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RESEARCH ARTICLE

Invasive brown spot needle blight caused by *Lecanosticta acicola* in Estonia

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Lecanosticta acicola (Thüm.) Syd., a serious foliage pathogen of pines in many regions of the world, is an emerging invasive species in northern Europe. After the first record of *L. acicola* on *Pinus ponderosa* Dougl. ex Laws. in northern Estonia in 2008, monitoring was started to investigate the spread and host range of the fungus in the country. By the beginning of 2015, *L. acicola* was also recorded on *P. uncinata* Mill. ex Mirb., *P. mugo* Turra and on *P. mugo* var. *pumilio* (Haenke) Zenari, being the northernmost records of the fungus in Europe. So far, the single native pine species *Pinus sylvestris* L. has not been found to be infected. Molecular analysis proved infection of *L. acicola* on pines in five different localities of Estonia: in Tallinn (mainly in the Botanic Garden, northern Estonia), in Tori and Kärdla (western Estonia), in Vasula and Kärevere (east-central Estonia). The sexual state (teleomorph) of the fungus was not found, but the existence of the both mating types, MAT1-1 and MAT1-2, was confirmed.

Keywords: *Mycosphaerella dearnessii*; exotic pine species; invasive pathogen; mating type

Introduction

During the second half of the last century, numerous invasive fungal pathogens, such as *Dothistroma septosporum* (Dorog.) Moreletand, *Diplodia sapinea* (Fr.) Fuckel on pines, and *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya on ash trees, have reached northern Europe (Drenkhan & Hanso 2009; Hanso & Drenkhan 2009; Rytkönen et al. 2011; Oliva et al. 2013). Invasions can be attributed not only to climate change, but also to human activities, e.g. global trade and introduction of new species (Woods et al. 2005; Watt et al. 2009; Stenlid et al. 2011; Hanso & Drenkhan 2013; Santini et al. 2013; Drenkhan et al. 2014b). Recently, a comprehensive analysis of databases of invasive forest pathogens by Santini et al. (2013) demonstrated that 22% of the alien pathogens in Europe, including the causal agent of the Brown Spot Needle Blight (BSNB), *Lecanosticta acicola* (Thüm.) Syd., originate from North America. The first report of *L. acicola* is from South Carolina, USA, in 1876 (Tainter & Baker 1996). Today, the fungus is known to be distributed on several continents: North America (Luttrell 1949), Asia (Suto & Ougi 1998), Africa (Patton 1997), and Europe (Lévy & Lafaurie 1994; Holdenrieder & Sieber 1995; Pehl 1995; Jankovský et al. 2009; Hintsteiner et al. 2012), colonizing several *Pinus* spp.: *P. mugo* Turra, *P. nigra* Arnold, *P. ponderosa* Dougl. ex Laws., *P. rotundata* Link, *P. sylvestris* L., *P. uncinata* Mill. ex Mirb, etc. (Luttrell 1949; Holdenrieder & Sieber 1995; La Porta & Capretti 2000; Diekmann et al. 2002; Jankovský et al. 2009).

Lecanosticta acicola has demonstrated its aggressiveness in North and Central America, in the central EPPO region (as an A2 quarantine disease, meaning that planting material of *Pinus* should come from an area free from *L. acicola*, i.e. that the region of production of that material should be free of the disease) and in Eastern Asia (Quaedvlieg et al. 2012). The first symptoms of the disease are expressed as resin-soaked, yellowish lesions, with a clear orange border on infected needles. Then, black stromata, visible as round black spots, develop under the epidermis in the dead parts of needles. Later on, protrusive fruit bodies (conidiomata) develop on these spots. As time progresses, the dead lesions coalesce, the needles die and drop down. A severe defoliation may lead to death of branches and whole trees (Anonymous 2005, 2008). Mature conidiomata produce mucilaginous spore masses, and the spreading of the conidia results in new infections. Rapid dissemination of the pathogen into new areas may cause major damages in tree populations (e.g. as it happened on *P. palustris* in the south-eastern USA, see Sinclair & Lyon 2005).

