clusters, but in Tallinn, one of the clusters (orange) is represented only by small proportions in a small number of haplotypes (Fig. 3). Although Principal Coordinates Analysis (Fig. 4) shows that some isolates from Tallinn (TLL) and the rest of Estonia (EST) are genetically close, most of the isolates from EST are distant from those of TLL. Those results strongly suggest that Tallinn most likely has not been the single source in the pathogen's colonization of Estonia. Since Tallinn was the first place in Estonia where the pathogen was found, it has been hypothesized that, after the primary arrival in Tallinn, the pathogen has spread in Estonia further from north to south (Adamson et al., 2015). Now, in the light of these results it seems more probable that some isolates in EST originate from TLL, while several other genotypes have been introduced in separate introductions. So, with the later appearance of the second mating type idiomorph (*MAT1-2*) and the distribution history of the pathogen in Estonia, this supports the hypothesis of several introductions of the fungus (Adamson et al., 2015, 2018a). In addition, results of AMOVA prove that the population of *L. acicola* in Tallinn is significantly different from those isolated from other parts of Estonia (EST). Additionally, the first samples collected from Estonia (in 2010–2015) are different by AMOVA from the samples collected later (in 2016–2017) (Table 2).

4.2. Reproductive mode

In vivo, so far, the sexual state of the pathogen has not been found in Estonia, although occurrence of both mating type idiomorphs was documented and even found coexisting in the same sampling sites, which should provoke sexual reproduction. High clonal fraction refers to mostly asexual reproduction, since all the analysed isolates were obtained from different trees, which should eliminate the possibility of analysing the same specimen twice. Mostly clonal distribution of the pathogen is also supported by the dominance of one mating type idiomorph (*MAT1-1*). Therefore, we cannot exclude that on some level the sexual recombination takes place in Estonia, but probably it is not frequent.

Thus, the situation with reproduction of *L. acicola* in northern Europe seems to be similar to *D. septosporum.* For this pathogen both mating types are also present, but the sexual state has not been found while sampling (Adamson et al., 2018b; Drenkhan et al., 2013). It is not known if *L. acicola* would even undergo sexual reproduction so far in the north. Until now, the northernmost documented record of *L. acicola* ascospores in the world originates from the state of Missouri, USA (Luttrell, 1949). There are no records of asci or ascospores having been found in Europe, although Janoušek et al. (2016) concluded that sexual reproduction of the pathogen probably takes place in Austria and Germany, based on the occurrence of both mating type idiomorphs and microsatellite analyses.

4.3. *Diversity of* L. acicola *in the mixed stand of* P. mugo *and* P. sylvestris

One objective of this study was to investigate if there are any differences in the population structure of *L. acicola* on different host species in a mixed stand. Adamson et al. (2018a) described morphological measures of the conidia of *L. acicola,* which were not significantly different between two hosts. Our analysis showed that the sub-populations (PSYL and PMUG), isolated from the same stand but different hosts, also shared similar genetic structure and diversity of the pathogen (see Table 2, Table 3, Fig. 3).

According to the clone corrected dataset, both of the mating types occurred at equal ratios in the mixed stand of *P. mugo* and *P. sylvestris*. That increases the possibility of sexual recombination to take place in that stand and it could explain why *L. acicola* infected Scots pine there. In the mixed stand four haplotypes (no. 9, 15, 19, 21 and 24) were found only on *P. sylvestris* and two (10 and 30) only on *P. mugo* (Fig. 5). From those, four haplotypes (9, 15, 19 and 24) were found only in the mixed stand on *P. sylvestris* and nowhere else in Estonia. The presence of haplotypes that are not found from *P. mugo* in the stand nor from other locations in Estonia may be the result of sexual reproduction or genetic mutations, which already have taken place in that stand. Those may be important and dangerous factors in increasing the genetic variance of pathogen's populations, assisting adaptation to new conditions and supporting host jumps (McDonald and Linde, 2002; Parker and Gilbert, 2004). Sexual recombination or mutations would also explain the appearance of the obviously more virulent haplotype no. 31 that was identified on both host species (non-native *P. mugo* and native *P. sylvestris*) and was isolated from more trees in this stand than any other haplotype. The same haplotype (no. 31) was also found in two other sampling sites (18 and 23, see Fig. 2) on *P. mugo*. Fortunately, so far, near those sites any damage (e.g. serious needle loss) to *P. sylvestris* has not been documented.

In northern Europe non-native pine species have mainly ornamental value and are not of high economic importance in silviculture. However, Scots pine, the only native pine species in the region, is one of the most economically and ecologically

important tree species in northern Europe. Therefore, it is essential to monitor the distribution and host range of the pathogen and establish its aggressiveness on this native host. In Europe BSNB has also been found from Scots pine in Austria (Cech and Krehan, 2008; EPPO, 2015), the Czech Republic (Jankovský et al., 2009), Ireland (Mullett et al., 2018), Lithuania (EPPO, 2012b), Slovenia (Jurc and Jurc, 2010) and Spain (Ortiz de Urbina et al., 2017). In the United States Scots pine is a non-native tree species and one of the species most harmed by that pathogen (Siggers, 1944; Skilling and Nicholls, 1974). Until now, the few reports of BSNB on Scots pine from Europe have described only localized occurrences that have not escalated into serious epidemics. It is possible that the pathogen will not harm Scots pine so much in Europe since it grows here in its natural environment and has high genetic diversity (Naydenov et al., 2007). However, it is still important to continue with the monitoring of BSNB.

5. Conclusions

Although *L. acicola* has been present in Estonia for only 10 years, it has been spreading here fast, and considering the fact that human activity influences the pathogen dispersal, its diversity can continuously rise. Possibly via several separate introductions of genetically different strains also both mating types have arrived. This is the first time for Estonia when the pathogen's dispersal by human activity is evidently proved. Therefore, it is important to continue monitoring of the pathogen. In the case of *L. acicola*, as with other pathogens, introduction of new genetic strains, which may accompany the introduced plants, should be avoided through better quarantine measures. Use of more resistant genotypes of Scots pine could be another potential control measure against this invasive pathogen in northern Europe. In the future the results of this study may hopefully provide an opportunity to observe and document the ongoing changes in the population genetic structure. However, the primary origin of the pathogen in the region remains unknown. Thus, wider scale population genetic studies of *L. acicola* in northern Europe are needed.

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First report of *Lecanosticta acicola* on pine and non-pine hosts in Turkey

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Abstract

Brown spot needle blight, caused by *Lecanosticta acicola*, is a serious disease of pines worldwide and has become of great concern in Europe over the last decade, with significantly increased outbreaks in pine forests. We examined native and non-native Pinaceae taxa (four *Cedrus* and 24 *Pinus*) in the Atatürk Arboretum, Istanbul, Turkey, for the presence of *L. acicola*. Needles were sampled from 37 trees at least twice between March 2017 and July 2018. Symptomatic occurrence of the disease was confirmed by isolations, followed by molecular identification via sequencing of the ITS region. *Lecanosticta acicola* was isolated from symptomatic needles of 10 trees from seven host taxa (*Cedrus* and six *Pinus*). Molecular diagnostics of isolates confirmed the identification of *L. acicola* on *Cedrus libani*, *Pinus sylvestris*, *P. nigra* subsp. *nigra*, *P. nigra* subsp. *laricio*, *P. nigra* subsp. *pallasiana*, *P. nigra* subsp. *pallasiana* var. *fastigiata* and *P. nigra* subsp. *pallasiana* var. *pallasiana* f. *şeneriana* in the arboretum.

This paper is the first report of *L. acicola* on *C. libani* and also the first report of the pathogen infecting a genus other than a *Pinus* spp. under natural conditions. Additionally, it is also the first report of *L. acicola* occurring in Turkey. The pathogen was clearly able to cause severe damage on native Turkish *Pinus* taxa, including *P. sylvestris* and *P. nigra* subsp. *pallasiana*, and endangered endemic forms of the host in Turkey.

KEYWORDS

Anatolian black pine, brown spot needle blight, *Cedrus libani*, emerging disease, invasive pathogen, *Mycosphaerella dearnessii*

1 | INTRODUCTION

Lecanosticta acicola (Thümen) H. Sydow, the causal agent of brown spot needle blight (BSNB), is among the most serious emerging diseases of pines globally and is of great concern in Europe where increased outbreaks have occurred in forests in Austria, Estonia and Spain (Adamson et al., 2018; EPPO, 2016; Laas et al., 2019; Ortíz de Urbina et al., 2017). *Lecanosticta acicola* was recently redefined as a North American taxon (Van Der Nest et al., 2019) with a geographical distribution covering Central America, Colombia, the USA, Canada, Asia and several European countries (EPPO, 2020; Van Der Nest et al., 2019). An almost twofold increase in reports of the disease has been published in the last ten years, and the pathogen is continuing to spread in Europe (Mullett et al., 2018; Van Der Nest, Wingfield, Janoušek, et al., 2019).

Severe blight symptoms resembling those of BSNB caused by *L. acicola* were observed in March 2017 in the Atatürk Arboretum, Istanbul, Turkey, on needles of Anatolian black pine (*Pinus nigra* J.F. Arnold. subsp. *pallasiana* [Lamb.]), along with two endemic forms of this black pine subspecies (var. *fastigiata* Businský and var. *pallasiana* f. *şeneriana* (Saatçioğlu) Kandemir & Mataracı) and on a Scots pine (*Pinus sylvestris* L.). In addition, needle blight symptoms, with

defoliation in the lower crown of a Lebanon cedar (*Cedrus libani* A. Rich) were noticed (Figure 1).

Lecanosticta acicola has been reported from 53 *Pinus* species and hybrids in the wild and from *Picea glauca* (Moench) Voss in a field inoculation experiment performed by Skilling and Nicholls, (1974) (Van Der Nest, Wingfield, Janoušek, et al., 2019). There have been no reports of *L. acicola* on Pinaceae other than *Pinus* spp. under natural conditions. Geographically, Sochi (Russia), on the eastern Black Sea coast, is the closest area to Turkey where *L. acicola* is known to occur on *Pinus mugo* Turra and *Pinus thunbergii* Parl. (Mullett et al., 2018). The aim of the work reported here was to identify the agent causing needle blight on *Cedrus libani* and various *Pinus* spp. and forms in the Atatürk Arboretum, Istanbul, Turkey.

2 | MATERIALS AND METHODS

2.1 | Sampling

The Atatürk Arboretum was established in 1949 in an area of deciduous forest in the northern part of Istanbul, Turkey (41°10'30.53"N, 28°59'5.22"E). The arboretum has a substantial collection of both native and non-native angiosperm and gymnosperm trees. One of the richest collections in this arboretum is that of the Pinaceae, including native and non-native species of *Pinus* L.

Following the initial observations of BSNB-like symptoms on a number of native *Pinus* spp. and on *C. libani* (March 2017), other pines and cedars located close to the symptomatic trees were examined for BSNB symptoms in March and July 2017 and July 2018. Needle samples were collected, mainly in July 2017 and 2018, from all examined trees. Some trees were sampled on three separate occasions, in March and July 2017, and in July 2018 (Table 1). Needles were sampled from the lower (up to 1.5 metres in height) canopies except for the needles of both *P. nigra* subsp. *laricio* and *P. nigra* subsp. *nigra*, which were collected from fallen branches with live green shoots since the crowns of these two hosts were out of reach. Needles were placed in sterile plastic bags, labelled and stored at −20°C.

A total of 37 trees from 28 taxa (four *Cedrus* and 24 *Pinus*) were examined for symptoms of BSNB during surveys conducted in March and July 2017 and 2018 in the arboretum (Table 1). Seven of the 28 taxa were native Turkish Pinaceae: *C. libani*, *P. brutia*, *P. nigra* subsp. *pallasiana* (including two varieties of the subspecies), *P. pinea* and *P. sylvestris* (Table 1).

Severity of BSNB on individual trees was assessed in July 2017 and 2018, following the methods of Bulman et al., (2004), using 10% increments for scoring the total crown volume with symptoms.

