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

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Adamson, K., Laas, M., Drenkhan, R. and 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.

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.

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,

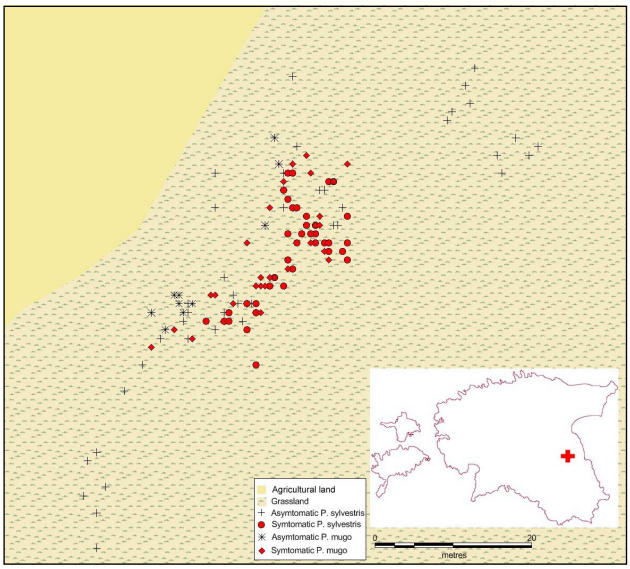

Figure 1. Map of the infected by *L. aciola* young mixed *P. mugo*/*P. sylvestris* stand in Estonia

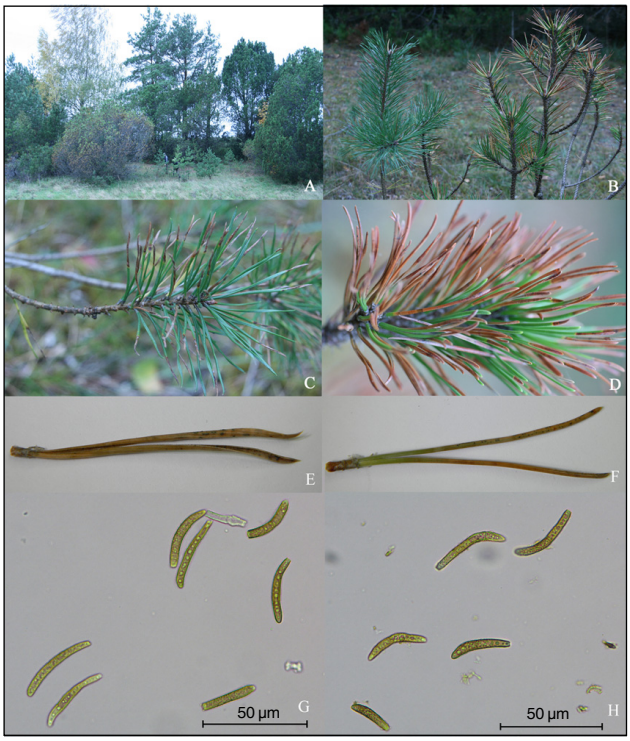

Figure 2. The investigated mixed stand of *P. mugo*/*P. sylvestris,* infected by *L. acicola.* A) the site; B) *P. sylvestris* (left) next to the heavily infected *P. mugo* tree (right); C) *P. sylvestris* shoot with the infected by *L. acicola* needles; D) *P. mugo* shoot with the heavily infected by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

Acknowledgements

This study was supported by the Estonian Science Foundation grants PSG136 and IUT21-04, EMU Project P170053MIMK, Euphresco project BROWNSPOTRISK and the Ministry of Rural Affairs of Estonia.We would like to thank Mr Terry Bush (Madison, Wisconsin, USA) for the English revision of the manuscript.

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