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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

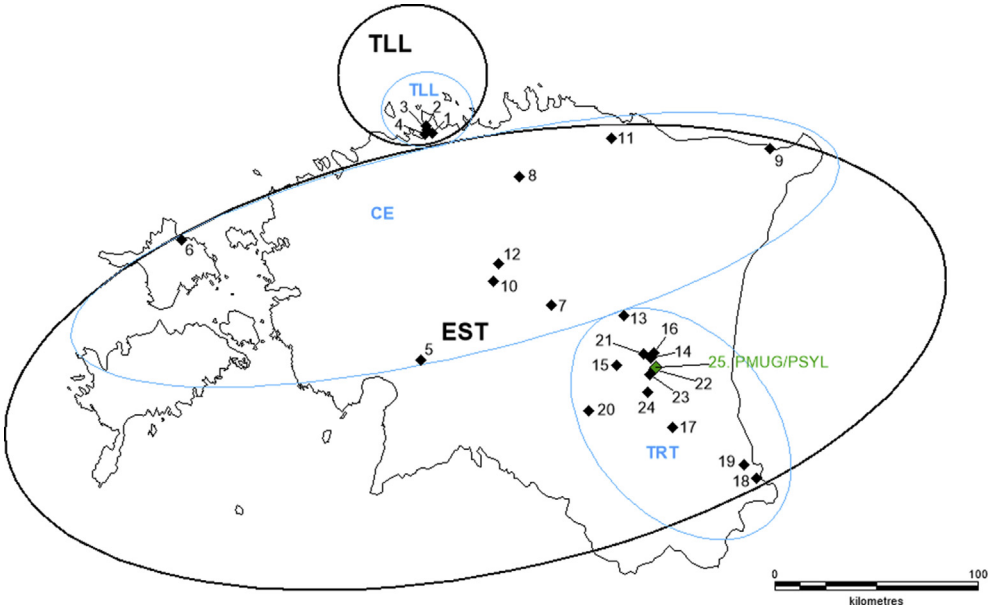

Fig. 1. Map of sampling sites (diamonds) in Estonia, where *Lecanosticta acicola* isolates were obtained and the composed populations of *L. acicola*. The blue lines indicate the geographically-separate populations, and the black lines indicate the populations that were merged according to the results 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

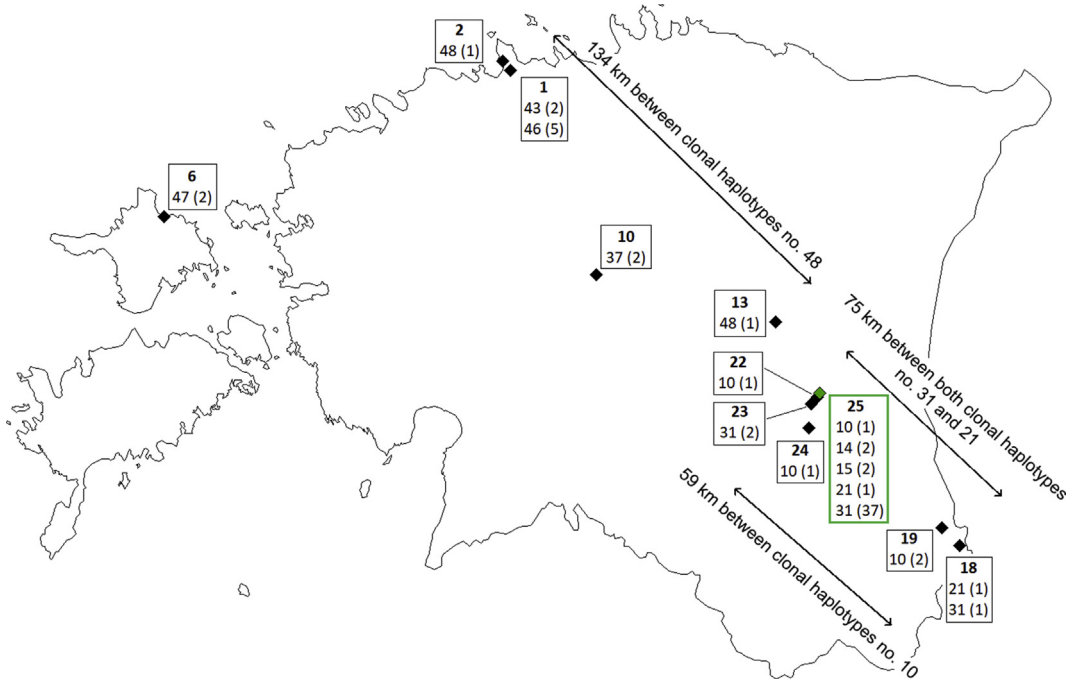

Fig. 2. The sampling sites (diamonds) of *Lecanosticta acicola* haplotypes that were identified several times and their quantity at the sites. In the boxes are the number of definite sampling site (in bold), the numbers of haplotypes and their quantity (in brackets). Numbers of the sampling sites correspond 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.