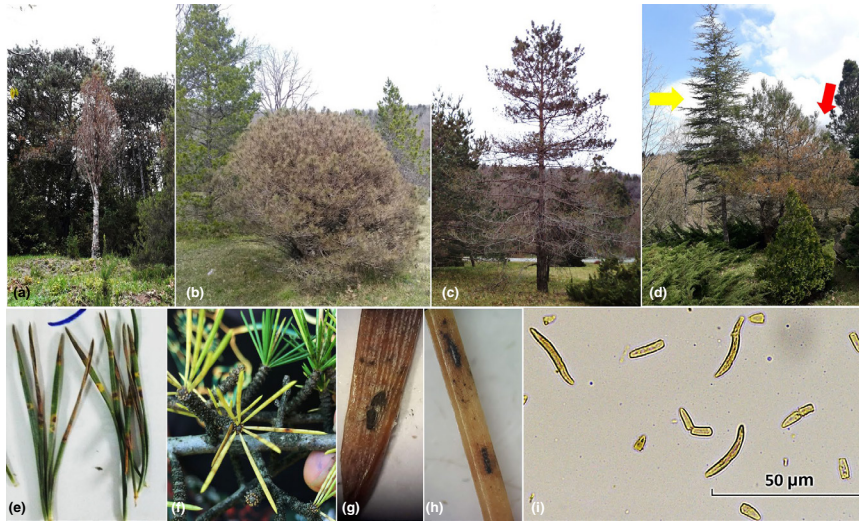

FIGURE 1 Damage caused by *L. acicola* on *P. nigra* subsp. *pallasiana* var. *fastigiata* (a), *P. nigra* subsp. *pallasiana* var. *pallasiana* forma *şeneriana* (b), *P. sylvestris* (c) and *C. libani* individual (Yellow arrow) infected with *L. acicola* located next to *P. brutia* var. *brutia* forma *agrophiotii* severely infected with *Dothistroma* sp. (Red arrow) (d). Symptoms and conidiomata of *L. acicola* on needles of *P. sylvestris* (e and g) and *C. libani* (f and h) and conidia of *L. acicola* from mature conidiomata on needles of *P. nigra* subsp. *laricio* (i) at 600× magnification

TABLE 1 Trees monitored for the presence of brown spot needle blight (BSNB) in the Atatürk Arboretum, Turkey between March 2017 and July 2018 (host taxa on which *L. acicola* infection was confirmed are shown in bold)

		Field observations and samplings			Laboratory examinations and identification			
Monitored tree no	Host taxon[a] Species Infraspecific taxa	Monitoring and sampling dates[b]	Symptoms of BSNB (±)	Severity of BSNB[c] (%)	BSNB causal agent confirmation	Culture collection numbers	GenBank Accession numbers by ITS	Other fungal pathogens detected on needles[d]
1	*Cedrus atlantica* (Endl.) Manetti ex Carrière	July 2017–2018	-					-
2	*Cedrus deodara* (Roxb. ex D.Don) G.Don	July 2017–2018	-					-
3	[e]***Cedrus libani* A. Rich**	**March**, July **2017**–2018	+	10→20	*L. acicola*	TFC101134	MK797045	-
4		July 2017–2018	-		-			-
5		July 2017–2018	-		-			*Diplodia sapinea*[g]
6	*Cedrus libani* var. *brevifolia* Hook.f.	July 2017–2018						-
7	*Pinus armandii* Franch.	July 2017–2018	-		-			-
8	[e]*Pinus brutia* Ten. var. *brutia*	**March**, July **2017**–2018	-		-			*Dothistroma* sp. *Diplodia* sp.
9		**March**, July **2017**–2018	-		-			*Dothistroma* sp. *Diplodia* sp.
10		**March**, July **2017**–2018	-		-			*Dothistroma* sp. *Diplodia* sp.
11	[f]var. *brutia* forma *agrophiotii* (Papaj.) Kandemir & Mataracı)	**March**, July **2017**–2018	-		-			*Dothistroma* sp. *Diplodia* sp.
12	*Pinus contorta* Douglas ex Loudon	July 2017–2018	-		-			-
13	*Pinus densiflora* Siebold & Zucc.	July 2017–2018	-		-			-
14	*Pinus engelmannii* Carrière	July 2017–2018	-		-			-
15	*Pinus greggii* Engelm. ex Parl.	July 2017–2018	-		-			-
16	*Pinus montezumae* Lamb.	July 2017–2018	-		-			-
17	*Pinus mugo* Turra	July 2017–2018	+	100→100	-			*Diplodia* sp.
18		July 2017–2018	-		-			-
19	*Pinus nelsonii* Shaw	July 2017–2018	-		-			-
20	[e]***Pinus nigra* J.F. Arnold. subsp. *nigra***	**March**, July **2017**–2018	+	Na	*L. acicola*	TFC101139	MT188701	
21	**subsp. *laricio* Palib. ex Maire**	**March**, July **2017**–2018	+	Na	*L. acicola*	TFC101140	MT188702	-
22	[e]**subsp. *pallasiana* (Lamb.) Holmboe**	**March**, July **2017**–2018	+	70→100	*L. acicola*	TFC101141	MT188703	*Diplodia* sp.
23		**July 2017**–2018	+	20→30	*L. acicola*	TFC101142		*Diplodia* sp.
24		**July 2017**–2018	+	30→40	*L. acicola*	TFC101143		*Diplodia* sp.
25		**July 2017**–2018	+	30→40	*L. acicola*	TFC101144		*Diplodia* sp.
26	[f]**subsp. *pallasiana* var. *fastigiata*** Businský	**March**, July **2017**–2018	+	80→100	*L. acicola*	TFC101133	MK797044	*Diplodia* sp.

(Continues)

TABLE 1 (Continued)

Monitored tree no	Host taxon[a] Species Infraspecific taxa	Field observations and samplings			Laboratory examinations and identification			
		Monitoring and sampling dates[b]	Symptoms of BSNB (±)	Severity of BSNB[c] (%)	BSNB causal agent confirmation	Culture collection numbers	GenBank Accession numbers by ITS	Other fungal pathogens detected on needles[d]
27	[f]**subsp. *pallasiana* var. *pallasiana*** (Lamb.) Holmboe **forma *seneriana*** (Saatçioğlu) Kandemir & Mataraci	**March**, July **2017**–2018	+	100→100	*L. acicola*	TFC101136	MK797047	*Diplodia* sp.
28	*Pinus parviflora* Siebold & Zucc.	July 2017–2018	-		-			-
29	*Pinus patula* Schiede ex Schltdl. & Cham.	July 2017–2018	-		-			-
30	*Pinus pinaster* Aiton	July 2017–2018	-		-			-
31	[e]*Pinus pinea* L.	July 2017–2018	-		-			-
32	*Pinus radiata* D.Don	July 2017–2018	-		-			-
33	*Pinus strobus* L.	July 2017–2018	-		-			-
34	[e]***Pinus sylvestris* L.**	**March**, July **2017**–2018	+	60→80	*L. acicola*	TFC101135	MK797046	*Diplodia* sp.
35		**March**, July **2017**–2018	+	20→40	-			*Diplodia* sp.
36	*Pinus taeda* L.	July 2017–2018	-		-			-
37	*Pinus thunbergii* Parl.	July 2017–2018	-		-			-

Note: Presence (+) or absence (-) of BSNB symptoms based on first monitoring date (no change was observed at following monitoring dates).

[a]The nomenclature follows catalogue of life (https://www.catalogueoflife.org/col/search/all) and Güner et al. (2018) for the native taxa.

[b]Monitoring and sampling dates in bold indicates the first time when symptoms of BSNB were observed and the pathogen(s) detected on collected samples.

[c]Severity of *L. acicola* infection on individual trees was assessed using the methodology of Bulman et al., (2004) in which 10% steps of scoring for the percentage of the total crown volume with BSNB symptoms were considered. Severity scores for July 2017 →July 2018 are given in the table. **Na:** Severity of BSNB Not assessed (Trees were too tall for crown observations).

[d]Samples were also examined for the presence of *Dothistroma* sp. and *Diplodia* sp. and involved only microscopic examinations (i.e. spores seen with compound microscope).

[e]Native taxon.

[f]Endemic taxon.

[g]*Diplodia sapinea* was confirmed in this tree (Oskay et al., 2018).

2.2 | Morphological diagnosis and fungal isolation

Morphological diagnosis of the causal agent was through examination of symptomatic needles for the presence of conidiomata and conidia of *L. acicola* using dissecting (Leica MZ6) and compound microscopes (BEL BIO2T, Italy and Nikon Eclipse 50i). Morphological measurements of 30 random conidia from *C. libani*, *P. nigra* subsp. *pallasiana* and *P. sylvestris* were carried out using software NIS-Elements D 4.12.01 (Nikon). Needle specimens examined during this work were deposited in the Fungal Culture Collection, Estonian University of Life Sciences and in the Forest pathology herbarium of Çankırı Karatekin University, Turkey. Other pathogenic fungi observed during microscopic examinations of needles were also noted.

Isolations were made from needles after gently wiping the surface with a tissue soaked in 96% ethanol. Well-developed conidiomata were excised and placed on pine needle agar medium (PNA) (Drenkhan et al., 2013) in 90-mm-diameter Petri dishes. Conidiomata were rolled over the surface of the medium to separate individual conidia (Mullett & Barnes, 2012) and incubated at room temperature in the dark for 14 days.

2.3 | Molecular identification of isolates

Pure colonies with morphological features similar to *L. acicola* were subcultured to fresh PNA and incubated in the dark at room temperature for 20 days. Approximately 0.04 g of mycelium from the colony edge was transferred to 2.0-ml micro centrifuge tubes, homogenized using a Retsch MM400 homogenizer (Retsch GmbH) with sterile metal beads (Ø 1.6 mm) and DNA extracted using the Thermo Scientific GeneJET Genomic DNA Purification Kit (Lithuania) following the manufacturer's instructions.

The species-specific primer pair developed for *L. acicola* (LAtef-F/R) targeting the EF1-α genes was used to identify the isolates. Conventional PCR was carried out in a TProfessional Thermocycler (Biometra) as described in Ioos et al. (2010), with modifications (Drenkhan et al., 2014). PCR products were visualized on a 1% agarose gel (SeaKem® LE Agarose) stained with ethidium bromide under UV light using a Quantum ST4-system (Vilber Lourmat SAS).

For additional confirmation of isolate identity, the internal transcribed spacer (ITS) region was amplified by PCR using ITS1-*F* (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) primers. PCR and ITS region sequencing were carried out as described by Drenkhan et al. (2014). Samples were sent for Sanger sequencing to the Estonian Biocentre in Tartu. Sequences were manually edited and aligned using BioEdit version 7.2.5. Sequences were then subjected to a Blast analysis on NCBI's databases.

3 | RESULTS

Dark olive green to black conidiomata, and conidia characteristic of *L. acicola* were found on needles of ten trees, in microscopic investigations. Conidia were light green or olivaceous to pale brown in colour with rough, verruculose thick walls. Fusiform to cylindrical conidia were straight or curved, (1–)3(–5)-septate with a truncate base and rounded apex. Dimensions of conidia varied on *P. sylvestris* between 22.15-(33.34)-38.38 × 3.59-(4.84)-7.32 μm, on *P. nigra* subsp. *pallasiana* between 20.17-(30.03)-41.11 × 3.11-(4.36)-5.76 μm, and on *C. libani* between 27.79-(34.90)-46.56 × 3.00-(4.51)-5.87 μm. The sexual stage of the pathogen was not observed on samples investigated during this work. Ten isolates, one isolate per tree, were obtained from *P. nigra* subsp. *nigra* (1 tree), *P. nigra* subsp. *laricio* (1 tree), *P. nigra* subsp. *pallasiana* (4 trees), *P. nigra* subsp. *pallasiana* var. *fastigiata* (1 tree), *P. nigra* subsp. *pallasiana* var. *pallasiana* f. *şeneriana* (1 tree), *P. sylvestris* (1 tree) and *Cedrus libani* (1 tree) (Table 1, Figure 1).

Lecanosticta acicola species-specific primers (LAtef-F/R) gave positive results for all ten isolates, confirming the identification as *L. acicola*. ITS sequences of seven isolates (one isolate per host taxon) were compared against the ITS sequence of the type material CBS 133791 (GenBank accession number NR_120239). All sequences showed 100% homology to the reference strain. ITS sequences obtained from these seven isolates were deposited in GenBank (Accession numbers: MK797045, MT188701, MT188702, MT188703, MK797044, MK797047, MK797046; Table 1). Fungal cultures were deposited in Fungal Culture Collection, Estonian University of Life Sciences (culture collection numbers: TFC101134, TFC101139, TFC101140, TFC101141, TFC101142, TFC101143, TFC101144, TFC101133, TFC101136, TFC101135; Table 1).

Lecanosticta acicola was not detected on the non-native cedar species (*C. atlantica*, *C. deodara*, *C. libani* var. *brevifolia*; Table 1), nor on the non-native pines (15 *Pinus* spp.; Table 1) except on the two *P. nigra* (subsp. *nigra* and *laricio*), which are not native to Turkey. Additionally, *L. acicola* infections were not detected on two of the native pines; *P. brutia* and *P. pinea* (Table 1). The pathogen was therefore confirmed causing brown spot needle blight on five native taxa (*C. libani*, *P. nigra* subsp. *pallasiana* including two varieties of the subspecies and *P. sylvestris*), and on two non-native taxa (*P. nigra* subsp. *nigra* and *P. nigra* subsp. *laricio*).

In addition to *L. acicola*, *Dothistroma* sp. Hulbary and *Diplodia* sp. Fr. were also found on sampled needles in microscopic examinations (Table 1). *Dothistroma* sp. was observed on *P. brutia*, but not on other hosts, including those infected with *L. acicola*. Conversely, *Diplodia* sp. was observed on most needle samples of hosts infected with *L. acicola* and also with *Dothistroma* sp. (Table 1).

Disease severity, assessed by estimating the percentage of symptomatic crown volume, ranged between 10% and 100%. Increases of at least 10% in the second year of assessment were observed (Table 1). Infection severity was highest on the endemic varieties of *P. nigra* subsp. *pallasiana* (80%-100%) and lowest on *C. libani*. The severity of disease on *P. nigra* subsp. *nigra* and *P. nigra* subsp. *laricio* was not assessed in this work, as the trees were over 17 m in height and detailed symptoms in the crown (and on needles) were not visible from ground level.

4 | DISCUSSION

This report is the first record of *L. acicola* in Turkey. It is also the first record of this pathogen on *Cedrus libani* and two varieties of *P. nigra* subsp. *pallasiana* (var. *fastigiata* and var. *pallasiana* f. *şeneriana*). The ability of *L. acicola* to cause disease on *Cedrus libani* is a new global record. Apart from *Picea glauca*, on which a trace of infection (less than 1% of needles) was found after 2 successive years of exposure to natural infection in field inoculations (Skilling & Nicholls, 1974), hitherto there were no reports of *L. acicola* on genera in the Pinaceae other than *Pinus* spp. Moreover, the known hosts for the remaining eight described species in the genus *Lecanosticta* do not include non-*Pinus* hosts (Van Der Nest, Wingfield, Ortiz, et al., 2019). In addition to *C. libani* and the two varieties of *P. nigra* subsp. *pallasiana*, the pathogen was also detected and confirmed on three additional known pine host taxa in the Atatürk arboretum: *P. sylvestris*, *P. nigra* subsp. *nigra* and *P. nigra* subsp. *laricio*. *Pinus nigra* and *P. sylvestris* were considered to be moderate to highly susceptible pine hosts of *L. acicola* in the early 1970s by Skilling and Nicholls (1974).