The pathogen *L. acicola* is a heterothallic species (Janoušek et al. 2014). The sexual state of *L. acicola*, *Mycosphaerella dearnessii*, is rarely found (Anonymous 2008). *M. dearnessii* (and *L. acicola*) is thought to be native in the mountain areas of Honduras and Guatemala (Bednářová et al. 2013). According to Quaedvlieg et al. (2012), the name *M. dearnessii* should no longer be applied; the correct name of this species should be *Lecanosticta acicola*.

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The first recorded occurrence of *L. acicola* in Estonia, and apparently also in the whole Baltic and Nordic region, was dated to the first half of 2008, when the fungus was recorded on *P. ponderosa* in Tallinn (Drenkhan & Hanso 2009; Drenkhan 2011). A year later, in 2009, the fungus was documented also in Lithuania (Markovskaja et al. 2011). To our knowledge, *L. acicola* has not yet been reported in Scandinavia, Finland, and/or north-western Russia.

The aim of this study was to monitor the distribution of *L. acicola* in Estonia during what was apparently the beginning phase of the colonization of this country by this fungus.

Materials and methods

Survey design

In order to specify the occurrence and host range of *L. acicola* in Estonia, needle samples were collected from different conifers: from 13 species of *Pinus* L., one from *Picea* A. Dietr. and one from *Abies* Mill. (see Table 1). From May 2010 to January 2015, samples of conifer needles were collected:

(1) At least six times per year from three definite locations: (1) Tallinn (Tallinn Botanic Garden, referred to here as TBG, and Pirita, both in northern Estonia) from 15 trees, (2) Vasula (Tartu County, east-central Estonia), from two trees, and (3) from three trees in Tori (Pärnu County, western Estonia), all from definite sample trees;
(2) Once from a total of 204 trees on 68 plots (Figure 1), distributed across Estonia and established for the monitoring of the Dothistroma Needle Blight (DNB, caused by *Dothistroma septosporum* (Dorog.) Morelet, see Drenkhan & Hanso 2009);
(3) Randomly in several parks and forest stands across Estonia.

Approximately 880 needle samples were surveyed. Each needle sample contained needles from one tree, except the samples which were collected during the monitoring of the DNB, where one sample contained needles from several (at least from three) trees. Needle samples (both symptomatic and asymptomatic) were collected from the lower (up to 1.5 meters height) parts of tree canopies. At sampling, needles were classified symptomatic if they had visible symptoms and conidia matching with the descriptions of EPPO (Anonymous 2005, 2008), were observed in microscopic studies (Nikon Eclipse 50i, Japan). Samples of symptomless needles were randomly collected for the molecular analyses of the possible early infections.

Morphological measurements, length and width, of 30 conidia were carried out from the needles of *P. mugo* growing in Tori (Table 1) according to the program NIS-Elements 4.12.01 (Nikon, Japan).

To verify visual and morphological identification of *L. acicola* on symptomatic needles, and to document the presence or absence of the pathogen in asymptomatic needles, molecular analyses of 72 needle samples were carried out. Species-specific PCR primers (SSPPs) were used to document the host range of *L. acicola* in Estonia, especially in TBG, where BSNB was first found and where *L. acicola* was documented on several exotic pine species.

Isolation of L. acicola

Pure cultures were isolated from the infected needles. After rinsing of needles in 96% ethanol, the well-developed conidiomata were placed on pine needle agar medium (PNA), rolled along its surface for separation of conidia onto the medium (Mullet & Barnes 2012) and incubated for 7–14 days at room temperature. Germinated conidia with some mycelia were transferred to fresh PNA plates. After incubation of 14–28 days, small amounts of hyphal mass from culture edges were transferred into 1.5-ml sterile micro-centrifuge tubes (MCT) for DNA extraction.

DNA extraction, PCR, and sequencing

Cell disruption of the samples, DNA extraction from mycelia, and PCR reactions were carried out as described by Drenkhan et al. (2013) and Drenkhan et al. (2014a). The identity of *L. acicola* 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 & Bruns 1993) and ITS4 (5′-TCCTCCGCTTATTG ATATGC-3′; White et al. 1990). PCR products were sent for sequencing to the Estonian Biocentre in Tartu.