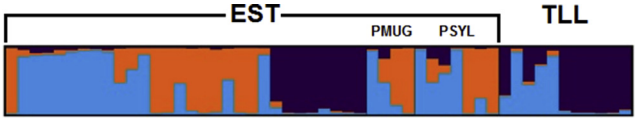

Fig. 3. Structure clustering of *Lecanosticta acicola* populations (EST and TLL) and sub-populations of the mixed stand (PMUG and PSYL), based on the clone corrected dataset. Optimal number of clusters (K = 3) by ln(Pr(X|K)). Abbreviations: EST – all of Estonia (except TLL), PMUG – *Pinus mugo* in the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

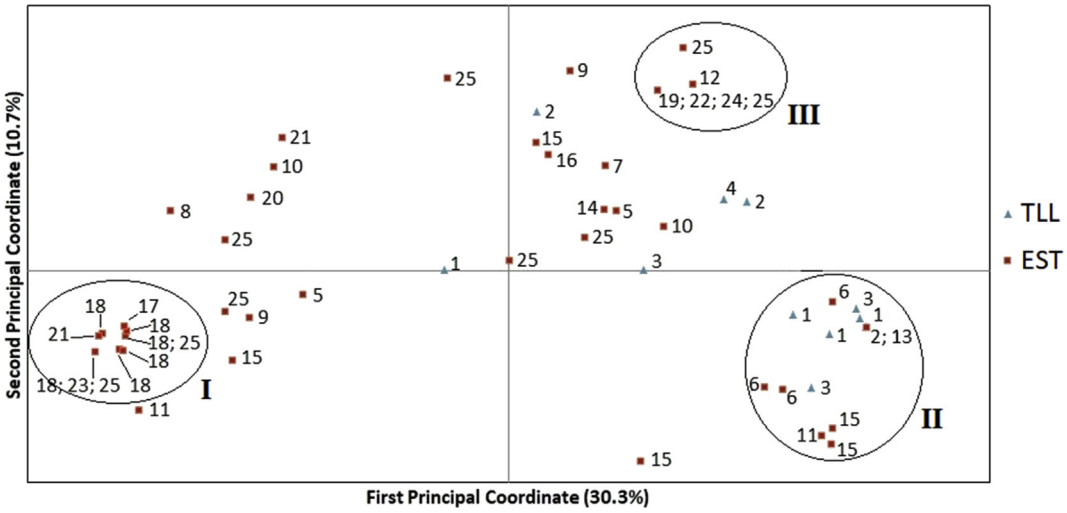

Fig. 4. Results of Principal Coordinates Analysis of the clone corrected dataset. Numbers represent the sampling sites of the isolates and correspond to Table 1. I – Group of isolates from Tartu and Värska area (central-eastern and south-eastern Estonia). II – Group of isolates from Tallinn and Kärdla (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).

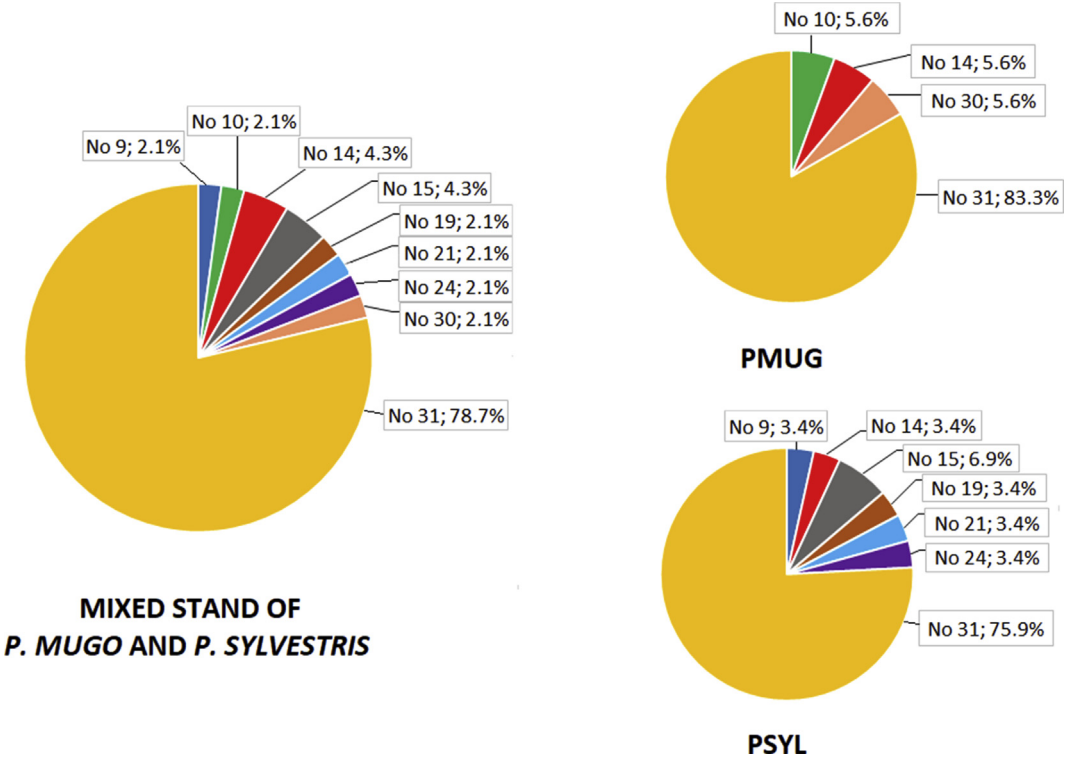

Fig. 5. Distribution of *Lecanosticta acicola* haplotypes in the mixed stand of *Pinus mugo* and *P. sylvestris* and in the sub-populations PMUG and PSYL. The haplotype numbers and their percentages are shown in the boxes.

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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