The closest location to Turkey with a verified record of *L. acicola* was on non-native pines in a Botanical Garden in Sochi (Russia) on the Black Sea coast (Mullett et al., 2018). The pathogen has also been reported from nearby Georgia (EPPO, 2020), Greece (Pantidou, 1973) and Bulgaria (Kovacevski, 1938). However, the EPPO record for Georgia cites Kizikelashvili (1987) who refers to *Dothistroma acicola* (Thüm.) A. Schischk. et N. Tzan. Shishkina & Tsanava produced a series of papers (Shishkina & Tsanava, 1966a, 1966b, 1967) on Dothistroma needle blight disease of *Pinus brutia* var. *pityusa* (Steven) Silba (as *Pinus pityusa*) in Georgia in the 1960s in which they clearly describe red banding associated with the disease as well as the hyaline conidia of the fungus, both typical of *Dothistroma* not of *Lecanosticta*. They also directly compared material from Great Britain, supplied by S. Murray who first described *Dothistroma* in Britain (Murray & Batko, 1962), with Georgian material and confirmed the fungus to be identical. Shishkina and Tsanava (1967) renamed *Dothistroma pini* as *D. acicola* due to finding the sexual stage of the fungus and confusion with the sexual stage of *L. acicola*. Therefore, it is probable that the report from Georgia was of *Dothistroma* sp. not *L. acicola*, a view confirmed by Gibson (1980) and Barnes et al. (2016). Recently, *D. septosporum* (Dorogin) M. Morelet was isolated and confirmed with molecular methods on naturally regenerated seedlings of *Pinus sylvestris* var. *hamata* in Georgia (Matsiakh et al., 2018). Nonetheless, due to Georgia's proximity to both Turkey and Sochi, where *L. acicola* is now known to be present, it would not be unexpected to find the pathogen in Georgia. Indeed, there is a recent unpublished finding and molecular confirmation of the pathogen in Georgia (M. Laas et al., unpublished data). The reports from Greece from the 1950s (Gibson, 1980; Sarejanni et al., 1954, 1955), however, remain unconfirmed and are therefore not considered reliable (EPPO, 2020), and may well also refer to *Dothistroma* sp. The original report from Bulgaria (Kovacevski, 1938) has subsequently been refuted and almost certainly refers to *Dothistroma* sp. not *Lecanosticta acicola* (Mullett et al., 2018; Van Der Nest, Wingfield, Janoušek, et al., 2019). However, its recent detection in a nursery, which was reported to be eradicated (EPPO, 2020) requires cautious consideration for the presence of *L. acicola* in this country.

In the Ataturk Arboretum, Istanbul, BSNB caused moderate (i.e. 20%–40% of crown infected) to severe (≥ 50% of crown infected) damage on native pines. Symptom severity on the single individual of *C. libani* was lower than on pines (10% reaching 20% in the following year). Not all *P. sylvestris* and *C. libani* individuals examined were affected by the disease.

Except for subspecies of *P. nigra*, BSNB was not found on non-native pines surveyed in this work in 2017–2018, including *P. mugo* or *P. radiata*. In Europe, *P. mugo* appears to be the most susceptible host of *L. acicola*, followed by *P. sylvestris* in central and northern Europe and the non-native *P. radiata* in southern Europe (Van Der Nest, Wingfield, Janoušek, et al., 2019). Reports from botanical gardens in different climatic regions across Europe also suggest the high susceptibility of *P. mugo*. *Lecanosticta acicola* was detected only on *P. mugo* with severe symptoms in Italy (La Porta & Capretti, 2000), in Sweden (Cleary et al., 2019) and in Germany (Van Der Nest, Wingfield, Janoušek, et al., 2019) and was found on both *P. mugo* and *P. sylvestris* in Ireland (Mullett et al., 2018). The disease was also reported on other non-native pines in Estonia, Latvia and Russia (Adamson et al., 2015, 2018; Mullett et al., 2018). In the present work, however, *L. acicola* was not detected on *P. mugo* at the time of sampling, despite the arboretum containing numerous *P. mugo* individuals (probably of various origins and cultivars).

Both native Turkish and non-native provenances of *P. sylvestris* were present in the arboretum, the local provenances representing the most southern populations of this pine species. It is possible that this southern provenance of *P. sylvestris* is more susceptible to *L. acicola* than more northern provenances. For example, previous inoculation work demonstrated that Spanish and French provenances of *P. sylvestris* were highly susceptible to *L. acicola*, whereas those from Germany and Austria were less susceptible (Phelps, Kais & Nicholls, 1978; Skilling & Nicholls, 1974). Additionally, in another study from central Europe, from the Czech Republic, the pathogen was not reported on *P. sylvestris* individuals despite the presence of high inoculum pressure from heavily infected adjacent *P. rotundata* Link individuals (Jankovský et al., 2009). It is likely that host species and provenances differ in response to *L. acicola* attack as reported for *D. septosporum* where both intra- and inter-specific susceptibility varies considerably (Fraser et al., 2015, 2016).

Although infection severity on the *P. sylvestris* individual was high (reaching up to 80%) in the Atatürk Arboretum, more severe *L. acicola* infections occurred on the two varieties of *P. nigra* subsp. *pallasiana* (100% for both). Natural occurrence of these varieties of *P. nigra* subsp. *pallasiana* (var. *fastigiata* and var. *pallasiana f. şeneriana*) are limited to small populations within the Anatolian black pine forests, where both varieties are in danger of extinction. Hence, the introduction of *L. acicola* may accelerate the disappearance of these trees from their native range.

In comparison with native pines, which were severely damaged by the pathogen, the intensity of *L. acicola* infection on *C. libani* was relatively low, causing defoliation only on lower suppressed branches. The cedar tree infected with *L. acicola* was immediately adjacent to a *Pinus brutia* Ten. var. *brutia* forma *agrophiotii* (Papaj.) Kandemir & Mataraci tree, which was heavily infected with *Dothistroma* sp. *Cedrus libani* is susceptible to infection by *Dothistroma septosporum*, especially under high inoculum pressure (Mullett & Fraser, 2016). Conidia of *L. acicola* can spread only short distances, with a maximum of up to 60 m (Wyka et al., 2018); for the infected specimen of *C. libani*, the nearest pines with *L. acicola* infections were approximately 50 m away. It was surprising, therefore, that this cedar tree was infected with *L. acicola* but not with *Dothistroma* sp.

Diplodia sp. infections were found on all pine host trees infected with *L. acicola*: the co-occurrence of these pathogens will accelerate the rate of damage, possibly leading to mortality. Although not confirmed on all individuals in this work, all *P. nigra* subsp. *pallasiana* var. *fastigiata* individuals were heavily damaged, some even killed, possibly by simultaneous attacks of *L. acicola* and *Diplodia* sp.. Diplodia shoot blight is common and known to cause severe damage in *P. nigra*, *P. sylvestris* (plantations) and *P. brutia* forests in Turkey (Aday Kaya et al., 2019; Doğmuş-Lehtijärvi et al., 2007). The causal agent was identified to be *Diplodia sapinea* (Fr.) Fuckel (syn. *Diplodia pinea* (Desm.) Kickx., Sphaeropsis sapinea/Fr.: Fr./ Dyko & Sutton) in above-mentioned studies on pines from Turkey. *Cedrus libani* is also among the known hosts of *D. sapinea* (Zlatkovic et al., 2017), and the pathogen was recently detected on this species in the Atatürk Arboretum (Oskay et al., 2018), however not on the particular *C. libani* tree infected with *L. acicola*. Thus, it is possible that the detected *Diplodia* sp. in the Arboretum can be *D. sapinea*. However, confirmation of this pathogen requires additional studies.

The work reported in this paper contributes to knowledge on the geographical and host range of *L. acicola*, including reporting the presence of the disease for the first time on a non-pine host. Clearly, the potential impact of the pathogen on all Pinaceae requires investigation. The finding that many Turkish pines, as well as Lebanon cedar, are very susceptible to *L. acicola* is of great concern for the future development of forest ecosystems and the forestry industry in Turkey. The additional co-occurrence with *Dothistroma* sp., and *Diplodia sapinea* adds to these concerns.

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PEER REVIEW

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IV

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ORIGINAL ARTICLE

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Diversity, migration routes, and worldwide population genetic structure of *Lecanosticta acicola*, the causal agent of brown spot needle blight

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Abstract

Lecanosticta acicola is a pine needle pathogen causing brown spot needle blight that results in premature needle shedding with considerable damage described in North America, Europe, and Asia. Microsatellite and mating type markers were used to study the population genetics, migration history, and reproduction mode of the pathogen,

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based on a collection of 650 isolates from 27 countries and 26 hosts across the range of *L. acicola*. The presence of *L. acicola* in Georgia was confirmed in this study. Migration analyses indicate there have been several introduction events from North America into Europe. However, some of the source populations still appear to remain unknown. The populations in Croatia and western Asia appear to originate from genetically similar populations in North America. Intercontinental movement of the pathogen was reflected in an identical haplotype occurring on two continents, in North America (Canada) and Europe (Germany). Several shared haplotypes between European populations further suggests more local pathogen movement between countries. Moreover, migration analyses indicate that the populations in northern Europe originate from more established populations in central Europe. Overall, the highest genetic diversity was observed in south-eastern USA. In Europe, the highest diversity was observed in France, where the presence of both known pathogen lineages was recorded. Less than half of the observed populations contained mating types in equal proportions. Although there is evidence of some sexual reproduction taking place, the pathogen spreads predominantly asexually and through anthropogenic activity.

KEYWORDS
forest pathology, introduction pathways, invasive pathogen, mating type, microsatellites, *Mycosphaerella dearnessii*, *Pinus*

1 | INTRODUCTION

The genus *Lecanosticta* includes nine species, among which *Lecanosticta acicola* is the oldest documented and most well known (van der Nest et al., 2019b). *L. acicola* (formerly *Mycosphaerella dearnessii*) is an important pathogen of *Pinus* spp. causing brown spot needle blight (BSNB) disease that results in premature needle cast, leading to growth reduction and possible death of the trees. Historically, the most prominent damage caused by *L. acicola* has been on *Pinus palustris* plantations in the south-eastern United States (Siggers, 1944; Sinclair & Lyon, 2005), where the pathogen was also first described (de Thümen, 1878). To date, *L. acicola* has been reported on 53 pine species and subspecies (van der Nest et al., 2019a) and on the non-pine host *Cedrus libani* (Oskay et al., 2020).

Due to the significant damage it causes, *L. acicola* is listed as a quarantine pathogen in numerous countries (EPPO, 2022) and extra measures for containment have been applied in the European Union, where the pathogen is classified as a regulated non-quarantine pest (European Commission, 2019). Overall, climate extremes, global trade, and failure to implement proper quarantine measures have been commonly considered as essential factors exacerbating the spread of invasive plant pathogens, including *L. acicola* (Adamson et al., 2018b, 2021; Drenkhan et al., 2014b, 2020; Fisher et al., 2012; Ghelardini et al., 2017; Hanso & Drenkhan, 2009; Jürisoo et al., 2021). Furthermore, climate change, especially warmer winters, has been thought to be one of the main reasons for northwards spread of several forest pathogens (Hanso & Drenkhan, 2013).

In the last decade, the distribution of *L. acicola* has increased, particularly in Europe, where the pathogen has been reported in numerous new countries (van der Nest et al., 2019a). Since 2008, the pathogen has spread into northern Europe and has been found in Estonia, Lithuania, Latvia, and Sweden (Adamson et al., 2015; Cleary et al., 2019; Markovskaja et al., 2011; Mullett et al., 2018). Several new reports of *L. acicola* have been documented in eastern Europe (EPPO, 2018; Georgieva, 2020; Golovchenko et al., 2020; Stamenova et al., 2018) and the pathogen has reached the British Isles (Mullett et al., 2018). *L. acicola* has also expanded its range in Asia and is now present in Turkey, western Asia (Oskay et al., 2020). To date, the pathogen has been documented in areas of North and South America, Europe, and West and East Asia (Oskay et al., 2020; van der Nest et al., 2019a) but is distinctly lacking in the Southern Hemisphere.

Initially, *L. acicola* was thought to originate from Central America (Evans, 1984). However, in a recent study by van der Nest et al. (2019b) using a large collection of isolates from Central America, several distinct *Lecanosticta* species were described, although *L. acicola* was not recovered. As a result, it was proposed that this fungus may originate from North America. Three lineages of *L. acicola* have been proposed. Kais (1972) and Huang et al. (1995) indicated the presence of two distinct lineages of the pathogen based on isolates originating from the northern and southern states of the United States. These two lineages were supported based on multigene sequence data, while a third lineage was also identified from Mexico (van der Nest et al., 2019b). Janoušek et al. (2016) suggested, based on evolutionary modelling, that there were separate introductions of the two lineages into Europe, one lineage introduced and spreading in south-western Europe and the other in central and northern Europe. In addition, Huang et al. (1995) proposed that the

pathogen populations in Asia originate from south-eastern United States. So far, the third lineage seems contained in Mexico (van der Nest et al., 2019b).

L. acicola is a heterothallic ascomycete (Janoušek et al., 2014), but the species predominantly reproduces asexually and spreads via conidia that are dispersed over short distances by rain splash and dew (Siggers, 1939; Skilling & Nicholls, 1974). Previous studies have indicated the presence of both mating types of *L. acicola* in several European countries (Janoušek et al., 2016; Laas et al., 2019; Ortíz de Urbina et al., 2017; Sadiković et al., 2019). Based on the proportion of mating types and genetic analyses, sexual reproduction probably takes place in Austria and Germany, and possibly also in Estonia (Janoušek et al., 2016; Laas et al., 2019). Sexual reproduction of the pathogen taking place in Europe was confirmed by a recent report by Mesanza et al. (2021) describing the presence of the sexual state on *Pinus radiata* in Spain. Genetic recombination by sexual reproduction is one of the main factors, along with mutations, migration, and genetic drift, that can increase genetic diversity, possibly changing its adaptive potential in new environments (McDonald & Linde, 2002). The occurrence of sexual reproduction would also produce airborne ascospores capable of long-distance spread (Kais, 1971), another potential reason for the fast and recent expansion of *L. acicola*.