The ITS region of samples was sequenced using the primer ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′; White et al. 1990). The sequences were edited using the BioEdit version 7.2.5 (Hall 1999). BLAST searches for the fungal taxa confirmation were performed at GenBank (NCBI). Altogether nine ITS sequences of *L. acicola* from Estonia were deposited in GenBank (see Table 1).

Species-specific priming PCR

SSPPs were used for the searching of *L. acicola* in symptomatic and asymptomatic needle samples. The sampled needles were washed using 96% ethanol for 1 min, cut into 2–3 mm pieces and transferred into 2 ml sterile MCT for DNA extraction. Samples were stored at –20°C.

The *L. acicola* species-specific primers LAtef-F (5′-GCAAATTTTCGCCGTTTATC-3′) and LAtef-R (5′-TGTGTTCCAAGAGTGCTTGC-3′); (Ioos et al. 2010) were used for the detection of the pathogen. The conventional PCR cycling conditions and reaction mixture were performed in 20 µl reaction volumes according to

Table 1. Records of *Lecanosticta acicola* in Estonia.

Samples		Results					
Host	Sampling date	Symptoms	Conidia	Isolate ID[a]	GENBANK accession no.	Mating type[b]	SSPP[c] of *L. acicola*
Samples from Tallinn Botanic Garden							
Pinus ponderosa	13.05.2010	+	+	139410	KF998580	MAT1-1	Positive
	17.11.2011	+	+	144742	KJ004505	MAT1-1	Positive
	19.08.2013	+	+	149460		MAT1-1	Positive
	20.09.2013	+	+	149461		MAT1-1	Positive
	17.01.2011; 29.04.2011; 07.06.2011	+	+				Positive
	15.09.2011; 18.06.2013	+	–				Negative
Pinus banksiana	28.03.2013	–	–				Negative
Pinus contorta	12.09.2012; 31.01.2013	–	–				Negative
Pinus leucodermis	25.05.2012; 10.04.2013	+	–				Negative
Pinus mugo	15.08.2011	+	–				Negative
	17.11.2011; 23.12.2011	+	+				Positive
	31.07.2012; 20.12.2012	+	–				Negative
Pinus mugo	15.08.2011	+	+	144738	KJ004506	MAT1-1	Positive
var. pumilio	15.09.2011	+	+	141009		MAT1-1	Positive
	29.04.2011; 24.10.2011;18.02.2012;	+	+				Positive
	10.04.2013	+	–				Positive
	18.04.2012; 25.09.2012	+	–				Negative
Pinus nigra	23.12.2011; 10.04.2013	–	–				Negative
	21.08.2012; 25.09.2012	+	–				Negative
Pinus rigida	15.08.2011	+	–				Negative
	31.07.2012; 28.02.2013	–	–				Negative
Pinus sibirica	25.05.2012	–	–				Negative
Pinus sylvestris	17.11.2011; 12.09.2012;31.01.2013	–	–				Negative
Pinus uncinata	15.08.2011	+	+	144741	KJ004507	MAT1-1	Positive
	7.06.2011;15.09.2011;17.11.2011;31.01.2013	+	–				Negative
	21.08.2012	+	–				Positive
Pinus x rhaetica	15.08.2012; 13.06.2013	–	–				Negative
Pinus x rotundata	7.06.2011; 15.08.2011;23.12.2011; 25.09.2012	+	–				Negative
Picea koyamae	17.11.2011	–	–				Negative
Samples from other locations of Estonia							
Pinus mugo[d]	17.11.2011	+	–				Negative
	11.12.2013	–	–				Negative
Pinus mugo[e]	20.06.2012	+	+	144716	KJ004504	MAT1-1	Positive
Pinus mugo[f]	15.10.2014	+	+	150042		MAT1-2	Positive
	20.01.2015	+	+	150940		MAT1-1	Positive
	20.01.2015	+	+	150943	KP872828	MAT1-2	Positive