Overall, a high number of clones and low genetic diversity of *L. acicola* have been registered in Europe in several country- or regional-based population studies (Adamčíková et al., 2021; Laas et al., 2019; Sadiković et al., 2019). There is, however, a lack of information regarding the genetic structure and origin of the recently recorded populations of *L. acicola* in northern Europe and western Asia. A combined population study incorporating all isolates from previous studies and including those from more recent outbreaks would shed light on the migration history of the pathogen in Europe and determine the distribution area of the lineages described by Janoušek et al. (2016). It would also provide information about the reproductive mode and genetic diversity in populations, attributes that are the basis for producing genetic variation and creating new genotypes of the pathogen, which are important for assessing evolutionary potential. Therefore, the objectives of this study were to (i) investigate the worldwide genetic diversity and population structure of *L. acicola* including recently found populations in northern Europe and western Asia, (ii) elucidate the possible migration history of the pathogen, and (iii) assess the possibility of sexual reproduction taking place in the studied populations.

2 | RESULTS

2.1 | Isolates and haplotype identification

In total, 650 isolates from 27 countries and 26 different host taxa were used in the study. With the exception of *Cedrus libani*, all isolates were obtained from *Pinus* hosts. Of the collected isolates, 524 originated from 19 countries in Europe, 103 from North America (Canada, Mexico, United States), 18 from West Asia (Turkey, Georgia), three from East Asia (China, Japan), and two from South America (Colombia) (Table S1, Figure 1). In all downstream analyses, the samples from West Asia were considered part of Europe due to their geographical closeness. The internal transcribed spacer (ITS) sequences obtained for isolates from Colombia matched 100% to the sequences of the epitype culture of *L. acicola* in GenBank (NR_120239), the sequences of the isolates from Mexico had 99.6%–99.8% identity match with NR_120239, and the isolate obtained from Georgia showed 100% similarity with NR_120239 based on ITS sequencing. This represents the first confirmed record of *L. acicola* in Georgia using molecular methods. A culture was deposited in the Fungal Culture Collection of the Estonian University of Life Sciences (culture collection number: TFC101254). The ITS sequence of the isolate was deposited in GenBank (MZ323309).

The obtained sequences of the translation elongation factor 1-α gene region (*TEF1*) were 501 bp in length. Of the 15 isolates sequenced, four different elongation factor (EF) haplotypes were obtained. The sequences for two isolates from France (original culture codes B1254 and B1599) were found to be identical with isolates from Germany, Lithuania, and Canada (codes 18313, 23677, 17787, and 23696, Table S1) and with a reference sequence obtained from GenBank (accession number KC013002.2) marked as the Northern lineage or lineage 1 in van der Nest et al. (2019a).

Isolates from Canada, Estonia, and the north-eastern United States (culture codes 16637, 17789, 15644, and 17853, Table S1) were described by another EF haplotype. Named isolates contained a unique single-nucleotide polymorphism at location 22 of the obtained alignment—adenine (A)—while all other observed sequences were characterized by thymine (T) at the position.

One EF haplotype was present in isolates from the south-eastern United States, Spain, and France (culture codes 18065, 14881, 17856, and 16628, Table S1) and it was identical to the reference sequence (GenBank accession number MK015399) marked as the Southern lineage or lineage 2 in van der Nest et al. (2019a). One haplotype was unique to the south-eastern United States (culture code 18071, Table S1). All obtained *TEF1* sequences were deposited in GenBank (MZ826765–MZ826779, Table S1).

Analyses across 11 microsatellite markers resulted in a total of 172 alleles (Table S2). All observed loci were polymorphic, with the number of alleles ranging from four at loci MD5 and MD11 to 54 at locus MD8. Locus MD1 was monomorphic in north-eastern United States, in western Asia, and all over Europe, except in south-western Europe. Locus MD11 was monomorphic in north-eastern United States, western Asia and all over Europe, including south-western Europe, showing polymorphism only in the south-eastern United States. Microsatellite marker MD6 did not amplify isolates from Mexico, Slovakia, Japan, and China. In the following analyses, missing microsatellite data were treated as unknown data according to instructions given for each software program used. In total, 284 different multilocus haplotypes (MLHs) were found in the collection of isolates. All populations, except Belarus ($N = 3$), Ireland ($N = 3$), Mexico ($N = 4$), and Japan ($N = 2$), contained clones. In total,

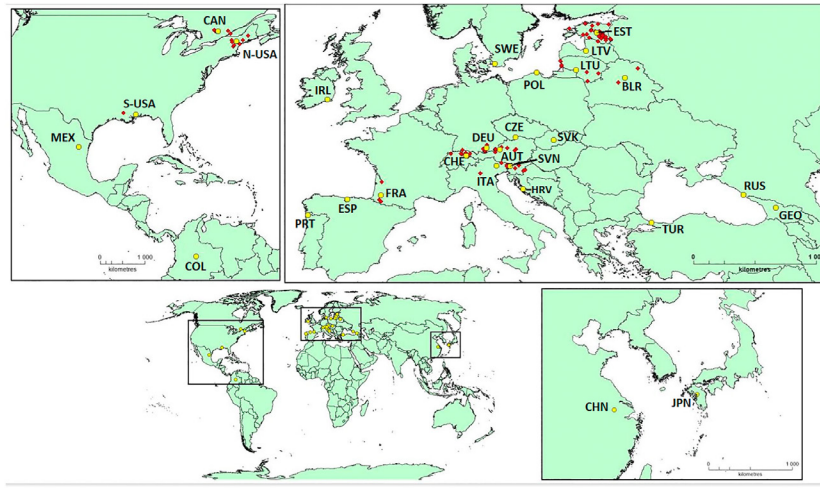

FIGURE 1 Map of the sampling locations (red dots) of *Lecanosticta acicola*. Yellow points indicate the weighted geographical midpoint of a particular sampling area and the representative population (Table S1). Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

100 haplotypes appeared more than once, and 16 haplotypes were found in more than one population (Table S3). The most common haplotype (MLH 196) appeared 45 times in four different populations (Turkey, Belarus, Lithuania, and Estonia). The second most frequent haplotype (MLH 125) was identified 23 times in two populations (Austria and Switzerland) and the third most frequent one (MLH 83) was identified 19 times in Slovenia. One haplotype (MLH 225) was found to be present on two continents, in Canada (North America) and Germany (Europe). One haplotype (MLH 257) was shared between China and Japan.

The global clonal fraction of *L. acicola* was 0.563. Overall, the clonal fraction index for Europe (0.600) was higher than for America (0.371) (Table 1), although for north-eastern America (CAN and N-USA combined) the clonal fraction was considerably higher (0.566, data not shown) and closer to the value found for Europe. In Europe, the clonal fraction ranged from 0.333 (ESP) to 0.818 (SVK). The population S-USA had a notably smaller clonal fraction (0.174) than other populations.

2.2 | Genetic diversity

Isolates from S-USA contained the highest number of unique alleles (36), followed by MEX (8) (Table 1, Table S2). Unique alleles were not found in N-USA and CAN. In Europe, unique alleles were found in eight of the 21 populations, with the highest number in AUT (six), followed by EST, HRV, and TUR (three). One allele was unique to East Asia, being present only in CHN and JPN.

In the East Asian populations of CHN and JPN, a total of 11 alleles was found. Eight of these alleles were also only found in S-USA, south-western Europe, or COL (Table S2).

The highest allelic richness (A_R) was recorded in S-USA (3.570). For Europe, the highest allelic richness was found in FRA (2.364), followed by SVN (2.290) and EST (2.233). The highest private allelic richness (PA_R) was also recorded in S-USA (1.968). In Europe, the highest private allelic richness was observed in TUR (0.442), followed by SVN (0.331) and RUS (0.302).

Likewise, the highest mean number of different alleles (Na), mean unbiased diversity (uh), and mean haploid genetic diversity (*h*) were observed in S-USA (Table 1). In Europe, the highest mean number of different alleles was recorded in LTU (4.545), EST (4.273), and SVN (3.909). Both the highest mean unbiased diversity and the highest mean haploid genetic diversity in Europe were observed in FRA, EST, and LTU.

2.3 | Population differentiation and genetic distance

According to the analysis of molecular variance (AMOVA), no significant differences were found between population pairs of

TABLE 1 Diversity statistics of *Lecanosticta acicola* populations based on 11 microsatellite markers

Region/ population code	N^a	No. of haplotypes[b]	No. of alleles	Unique alleles	Allelic richness A_R (SE^c) cc[d]	Private allelic richness PA_R (SE^c) cc[d]	Mean number of different alleles Na (SE^c) cc[d]	Mean unbiased diversity uh (SE^c) cc[d]	Mean haploid genetic diversity h (SE^c) cc[d]	Clonal fraction
America	**105**	**66**	**125**	**61**	**3.827 (0.337)**	**2.718 (0.353)**	**11.364 (2.028)**	**0.775 (0.046)**	**0.762 (0.045)**	**0.371**
CAN	19	12	31	0	2.248 (0.412)	0.161 (0.126)	2.818 (0.672)	0.407 (0.102)	0.372 (0.093)	0.368
COL[e]	2	1	11	3	–	–	–	–	–	–
MEX[e]	4	4	17	8	–	–	–	–	–	–
N-USA	34	11	21	0	1.730 (0.339)	0.134 (0.118)	1.909 (0.436)	0.251 (0.108)	0.223 (0.095)	0.676
S-USA	46	38	98	36	3.570 (0.469)	1.968 (0.365)	8.909 (1.856)	0.672 (0.096)	0.653 (0.093)	0.174
East Asia	**3**	**2**	**11**	**1**	–	–	–	–	–	–
CHN[e]	1	1	10	0	–	–	–	–	–	–
JPN[e]	2	2	11	0	–	–	–	–	–	–
Europe	**542**	**217**	**103**	**46**	**2.549 (0.434)**	**1.440 (0.461)**	**9.455 (3.864)**	**0.460 (0.097)**	**0.458 (0.097)**	**0.600**
AUT	31	15	30	6	1.902 (0.432)	0.253 (0.253)	2.727 (1.054)	0.263 (0.097)	0.245 (0.097)	0.516
BLR[e]	3	3	17	0	–	–	–	–	–	–
CHE	50	12	22	0	1.766 (0.350)	0.050 (0.034)	2.000 (0.467)	0.258 (0.110)	0.234 (0.100)	0.760
CZE	16	6	19	0	1.727 (0.384)	0.068 (0.061)	1.727 (0.384)	0.242 (0.111)	0.199 (0.091)	0.625
DEU	33	19	33	1	1.918 (0.410)	0.188 (0.128)	3.000 (0.982)	0.267 (0.099)	0.251 (0.093)	0.424
ESP	9	6	15	0	1.364 (0.152)	0.044 (0.044)	1.364 (0.152)	0.176 (0.077)	0.145 (0.064)	0.333
EST	127	62	47	3	2.233 (0.355)	0.172 (0.111)	4.273 (1.294)	0.421 (0.092)	0.414 (0.090)	0.512
FRA	10	6	26	0	2.364 (0.244)	0.028 (0.028)	2.364 (0.244)	0.524 (0.064)	0.431 (0.053)	0.400
GEO[e]	1	1	11	0	–	–	–	–	–	–
HRV	24	8	17	3	1.477 (0.319)	0.271 (0.194)	1.545 (0.455)	0.126 (0.091)	0.108 (0.079)	0.667
IRL[e]	3	3	15	0	–	–	–	–	–	–
ITA[e]	4	2	15	0	–	–	–	–	–	–
LTU	106	52	49	1	2.164 (0.388)	0.115 (0.072)	4.545 (1.734)	0.377 (0.102)	0.369 (0.100)	0.509
LTV[e]	1	1	11	0	–	–	–	–	–	–
POL	16	6	16	0	1.455 (0.207)	0.009 (0.009)	1.455 (0.207)	0.200 (0.084)	0.167 (0.070)	0.625
PRT[e]	2	1	11	0	–	–	–	–	–	–
RUS	17	8	18	2	1.455 (0.455)	0.302 (0.302)	1.636 (0.636)	0.091 (0.091)	0.080 (0.080)	0.529
SVK	11	2	10	0	–	–	–	–	–	0.818
SVN	58	16	43	1	2.290 (0.530)	0.331 (0.202)	3.909 (1.289)	0.331 (0.117)	0.309 (0.110)	0.724
SWE[e]	3	1	11	0	–	–	–	–	–	–

TABLE 1 (Continued)

Region/ population code	N^a	No. of haplotypes[b]	No. of alleles	Unique alleles	Allelic richness A_R (SE[c]) cc[d]	Private allelic richness PA_R (SE[c]) cc[d]	Mean number of different alleles Na (SE[c]) cc[d]	Mean unbiased diversity uh (SE[c]) cc[d]	Mean haploid genetic diversity *h* (SE[c]) cc[d]	Clonal fraction
TUR	17	6	24	3	2.182 (0.464)	0.442 (0.219)	2.182 (0.464)	0.315 (0.105)	0.263 (0.087)	0.647

Notes: Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

[a]Number of isolates.

[b]Equivalent to the number of isolates in the clone-corrected dataset (*N* [cc]).

[c]*SE*, standard error.

[d]cc, based on a clone-corrected dataset.

[e]Due to small sample size (*N* [cc] <6), these populations were excluded from population genetic analyses.

neighbouring European countries FRA–ESP, AUT–CZE, and CZE–DEU ($p > 0.05$) (Table 2). All other populations were significantly differentiated from each other.

Nei's genetic distance indicated that the populations N-USA and CAN are genetically rather similar to most populations in Europe (Table S4, Figure 2). The population S-USA is genetically similar only to ESP and genetically distant from all other populations. Similarly, ESP is distinct from all other European populations included in the analysis of Nei's genetic distance, except for FRA.

2.4 | Isolation by distance, phylogenetic tree, and population structure

The Mantel test for isolation by distance among 16 American and European populations represented by at least six isolates revealed significant correlation between geographical distance and Nei's genetic distance ($R^2 = 0.1679$, $p = 0.030$, Figure 3a). Isolation by distance was also supported in Europe ($R^2 = 0.2292$, $p = 0.010$, Figure 3b), but rejected in North America ($R^2 = 0.9975$, $p = 0.166$, Figure 3c).

The ln(Pr(X|*K*)) method of choosing the best number of STRUCTURE clusters indicated that seven clusters describe the dataset best (Figure S1), whereas the Δ*K* statistic indicated that two clusters explained the data best (Figure S2). At $K = 2$ one of the clusters (indicated in red) dominates not only in S-USA, MEX, COL, and the East Asian populations JPN and CHN, but also in the south-western European populations FRA, ESP, and PRT (Figure 4). The other cluster (indicated in light blue) dominates in N-USA, CAN, western Asia, and most of Europe, whilst also occurring in the south-western European population FRA. At $K = 4$ a clear central European cluster is differentiated (indicated in green) and from $K = 5$ up to $K = 7$ a single cluster (brown) dominates in HRV, W-ASIA, and part of CAN.

In populations POL, BLR, IRL, CZE, HRV, ESP, RUS, S-USA, MEX, and JPN, all isolates were dominated by a single cluster, whereas other populations contained isolates belonging to multiple different clusters ($K = 7$, Figure 4). The proportion of the STRUCTURE clusters in populations indicates differences between geographical regions (Figure 5). The northern European populations EST, LTV, LTU, POL, and BLR shared a roughly similar structure, with the light blue cluster dominating. Isolates from the Curonian Spit region in LTU belonged to the same cluster (green, $K = 4$–7) as those in central Europe. In central Europe, CHE and SVN show a more diverse structure with all the previously mentioned clusters represented in small proportions without a single dominating cluster. Populations from HRV, RUS, and TUR belong primarily to the brown cluster, which also occurs in CAN and N-USA. Up to $K = 5$, isolates from ESP, FRA, and PRT were mostly placed into the red cluster that is also dominating in S-USA; however, at $K = 6$ and $K = 7$ isolates from south-western Europe were mostly placed into a cluster (pink) that has only a marginal proportion in S-USA.

The neighbour-joining (NJ) dendrogram covering 28 populations indicates the presence of four groups: the first group

TABLE 2 Population differentiation according to analysis of molecular variance (AMOVA) between 16 populations of *Lecanosticta acicola*

Population code	AUT	CAN	CZE	DEU	ESP	EST	FRA	HRV	LTU	S-USA	POL	RUS	CHE	SVN	TUR
AUT	0.000														
CAN	0.001	0.000													
CZE	**0.335**	0.002	0.000												
DEU	0.003	0.001	**0.136**	0.000											
ESP	0.001	0.001	0.003	0.001	0.000										
EST	0.001	0.001	0.002	0.001	0.001	0.000									
FRA	0.001	0.001	0.013	0.001	**0.240**	0.001	0.000								
HRV	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000							
LTU	0.001	0.001	0.031	0.001	0.001	0.001	0.001	0.001	0.000						
S-USA	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000					
POL	0.001	0.001	0.005	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.000				
RUS	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000			
CHE	0.036	0.001	0.044	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		
SVN	0.001	0.002	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.011	0.000	
TUR	0.001	0.001	0.006	0.001	0.004	0.001	0.011	0.001	0.001	0.001	0.006	0.001	0.001	0.001	0.000
N-USA	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Notes: In the table the AMOVA *p*-values are shown. The populations that are similar according to AMOVA ($p > 0.05$) are indicated in bold. Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

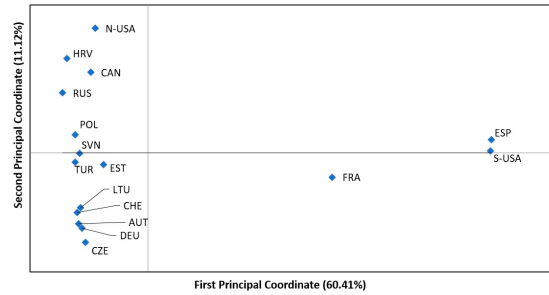

FIGURE 2 Principal coordinate analysis of Nei's genetic distance of 16 populations of *Lecanosticta acicola*. Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

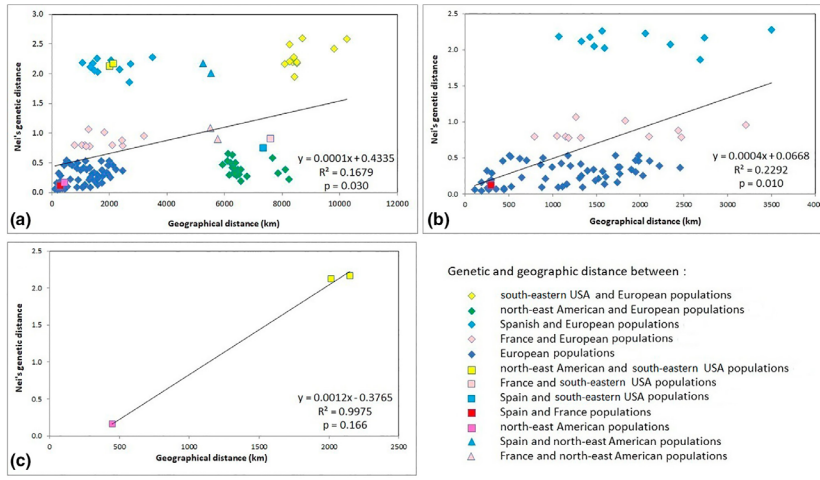

FIGURE 3 Results of the Mantel test on geographical and Nei's genetic distances of populations with at least six samples (*N* [cc] ≥ 6). Each point represents the combination of geographical and genetic distance values for each pair of populations compared. Legend (bottom right corner) explains symbols and colours representing the population pairs on the figures. (a) All 16 populations from North America and Europe (including western Asia). (b) Thirteen populations from Europe (including western Asia). (c) Three populations from North America.

includes populations from East Asia (CHN and JPN), S-USA, and COL; the second group includes populations from north-eastern America (N-USA and CAN) and most of the populations from Europe (Figure 6); MEX stands out as an independent clade (indicated as group 3, Figure 6); and the last group includes populations from south-western Europe (FRA, ESP, and PRT). However, the bootstrap support for most of the specific nodes is weak. Close genetic relationships, supported by the high bootstrap values, were observed between CHN and JPN and between FRA, ESP, and PRT in the dendrogram.

The NJ tree based on Nei's genetic distance between isolates showed an overall similar clustering into clades as defined by the STRUCTURE analyses (Figure S3). From the isolate-based figure it is evident that most isolates from south-western Europe (FRA, ESP, PRT) are genetically close but two isolates from France cluster together with samples from central Europe. Isolates from central and northern Europe indicated a mixed migration history with isolates from the same country being distributed among several clades.

2.5 | Modelling of population history

The first set of approximate Bayesian computation (ABC) scenarios was used to investigate the demographic history between

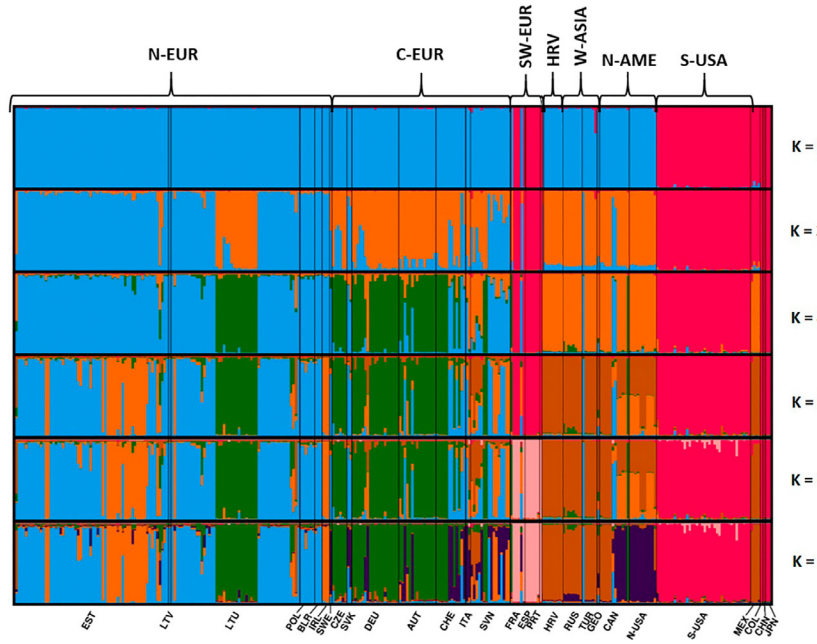

FIGURE 4 STRUCTURE clustering of the *Lecanosticta acicola* clone-corrected dataset, representing $K = 2$–7. Optimal number of clusters ($K = 2$) by ΔK and ($K = 7$) by ln(Pr(X|K)). Population codes are displayed under the figure; above the figure division into regions as analysed in the migration analyses is displayed. Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

the three main clusters indicated in America and Europe (Figure 4, $K = 3$). In Analysis 1, the posterior probabilities were highest for Scenario 17 ($p = 0.3760$, Table S5), where SW-EUR originated from N-AME and S-USA, while the merged population EUR originated from an admixture event between N-AME and an unsampled population. According to the estimated parameters N-AME was derived from the ancestral population a median of 30,600 and a mode of 35,200 generations ago and S-USA was derived from N-AME a median of 6880 and a mode of 4970 generations ago (Table S6). From the European populations, SW-EUR was derived from S-USA and N-AME a median of 512 and a mode of 628 generations ago and EUR was derived from N-AME a median of 99 and a mode of 51 generations ago. The scenario suggesting that the American populations originate from Europe was not supported (S1.20, $p = 0.000$), neither was the scenario suggesting that all named regions were derived separately from the ancestral population (S1.1, $p = 0.000$).

The most supported scenario in Analysis 2 (S2.3) suggested that N-EUR was derived from EUR ($p = 0.5026$, Table S5) a median of 40 and a mode of 32 generations ago (Table S6) with a weak bottleneck occurring (short duration and high number of founders).

The scenario with the highest support in Analysis 3 revealed that the populations in Croatia and western Asia (HRV and W-ASIA) originated from N-AME (S3.5, $p = 0.7077$, Table S5) a median of 205 and a mode of 235 generations ago (Table S6). The population of C-EUR originated from an admixture event between N-AME and an unsampled population a median of 115 and a mode of 77 generations ago.

A graphical representation of the winning historical scenarios showing the most supported historical events in the demographic history of *L. acicola* based on the observed populations is presented in Figure 7. Confidence in scenario choice with 95% credibility intervals for each analysis is presented in Table S5 and posterior distributions of parameters are presented in Table S6. Figures of the model checking results are presented in Figure S4.

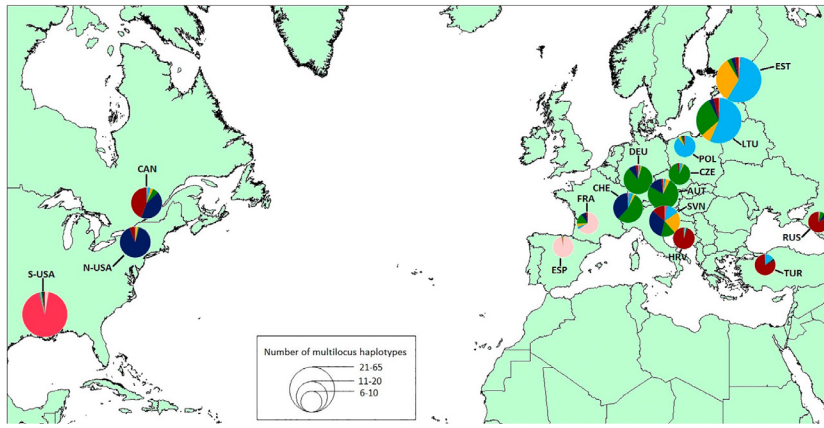

FIGURE 5 The proportion of STRUCTURE clusters ($K = 7$) in *Lecanosticta acicola* populations with at least six samples (N [cc] ≥ 6). Definition of population codes: AUT, Austria; CAN, Canada; CHE, Switzerland; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; HRV, Croatia; LTU, Lithuania; N-USA, north-eastern United States; POL, Poland; RUS, Russia; S-USA, south-eastern United States; SVN, Slovenia; TUR, Turkey.

2.6 | Mating type distribution and haploid linkage disequilibrium

The mating type idiomorphs were successfully identified for 629 isolates. Both mating type idiomorphs were present in 14 populations out of 28 (Table 3). In both East Asian populations (CHN and JPN) only *MAT1-2* was identified. The exact binomial test on the mating type ratios indicated that in six populations (ESP, FRA, IRL, MEX, SVN, and S-USA) equal ratios of the mating type idiomorphs ($p > 0.05$) were found based on the non-cc dataset and in 10 populations (CHE, DEU, ESP, FRA, IRL, MEX, N-USA, POL, SVN, and S-USA) based on the cc dataset. Therefore, in these populations, sexual reproduction is possible.

The index of association indicated that random mating occurred only in ESP and S-USA populations based on the non-cc dataset and additionally in SVN, HRV, and CZE populations based on the cc dataset, the last two being unexpected because only one mating type was identified in those populations (Table 3). The calculation of the index of association and the standardized index of association was not successful for the RUS population.

3 | DISCUSSION

This study, which includes 650 isolates from 27 countries, represents the most comprehensive population genetics analysis of *L. acicola* to date. The objective was to combine previously studied populations with newly collected data, particularly from northern Europe and western Asia, to determine the global diversity and pathways of movement of the pathogen. STRUCTURE clustering roughly corresponded with the geographic distribution of the isolates and revealed a subdivided population structure in several regions. The results provide evidence for several separate pathogen introductions from America into Europe and suggest that the recently discovered populations in northern Europe originate from previously described *L. acicola* populations in Europe. However, the populations in western Asia and Croatia appear to originate from a separate introduction event from North America. Despite quarantine efforts, *L. acicola* is now widespread in Europe and seems to be spreading via anthropogenic activity and both asexual and sexual natural dispersal.

The global set of *L. acicola* isolates can be divided into two main groups supported by STRUCTURE and genetic distance analyses. The distribution areas of those groups correspond with the results of previous studies describing lineages within the species (Huang et al., 1995; Janoušek et al., 2016; Kais, 1972; van der Nest et al., 2019a). Most of Europe, western Asia, and north-eastern North America comprise one genetically similar group, while south-western Europe, southern USA, and East Asia comprise the second group. Finer levels of substructure could be observed in northern and central Europe, while populations in Croatia, Russia, and Turkey stand out with rather homogeneous structure.

Overall, in central Europe the populations were genetically similar. Results suggest genetic exchange between countries with several shared haplotypes being found. The modelled population history suggests that the central European populations originate from north-eastern North America and an unsampled population. The fact that numerous alleles that were documented in several

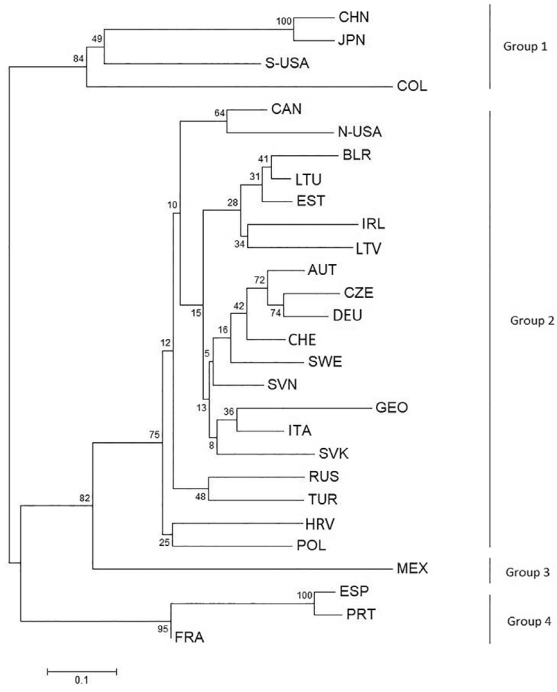

FIGURE 6 Neighbour-joining tree of genetic distances (Da, Nei, 1972) for 28 populations as implemented in POPTREE v. 2 with 10,000 bootstraps used to generate confidence at branch points. Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

European populations were not found in America (Table S2) highlights that some of the populations in Europe historically originate from unsampled populations. Sampling additional populations in North America could bring a better understanding of the origin of the European populations and about how the American populations themselves might have emerged.

This study revealed that the highest allelic diversity occurs in the south-eastern United States, where sexual reproduction takes place, supporting the findings of Janoušek et al. (2016). However, in most of the European populations, the genetic diversity was not much lower, and in some cases even higher, than in north-eastern American populations (N-USA and CAN), which probably represent the native range of the pathogen, and which are possibly the source of most of the European populations. In general, species are thought to have higher diversity in their native area when compared to regions where they were recently introduced (McDonald & McDermott, 1993). Additional sampling in northern America would, however, almost certainly reveal more diversity, filling the gap due to the currently more thorough sampling in Europe.

Up to $K = 5$, the populations of south-western Europe were dominated by the same cluster as were those of south-eastern United States, which is in accordance with the previous study by Janoušek et al. (2016). In south-western Europe, one haplotype was found to be shared between France, Spain, and Portugal, indicating shared origin or close connections between the populations. Surprisingly, the microsatellite data and the EF sequences revealed that both described lineages of the pathogen are present in France. Overall, the analyses of population history gave most support to the scenario where the south-western European populations were formed through an admixture event between south-eastern United States and north-eastern North America and are older than other populations in Europe. However, the NJ tree based on genetic distance placed isolates from France closer to samples originating from Germany and Switzerland. The presence of both N-AME and S-USA STRUCTURE clusters (at $K = 2$) and both North and South lineages in south-western Europe strongly suggest at least two independent introductions to the region, either directly from North America, or via spread from European countries. The high levels of genetic diversity found in France, indicated by high allelic richness, mean unbiased diversity, and mean haploid genetic diversity, are most probably due to isolates from genetically different populations being present there.

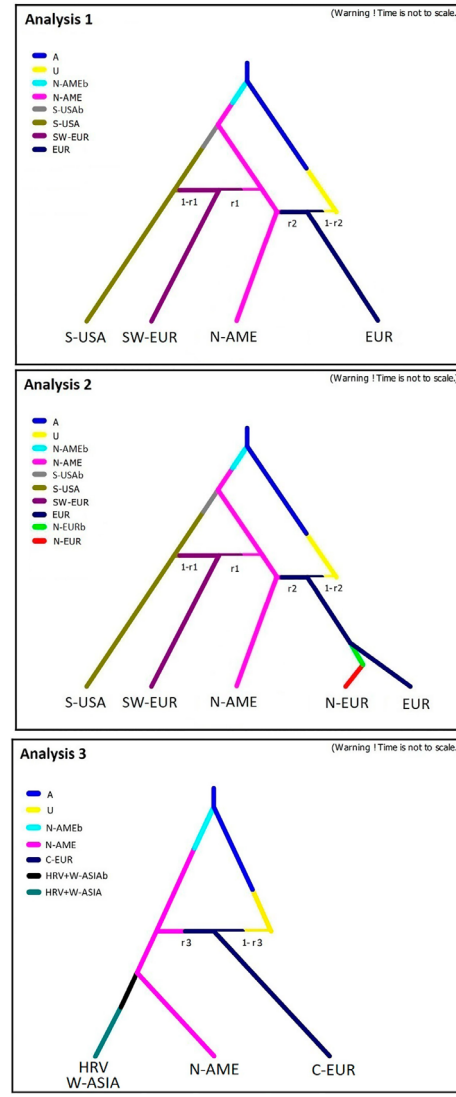

FIGURE 7 A graphical representation of the historical scenarios, most supported by the approximate Bayesian computation (ABC) analyses. A, ancestral population; U, unsampled population; N-AME, north-eastern America (N-USA + CAN); S-USA, south-eastern United States; SW-EUR, south-western Europe; EUR, combined population of C-EUR, HRV, and W-ASIA; C-EUR, central Europe; N-EUR, northern Europe; HRV, Croatia; W-ASIA, western Asia; b, bottleneck event; r1, r2, and r3, rates of admixture; thickness of line indicates the contribution from populations (r and r − 1).

Microsatellite diversity was surprisingly high in the northern European populations of Estonia and Lithuania, considering that *L. acicola* has been known to be present there for only little longer than a decade. Based on the results of the current analyses, modelling of population history suggests that *L. acicola* in northern Europe originates from other populations in Europe and not from a separate

TABLE 3 A summary of the mating type distribution and index of association results

Population	*MAT1-1-1* non-cc[a]	*MAT1-2* non-cc[a]	*p*-value of exact binomial test non-cc[a]	*MAT1-1-1* cc[b]	*MAT1-2* cc[b]	*p*-value of exact binomial test cc[b]	I_A[c] non-cc[a]	$\bar{r}_d$[e] non-cc[a]	*p*-value of I_A[c] and $\bar{r}_d$[e] non-cc[a]	I_A cc[b]	$\bar{r}_d$ cc[b]	*p*-value of I_A and $\bar{r}_d$ cc[b]
AUT	2	29	0.000	2	13	0.007	2.582	0.543	0.001	1.901	0.386	0.001
BLR	3	0	–	3	0	–	–	–	–	–	–	–
CAN	17	2	0.001	10	2	0.039	1.708	0.250	0.001	1.053	0.157	0.001
CHE	3	47	0.000	3	9	0.146	2.025	0.678	0.001	0.558	0.186	0.001
CHN	0	1	–	0	1	–	–	–	–	–	–	–
COL	0	2	–	0	1	–	–	–	–	–	–	–
CZE	0	16	–	0	6	–	1.173	0.403	0.001	0.109	0.036	0.509
DEU	24	9	0.014	13	6	0.167	0.987	0.177	0.001	0.921	0.157	0.003
ESP	3	6	0.508	2	4	0.688	−0.173	−0.058	0.848	−0.376	−0.126	1.000
EST	102	24	0.000	44	17	0.001	1.251	0.163	0.001	0.309	0.039	0.003
FRA	7	2	0.180	4	1	0.375	6.516	0.731	0.001	5.440	0.609	0.001
GEO	1	0	–	1	0	–	–	–	–	–	–	–
HRV	0	24	–	0	8	–	0.409	0.410	0.004	0.106	0.106	0.543
IRL	2	1	1.000	2	1	1.000	–	–	–	–	–	–
ITA	4	0	–	2	0	–	–	–	–	–	–	–
JPN	0	2	–	0	2	–	–	–	–	–	–	–
LTV	1	0	–	1	0	–	–	–	–	–	–	–
LTU	63	29	0.001	30	13	0.014	0.673	0.104	0.001	0.516	0.078	0.001
MEX	2	2	1.000	2	2	1.000	–	–	–	–	–	–
N-USA	31	3	0.000	8	3	0.227	2.041	0.689	0.001	0.968	0.324	0.001
POL	13	3	0.021	4	2	0.688	1.317	0.439	0.001	1.189	0.396	0.014
PRT	1	0	–	1	0	–	–	–	–	–	–	–
RUS	0	17	–	0	8	–	–	–	–	–	–	–
S-USA	26	20	0.461	21	17	0.627	0.090	0.010	0.223	−0.068	−0.007	0.809
SVK	11	0	–	2	0	–	–	–	–	–	–	–
SVN	26	28	0.892	4	9	0.267	2.001	0.404	0.001	0.139	0.030	0.260
SWE[d]	0	3	–	0	1	–	–	–	–	–	–	–
TUR	17	0	–	6	0	–	2.956	0.602	0.001	2.534	0.634	0.001

Notes: Definition of population codes: AUT, Austria; BLR, Belarus; CAN, Canada; CHE, Switzerland; CHN, China; COL, Colombia; CZE, Czech Republic; DEU, Germany; ESP, Spain; EST, Estonia; FRA, France; GEO, Georgia; HRV, Croatia; IRL, Ireland; ITA, Italy; JPN, Japan; LTU, Lithuania; LTV, Latvia; N-USA, north-eastern United States; MEX, Mexico; POL, Poland; PRT, Portugal; RUS, Russia; S-USA, south-eastern United States; SVK, Slovakia; SVN, Slovenia; SWE, Sweden; TUR, Turkey.

[a]non-cc, nonclone-corrected dataset.

[b]cc, clone-corrected dataset.

[c]I_A, index of association.

[d]Due to small sample size (N [cc] < 6), these populations were excluded from population genetic analyses.

[e]$\bar{r}_d$, standardized index of association.

introduction event from America. Strong support is given for several introductions of the pathogen. Based on the STRUCTURE results, isolates with different genetic origins are present in Lithuania, with samples from the Curonian Spit belonging to a different cluster than isolates from other sites in the country. Two haplotypes were found to be shared between Germany (Bavaria region) and Lithuania (Palanga Botanical Garden and Curonian Spit region), supporting introduction of the pathogen from central Europe. Plant trade has also probably contributed to the spread the pathogen throughout northern Europe, as populations shared similar structure and haplotypes between the Baltic countries. In addition, one haplotype identified from the first *L. acicola* report in Belarus was also present in Estonia and Lithuania, and another in Estonia and Ireland. It is assumed that there have been several introduction events into Estonia, because for several years after the first report of the pathogen, only isolates with the MAT1-1-1 mating type were found in the country before those with MAT1-2 appeared (Adamson et al., 2015). Therefore, introduction of genetically different strains of the pathogen is probably the reason for the higher diversity observed in northern Europe.

In this paper the presence of *L. acicola* in Georgia was confirmed with sequence data, demonstrating the pathogen's continuing range expansion in western Asia. A previous report from Georgia (Kizikelashvili, 1987) was considered to be due to taxonomic confusion with the red band needle blight pathogen *Dothistroma* (Barnes et al., 2016; Matsiakh et al., 2018; Oskay et al., 2020). A unique allele shared only between Turkey and Georgia (allele 159, locus MD6, see Table S2) indicates a connection between these geographically close populations. The results of the STRUCTURE analyses show that isolates from the Black Sea coast of Russia, Turkey, and Georgia belong to the same cluster that also predominates in Croatia, Canada, and Mexico. Also, the isolate-based NJ tree indicates that strains from the western Asian populations are genetically close to the Mexican and north-east American populations. The ABC analyses supported the scenario where populations from western Asia and Croatia originated from northern America.

Croatia is known to host one of the oldest populations of *L. acicola* in Europe, with the first description dating from 1975 (Milatović, 1976). The results of the ABC analyses suggested that the cluster containing Croatia and western Asia is older than the central European one. It was unexpected that the Croatian and western Asian populations clustered together because of the distance between them. It is possible that there has been natural spread of the pathogen across the Balkan peninsula to Turkey and the Black Sea coast of Russia from Croatia. However, the spread of the pathogen is limited when reproducing asexually, and even if sexual recombination takes place there should be records of diseased pine stands from Croatia through to Turkey. Recently, *L. acicola* has been reported from Romania and Bulgaria (EPPO, 2018; Georgieva, 2020; Stamenova et al., 2018) but to date, there have not been any reports from Balkan countries that would indicate the overland dispersal of the pathogen. Possibly only one of the populations in the western Asian region could have a link with Croatia. In Croatia, as in Russia, only the *MAT1-2* idiomorph has been found. However, in Turkey and Georgia only the *MAT1-1-1* idiomorph was found. Therefore, it is possible that the Croatian and western Asian populations originated from separate introduction events and the similarities between these populations are due to their origin being genetically similar populations in America.

The presence of only one mating type (MAT1-2) and a shared haplotype between China and Japan from isolates obtained decades apart suggests that the spread of the pathogen in Asia is strongly affected by human activity and by the introduction of a limited number of strains. However, a larger sample size of the Asian populations is needed to confirm this. Huang et al. (1995) suggested that the East Asian populations have a south-east American origin, but Janoušek et al. (2016) found that the isolates from East Asia formed a unique group that is not part of the Southern lineage based on EF sequences. However, a more recent global phylogenetic study also placed East Asian *L. acicola* isolates (China, Japan, and Korea) together with isolates originating from South America, southern United States, and south-western Europe (van der Nest et al., 2019b). The microsatellite data obtained in the current study indicated similarities between samples from East Asia and Southern lineage populations (south-eastern USA and south-western Europe) as several alleles were present only in the named regions (see Table S2). Although the number of isolates from East Asia was too low to use in the ABC analyses, STRUCTURE placed East Asian and south-east United States samples into the same cluster, and both NJ trees, based on genetic distances between populations and individual isolates, indicated a close connection between East Asian and south-east United States isolates.

Only half of the observed populations contained both mating types and an even smaller number of populations contained mating types in equal proportions (see Table 3). Additionally, all populations with more than four isolates contained clones, highlighting the predominantly asexual reproductive mode of the pathogen. As is expected after an initial introduction event, in most cases the new, recently introduced populations were found to contain only one mating type. However, in Ireland and Poland, both mating types were found, although the pathogen was only recently found in these countries. Several studies have concluded that sexual recombination could take place in some European populations based on the occurrence of both mating types and in some cases supported by microsatellite analyses (Janoušek et al., 2016; Laas et al., 2019; Sadiković et al., 2019). Based on the isolates used in this study, random mating was indicated in Spain, south-eastern United States, Slovenia, and, interestingly, also in Croatia and the Czech Republic, although only the *MAT1-2* idiomorph was documented there. Recently, the sexual state of *L. acicola* was found in *P. radiata* in Spain (Mesanza et al., 2021), proving that sexual reproduction of the pathogen takes place in Europe. The occurrence of sexual reproduction, together with the presence of both pathogen lineages, in south-western Europe raises concerns about whether sexual recombination between the two lineages could take place, particularly as it has been suggested that the lineages could represent distinct species (Janoušek et al., 2016).

The occurrence of shared haplotypes between countries illustrates the importance of human activity in the spread of *L. acicola* in Europe and between continents. Identical haplotypes being found in Canada and the Munich Botanical Garden in Germany provide evidence for direct anthropogenic transmission of the pathogen across the Atlantic Ocean. Overall, most European populations demonstrated a subdivided structure, the cause of which could be multiple introduction events. In cases where the pathogen is already widespread in the country, it is increasingly important to avoid new introductions and mixing of pathogen strains that originate from genetically different populations. Repeated introductions from genetically different populations could have serious outcomes as they increase the genetic diversity of the pathogen populations and create possibilities for the emergence of new haplotypes that may be more virulent or adapted to certain climate conditions (McDonald & Linde, 2002; Molofsky et al., 2014). Therefore, proper phytosanitary control measures, particularly effective quarantine rules and diagnostic methods, are needed to avoid pathogen introductions via plant trade.

In some cases, phytosanitary measures have proved to be effective in controlling pathogen spread, although it is difficult to eradicate the pathogen completely. In the Tallinn Botanic Garden, northern Estonia, fungicides were regularly used after the first detection of *L. acicola* (Kaur & Hermann, 2021), and although the pathogen is now widespread across Estonia, the haplotypes found in the botanical garden have not been detected in other Estonian locations (Laas et al., 2019). In Lithuania, after the first identification of the disease on the Curonian Spit, all heavily infected trees were felled and burned (Markovskaja et al., 2011). From the samples included in this study, the cluster present in the Curonian Spit region is contained there, although two isolates in the Palanga Botanical Garden, on the Lithuanian mainland, showed similar STRUCTURE clustering.

In numerous countries, the first records of *L. acicola* originate from non-native host species in city greeneries, botanical gardens, and arboreta (for example, see Cleary et al., 2019; Drenkhan & Hanso, 2009; Golovchenko et al., 2020; Mullett et al., 2018; Oskay et al., 2020). However, recently there have been a growing number of records of *L. acicola* from native *Pinus sylvestris* stands across Europe (Adamson et al., 2018a; Cech & Krehan, 2008; EPPO, 2012, 2015; Georgieva, 2020). In Bulgaria severe damage with defoliation from 50% to 100% has been reported from several *P. sylvestris* and *Pinus nigra* stands near the initial outbreak site, despite control measures being implemented after the initial discovery of the disease (Georgieva, 2020). A population study carried out by Laas et al. (2019) recorded the presence of potentially more pathogenic haplotypes in Estonia infecting both non-native *Pinus mugo* and native *P. sylvestris*. The results of the current analyses show that one of the potentially more aggressive haplotypes (MLH 196) is present in Estonia, Lithuania, Belarus, and Turkey. In Turkey, it was found on a *P. sylvestris* tree suffering high infection severity, with up to 80% of the canopy affected (Oskay et al., 2020). However, although this haplotype may have the potential to be a threat to the extensive natural stands of *P. sylvestris* in northern Europe, it may not demonstrate the same effect in southern regions because several reports and inoculation tests have indicated that southern and northern provenances of *P. sylvestris* have differing susceptibility to *L. acicola* (Jankovský et al., 2009; Phelps et al., 1978; Skilling & Nicholls, 1974).

The results of this study have indicated that pathogen strains with different origins exist in proximity in Europe. In addition, strong support is given for human activity (i.e., plant transportation) supporting the range expansion of the pathogen and leading to the co-existence of genetically different strains. At some level, sexual reproduction also takes place in the European populations. Recombination of different strains could lead to further increases in genetic diversity and produce more virulent strains. Many populations in Europe still contain a single mating type, are structurally homogeneous, have low genetic diversity, and only comprise one lineage. Therefore, it is important not only to avoid further human-mediated spread of the pathogen, but also to avoid mixing of populations. Continual monitoring of *L. acicola* will be needed to follow developments in the geographic spread, host range expansions, and ongoing changes in the genetic diversity, with a special focus on maintaining the extensive stands of native pine species in Europe.

4 | EXPERIMENTAL PROCEDURES

4.1 | Sample collection, fungal isolation, DNA extraction, and isolate identification

Needle samples with typical symptoms of BSNB were collected from a variety of *Pinus* taxa and from *C. libani*. Samples were obtained from a maximum of 30 sampling sites per country from a total of 27 countries in North and South America, Europe, and Asia (Figure 1, Table S1). Sampling sites in the same country were merged and referred to as populations, except for the United States, where samples were divided into two populations—north-eastern United States (N-USA) and south-eastern United States (S-USA).

Samples were collected from the lower parts of the tree canopy, placed in paper or plastic bags, and kept dry or stored at −20°C until pathogen isolation. Some isolates were obtained from culture collections (see Table S1). In most cases, one fungal isolate per sampled tree was obtained, except for Croatia, Munich Botanical Garden in Germany, Italy, Russia, Slovenia, Sweden, Turkey, Curonian Spit sampling sites in Lithuania, and the Mississippi sampling site in the United States, where up to six isolates per tree were obtained. Some of the data of isolates used in this study have been previously published in the context of country-specific population studies or new country and host records (Table S1), and DNA or mycelium for these isolates was received to be included in this study.

Isolations to pure culture were made according to Mullett and Barnes (2012). Isolates were grown in the dark at room temperature (21°C) on pine needle agar medium. The medium consisted

of 1 L filtered Scots pine needle extract (50 g fresh weight/L tap water boiled for 20 min), 15 g malt extract (Oxoid), and 15 g technical agar (Biolife) autoclaved at 106°C for 30 min (see Drenkhan et al., 2013). Approximately 0.04 g of mycelium from the colony edge was transferred to a 2-ml microcentrifuge tube and stored at −20°C until DNA extraction. Mycelium was homogenized with an MM400 homogenizer (Retsch GmbH) using metal beads (2.5 mm diameter). DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific) or as specified in Sadiković et al. (2019) for Croatian and Slovenian samples, in Raitelaitytė et al. (2020) for Polish samples, and in Mullett et al. (2018) for Portuguese samples.

L. acicola identity was confirmed by PCR with species-specific primers Latef-F and Latef-R (Ioos et al., 2010). The PCRs were performed in 20-µl reaction volumes. Cycling conditions were chosen according to Ioos et al. (2010) with modifications according to Drenkhan et al. (2014a). All PCRs were carried out using a Tprofessional thermocycler (Biometra). PCR products were visualized on a 1% agarose gel (SeaKem LE agarose) under UV light using a Quantum ST4-system (VilberLourmat SAS).

The ITS region of the *L. acicola* isolates obtained from Georgia, Mexico, and Colombia was sequenced in order to confirm the species identity and exclude the presence of other *Lecanosticta* species (Theron et al., 2022; van der Nest et al., 2019b). The ITS PCR was performed using the fungal-specific PCR primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). ITS PCR products were sequenced in a single direction using the primer ITS5 (White et al., 1990).

In addition, the *TEF1* region of a random selection of 15 isolates from Europe and North America was sequenced. PCR amplification and sequencing in both directions was done using the primers EF1-728F (Carbone & Kohn, 1999) and EF2 (O'Donnell et al., 1998). All PCR products were sequenced at the Estonian Biocentre in Tartu. Sequences were edited using BioEdit v. 7.2.5. and BLAST searches for the fungal taxa were performed in GenBank (NCBI).

4.2 | Haplotype identification

For multilocus haplotyping, 11 microsatellite markers were used: MD1, MD2, MD4, MD5, MD6, MD7, MD8, MD9, MD10, MD11, and MD12 (Janoušek et al., 2014). The PCR amplification conditions were as described in Janoušek et al. (2014, 2016). For fragment analysis, PCR products were pooled into two panels according to Janoušek et al. (2014) and run on a 3130XL genetic analyser (Applied Biosystems) with 500 LIZ Size Standard (Applied Biosystems) at the Estonian Biocentre in Tartu. Alleles were scored using GeneMapper v. 5.0 (Applied Biosystems).

Isolates with identical alleles at all microsatellite loci were considered clones. Two datasets were created: one containing all isolates (non-cc) and the other containing only one of each haplotype (cc) per population as defined in Table 1.

4.3 | Genetic data analyses

4.3.1 | Genetic diversity

The non-cc dataset was used to calculate the total number of haplotypes using GenAlEx v. 6.5 (Peakall & Smouse, 2012). The cc dataset was used to calculate the total number of alleles and unique alleles, the mean number of different alleles (Na), the mean haploid genetic diversity (*h*), and the mean unbiased diversity (uh) for each population using GenAlEx 6.5. The cc dataset was used to calculate the allelic richness (A_R, the number of distinct alleles in the population) and the private allelic richness (P_{AR}, the number of unique alleles in the population) in ADZE v. 1.0 (Szpiech et al., 2008). Because sample sizes across populations differed, a rarefaction approach was used with population sizes standardized to six (Szpiech et al., 2008). The clonal fraction was calculated for each population according to Zhan et al. (2003).

Due to low sample size (N [cc] < 6), 12 populations (BLR, CHN, COL, GEO, IRL, ITA, JPN, LTV, MEX, PRT, SVK, and SWE) were excluded from further population genetic analyses, unless otherwise stated.

An AMOVA was performed in GenAlEx v. 6.5 on the cc dataset to test for significant differentiation between populations.

4.3.2 | Isolation by distance

Mantel tests, conducted in GenAlEx v. 6.5, were used to test for isolation by distance on the cc dataset using Nei's genetic distance (Nei, 1972, 1978) and geographic distances. In total, three different analyses were performed for separate sampling regions. First, isolation by distance was tested among all populations. Next, isolation by distance was tested separately for the populations in North America and then for populations in Europe, in order to assess if the genetic distance between populations increases with distance. Generally, we would expect genetic differentiation to increase with distance in native populations. For introduced populations, we would expect a lack of isolation by distance.

For visualization of Nei's genetic distances and geographic distances, principal coordinates analysis (PCoA) was carried out in GenAlEx v. 6.5 using the covariance standardized method.

4.3.3 | Population clustering

The program STRUCTURE v. 2.3.4 (Falush et al., 2003) was used to estimate the most likely number of population clusters (*K*), assign isolates into genetically different groups, and thereby determine the structure within populations without any prior data on geographic location or host provided. For the STRUCTURE analysis the cc dataset was used. Each of 20 independent runs of $K = 1–25$ were carried out with 10,000 burn-in iterations followed by a run of 100,000. The most likely number of clusters (*K*) was determined using the

ln(Pr(X|*K*)) method (Pritchard et al., 2000, 2010) and the Δ*K* statistic (Evanno et al., 2005) in CLUMPAK (Kopelman et al., 2015). The final assignment of individuals to clusters was carried out on the optimum *K* by applying 100,000 burn-in iterations, followed by 1,000,000 runs. For each number of clusters (*K*), 20 independent runs were performed.

4.3.4 | Phylogenetic analysis

Phylogenetic relationships among the populations were inferred with POPTREE v. 2 (Takezaki et al., 2010) using the NJ method (Saitou & Nei, 1987) based on allele frequency of a cc dataset containing all 28 populations. A bootstrap test with 10,000 replications was run for the NJ tree to generate confidence at branch points.

Additionally, an NJ tree was constructed using all isolates to assess the genetic distance between isolates, independent of the geographic origin or population. For that purpose, the genetic distance between all isolates was calculated in POPULATIONS v. 1.2.31 (Langella, 2002) using Nei's standard distance D_A (Nei et al., 1983). The obtained distance matrix was used to construct an NJ tree in MEGA X (Kumar et al., 2018). Isolates were coloured as indicated by the population clustering results obtained with STRUCTURE according to $K = 7$ (Figure 4).

4.3.5 | Modelling of demographic history

To reconstruct the history of divergence among the observed *L. acicola* populations, ABC was performed on the cc dataset using DIYABC v. 2.1.0 (Cornuet et al., 2014). The STRUCTURE clusters (Figure 4) were considered to develop scenarios describing the demographic history between the main regions: north-eastern America (N-AME), south-eastern United States (S-USA), northern Europe (N-EUR), central Europe (C-EUR), south-western Europe (SW-EUR), Croatia (HRV), and western Asia (W-ASIA) (Figure 4). The populations from East Asia, Mexico, and Colombia were excluded from the analysis due to their small sample size.

As there are numerous possibilities for the origin of *L. acicola* in the observed regions, a stepwise procedure was used to address questions around historical scenarios (Konečný et al., 2013). The best scenario obtained in the first step was used to inform the scenarios of the next step. In total, three different analyses with various scenarios were performed. In Analysis 1 the relationship among the three main clusters in America and Europe was investigated, Analysis 2 was conducted to elucidate the origin of northern European populations, and Analysis 3 was conducted to investigate the origin of central Europe and the cluster dominating in Croatia and western Asia. In all scenarios, a bottleneck was modelled at the foundation of populations and the introduced populations were assumed to be isolated from each other and from source populations after the introduction events. A description of the analyses performed and scenarios is presented in Table S5.

Analysis 1: As Europe breaks into two clusters at $K = 3$, for the first analysis central Europe (C-EUR), Croatia (HRV), and western Asia (W-ASIA) were merged into EUR (Figure 4). The presence of an unsampled population was also considered as a possibility of populations originating from areas not represented in this study. Therefore, in Analysis 1 a set of 20 scenarios were included to test whether N-AME and S-USA were derived separately from an ancestral population (S1.1–S1.7, S1.19) or if one region was derived from the other (S1.8–S1.18). For the European populations the following were tested: if SW-EUR and EUR originated from an ancestral population (S1.1, S1.20), if SW-EUR originated from S-USA (S1.2, S1.5, S1.8, S1.9, S1.14, S1.15), from an admixture event between S-USA and N-AME (S1.3, S1.6, S1.10, S1.11, S1.16, S1.17), or from an admixture event between S-USA and EUR (S1.4, S1.7, S1.12, S1.13, S1.18, S1.19), and whether EUR was derived directly from N-AME (S1.2, S1.3, S1.4, S1.8, S1.10, S1.12, S1.14, S1.16, S1.18) or from an admixture event between N-AME and an unsampled population (S1.5, S1.6, S1.7, S1.9, S1.11, S1.13, S1.15, S1.17, S1.19) or if the American populations were derived from EUR and SW-EUR (S1.20).

Analysis 2: According to STRUCTURE clustering and their close geographical distance, populations EST, LTV, LTU, POL, BLR, IRL, and SWE were merged into the northern Europe (N-EUR) region for migration analyses. The question about the origin of *L. acicola* in N-EUR was tested in Analysis 2 via a set of six scenarios. The scenarios tested if N-EUR originated from N-AME (S2.1), EUR (S2.3), an unsampled population (S2.2), or an admixture event between these regions (S2.4, S2.5, S2.6).

Analysis 3: Subsequently, as the origin of the main clusters in Europe according to $K = 3$ was revealed, the demographic history between subclusters of central Europe (C-EUR), Croatia (HRV), and western Asia (W-ASIA) was explored based on seven scenarios in Analysis 3. The scenarios tested whether HRV and W-ASIA originated from N-AME (S3.5, S3.6), C-EUR (S3.2), an unsampled population (S3.1, S3.4), or an admixture event between these regions (S3.3, S3.7), and similarly whether C-EUR originated directly from N-AME (S3.1, S3.3), from an admixture event between N-AME and an unsampled population (S3.2, S3.4, S3.5, S3.7), or from an admixture event between HRV or W-ASIA with an unsampled population (S3.6).

Initially, the demographic priors of the tested scenarios were set with a broad range. After 100,000 preliminary runs, the prior checking option was used according to the DIYABC manual and prior distributions adjusted step by step and finally set up as mentioned in Table S6. The generalized stepwise model was followed for the microsatellite loci and default values for the mutation model parameters were used (Cornuet et al., 2014). Ten microsatellite markers (MD1, MD2, MD4, MD5, MD7, MD8, MD9, MD10, MD11, and MD12) were used in the ABC analyses.

For each simulation the commonly used genetic summary statistics were used (i.e., mean number of alleles and mean size variance for one sample and mean number of alleles, mean genetic diversity, F_{ST}, classification index, and $(d\mu)^2$ distance for two sample summary statistics). The obtained times of events are in generations and roughly one year represents one generation. Other statistics available in DIYABC were later used in model checking.

One million datasets were simulated for each scenario. The posterior probability of each scenario was estimated by polychotomous logistic regression on 1% of the simulated datasets closest to the observed dataset, transformed by linear discriminant analysis (Cornuet et al., 2014). Posterior distributions of parameters were estimated for the most supported scenario by the logit transformation of parameters and linear regression on 1% of the closest simulated datasets. Model checking was done using the summary statistics not used in model selection as recommended by Cornuet et al. (2010). Confidence in scenario choice was evaluated by analysing simulated pseudo-observed datasets with the same number of loci and individuals as a real dataset. One hundred pseudo-observed datasets were simulated for each scenario with parameter values taken from the same distributions as for previous ABC analyses.

4.4 | Mating type determination and mode of reproduction

Mating types of the isolates were determined using a set of primers developed by Janoušek et al. (2014). PCRs were carried out in 20-μl volumes according to Janoušek et al. (2014), with an initial denaturation step at 95°C for 12 min as described in Adamson et al. (2015). PCR products were visualized with gel electrophoresis. The presence of the *MAT1-1-1* and the *MAT1-2* idiomorph was indicated by an amplicon size of 560 bp and 288 bp, respectively.

To evaluate the possibility of sexual recombination in the populations, three tests were carried out on both the non-cc and cc datasets for populations with at least six isolates. In order to assess if the populations deviate significantly from the null hypothesis of a 1:1 ratio of mating types, the exact binomial test was used as described in Barnes et al. (2014). In addition, the index of association (I_A) and the standardized index of association ($\bar{r}_d$) were calculated in the R package poppr (Kamvar et al., 2014) to test for random mating in the populations. Both analyses involved comparing the values for the observed dataset with the values for 1,000 artificially recombined datasets.

AUTHOR CONTRIBUTIONS

Sample collection: M.L., K.A., I.B., J.J., M.S.M., K.A., M.A., L.B., H.B., T.S.B., P.C., T.C., M.C., L.G., L.J., S.M., I.M., J.B.M., F.O., B.P., K.R., D.S., and R.D. Laboratory and data analyses: M.L., K.A., and K.R. Manuscript preparation and writing: M.L., K.A., I.B., J.J., M.S.M., M.C., R.E, D.S., and R.D. All authors have read and agreed to the submitted version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

All the ITS and EF sequences are deposited into GenBank (https://www.ncbi.nlm.nih.gov/genbank/) with accession numbers listed in Table S1. Isolates of *L. acicola* are stored in the Laboratory of Forest Pathology in the Estonian University of Life Sciences and in the Fungal Culture Collection, Estonian University of Life Sciences. All the relevant data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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2018	Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops. Workshop. University of Catania, Itaalia.

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2022-2025	PM220078MIME, Eesti Maaülikool. "Uus võimalus biomajanduse edendamisel: parimad seenetüved Põhja-Euroopale".
2021–2024	T210087MIME, Keskkonnaministeerium. "Olulist majanduslikku ja ökoloogilist kahju põhjustavate metsapatogeenide leviku analüüs ning kahjustuste modelleerimine ja kahjude prognoos".
2020-2023	F200102PKDP, SA Archimedes. "Eesti osalus Euroopa loodusteaduslike kollektsioonide võrgustikus".
2018-2021	PSG136, SA Eesti Teadusagentuur. "Metsapatogeenide mass-invasioonid Põhja-Euroopasse: uute patogeenide varane tuvastamine, nende saabumisteede ja -viiside määratlemine ning tõkestusvõimaluste otsimine".

LIST OF PUBLICATIONS

Publications indexed in the Web of Science database

1. Adamson, K., **Laas, M.,** Drenkhan, R., Hanso, M. 2018. Quarantine pathogen *Lecanosticta acicola*, observed at its jump from an exotic host to the native Scots pine in Estonia. Baltic Forestry. 24 (1), 36-41.

2. Mullett, M.S., Adamson, K., Bragança, H, Bulgakov, T.S., Georgieva, M., Henriques, J., Jürisoo, L., **Laas, M.,** Drenkhan, R. 2018. New country and regional records of the pine needle blight pathogens *Lecanosticta acicola*, *Dothistroma septosporum* and *Dothistroma pini*. Forest Pathology. 48 (5), e12440. DOI: 10.1111/efp.12440.

3. **Laas, M.,** Adamson, K., Drenkhan, R. 2019. A look into the genetic diversity of *Lecanosticta acicola* in northern Europe. Fungal Biology. 123 (10), 773−782. DOI: 10.1016/j.funbio.2019.06.012.

4. Cleary, M., **Laas, M.,** Oskay, F., Drenkhan, R. 2019. First report of *Lecanosticta acicola* on non-native *Pinus mugo* in southern Sweden. Forest Pathology. 49 (3), e12507. DOI: 10.1111/efp.12507.

5. Oskay, F., **Laas, M.,** Mullett, M., Lehtijarvi, A., Dogmus-Lehtijarvi, H. T., Woodward, S., Drenkhan, R. 2020. First report of *Lecanosticta acicola* on pine and non-pine hosts in Turkey. Forest Pathology. 50 (6), ARTN e12654. DOI: 10.1111/efp.12654.

6. Adamson, K., **Laas, M.,** Blumenstein, K., Busskamp, J., Langer, G.J., Klavina, D., Kaur, A., Maaten, T., Mullett, M.S., Müller, M.M., Ondrušková, E., Padari, A., Pilt, E., Riit, T., Solheim, H., Soonvald, L., Tedersoo, L., Terhonen, E., Drenkhan, R. 2021. Highly clonal structure and abundance of one haplotype characterise the *Diplodia sapinea* populations in Europe and Western Asia. Journal of Fungi. 7 (634). DOI: 10.3390/jof7080634.

7. **Laas, M.,** Adamson, K., Adamčíková, K., Akiba, M., Barnes, I., Beenken, L., Braganca, H., Bulgakov, T., Capretti, P., Cech, T., Cleary, M., Enderle, R., Gheraldini, L., Jankovský, L., Janoušek, J., Markovskaja, S., Matsiakh, I., Meyer, J., Mullett, M., Oskay, F., Piškur, B., Raitelaitytė, K., Sadiković, D., Drenkhan, R. 2022. Diversity, migration routes and worldwide population genetic structure of *Lecanosticta acicola*, the causal agent of Brown spot needle blight. Molecular Plant Pathology. 23 (11), 1620-1639. DOI: 10.1111/mpp.13257.

Publications in other peer-reviewed journals

1. Drenkhan, R., Adamson, K., Drenkhan, T., Agan, A., **Laas, M.** 2017. Uus probleemistik dendropatoloogias – uued ja invasiivsed patogeenid. Forestry Studies / Metsanduslikud Uurimused. 67, 50−71. DOI: 10.1515/fsmu-2017-0012.

Popular-scientific publications

1. Drenkhan, T., Padari, A., **Laas, M.,** Jürimaa, K., Drenkhan, R. 2018. Juuremädanikud levivad männikutes märkimisväärses ulatuses. Eesti Mets. 4, 28−33.

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VIIS VIIMAST KAITSMIST

INDREK KERES
COMBINED EFFECTS OF GROPPING SYSTEM AND N-FERTILIZATION ON WINTER WHEAT YIELD AND BAKING QUALITY
VILJELUSVIISI JA N-VÄETAMISE MÕJU TALINISU SAAGI JA TAINA KVALITEEDILE
Kaasprofessor **Tiina Tosens**, professor **Ülo Niinemets**, dotsent **Evelin Loit**
27.oktoober 2022

THAISA FERNANDES BERGAMO
COMBINING UNMANNED AERIAL VEHICLES AND A MESOCOSM EXPERIMENT TO UNVEIL PLANT COMMUNITIES SHIFTS UNDER GLOBAL CHANGE CONDITIONS IN COASTAL MEADOWS
KLIIMAMUUTUSE MÕJU HINDAMINE RANNANIIDU TAIMEKOOSLUSELE MESOKOSMI KATSE JA MEHITAMATA ÕHUSÕIDUKIGA KOGUTUD ANDMETE PÕHJAL
Professor **Kalev Sepp**, Dr. **Raymond D. Ward**, professor **Christopher B. Joyce** (University of Brighton)
22. november 2022

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ALTERNATIVE BIOCONTROL STRATEGIES IN THE POTATO-PHYTOPHTHORA INFESTANS PATHOSYSTEM FOR INTEGRATED MANAGEMENT OF LATE BLIGHT
ALTERNATIIVSED BIOTÕRJE STRATEEGIAD KARTULI-LEHEMÄDANIKU INTEGREERITUD TÕRJEKS
Professor **Marika Mänd**, professor Dr. ir. **Geert Haesaert** (Ghent University), professor Dr. ir. **Kris Audenaert** (Ghent University)
28. november 2022

CARMEN KIVISTIK
ECOPHYSIOLOGICAL MECHANISMS CHARACTERIZING THE FRESH- AND BRACKISH MICROBIOTA
MAGE- JA RIIMVEELIST MIKROOBSET ELUSTIKKU MÕJUTAVAD ÖKOFÜSIOLOOGILISED MEHHANISMID
Professor **Daniel Philipp Ralf Herlemann**, Dr. **Kairi Käiro**, teadur **Helen Tammert**
2. detsember 2022

MIHKEL MÄESAAR
PREVALENCE AND COUNTS OF *LISTERIA MONOCYTOGENES* AND *CAMPYLOBACTER* SPP. IN FOOD AND MOLECULAR CHARACTERISATION OF THE ISOLATES IN ESTONIA
LISTERIA MONOCYTOGENES'E JA *CAMPYLOBACTER* SPP. LEVIMUS JA ARVUKUS TOIDUS NING TÜVEDE MOLEKULAARNE ISELOOMUSTUS EESTIS
Professor **Mati Roasto**
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