Table 1. (Continued)

Samples		Results				
Host	Sampling date	Symptoms	Conidia	Isolate ID[a]	GENBANK accession no.	Mating type[b]	SSPP[c] of *L. acicola*
Pinus mugo[g]	4.07.2013	+	+	145069	KJ004508	MAT1-1	Positive
Pinus mugo[h]	4.08.2014	+	+	149458	KP872826	MAT1-1	Positive
	4.08.2014	+	+	149456	KP872827	MAT1-2	Positive
	4.08.2014	+	+	149454		MAT1-1	Positive
	4.08.2014	+	+	149452		MAT1-1	Positive
Abies concolor[i]	4.03.2012	+	–				Negative

[a]Local register numbers in the laboratory of forest pathology of the Estonian University of Life Sciences.
[b]Mating type priming PCR (Janoušek et al. 2014).
[c]Species-specific priming PCR (Ioos et al. 2010).
[d]North Estonia, Pirita.
[e]East-central Estonia, Vasula.
[f]East-central Estonia, Kärevere.
[g]Western Estonia, Tori.
[h]Western Estonia, Hiiumaa Island.
[i]South Estonia, Haabsaare.

Ioos et al. (2010), with some modifications (see Drenkhan et al. 2014a). The PCR products were visualized on 1% agarose gel (SeaKem® LE Agarose, Lonza) under UV light using the Quantum ST4-system (VilberLourmat SAS, Marne-la-Vallée, France). All amplifications were made at least twice to ensure consistent banding patterns in gel. A strain of *L. acicola* (accession no. KJ004507) was isolated from the needles of *Pinus uncinata* and used as the reference culture for SSPP.

Mating types of L. acicola

Pure cultures were used to determine the distribution of mating types of *L. acicola* in the samples from Estonia. The PCR was performed using the PCR mix 5× HOT FIREPol Blend Master Mix with BSA and 7.5 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), and two pairs of species-specific mating type primers: Md MAT1-1F (5′-CGCATTCGCACATCCCTTTGT-3′), Md MAT1-1R (5′-ATGACGCCGATGAGTGGTGCG-3′) and Md MAT1-2F (5′-GCATTCCTGATCTACCGTCT-3′), MdMAT1-2R (5′-TTCTTCTCGGATGGCTTGCG-3′; Janoušek et al. 2014). PCR cycling conditions were carried out similar to those described in Janoušek et al. (2014), but with the initial denaturation step at 95°C increased to 12 min. Two strains of *L. acicola* DNA from USA and Canada were used as the references in the mating type analyses.

Results

The identity and presence of *L. acicola* were confirmed by sequencing of nine pure cultures, sampled from four different pine species: *P. ponderosa*, *P. uncinata*, *P. mugo,* and on *P. mugo* var. *pumilio* (Table 1). Species-specific priming PCR verified earlier visual diagnoses of *L. acicola* in November 2011 on *P. mugo* in Pirita (eastern Tallinn) and on other pine species in TBG since January 2011 (Table 1).

Molecular analyses of 72 specimens, sampled from April to December, 2011, revealed that *L. acicola* was not widely distributed in Estonia. Up to November 2012, this fungus was found only in Tallinn (Table 1). A month later, the first occurrence of *L. acicola* outside Tallinn was documented in east-central Estonia, and, by July 2013, in western Estonia (see Figure 1). In the new locations only a single exotic pine species was found to be infected. Until the beginning of 2015, the native pine species *P. sylvestris*, as well as several exotic conifer species *P. x rotundata*, *P. rigida* P. Mill, *P. nigra*, *P. sibirica* Tu Tour, *P. leucodermis* Ant., *P. contorta* Dougl. ex Loud, *P. banksiana* Lamb., *P. x rhaetica* Brügger, *Picea koyamae* Shiras., and *Abies concolor* (Gord. & Glend.) Hildebr.) were found uninfected (Table 1).

In Tori (western Estonia), three *P. mugo* trees at the age of about 25 years were found infected. Trees were growing under the shelter of mature broadleaved trees

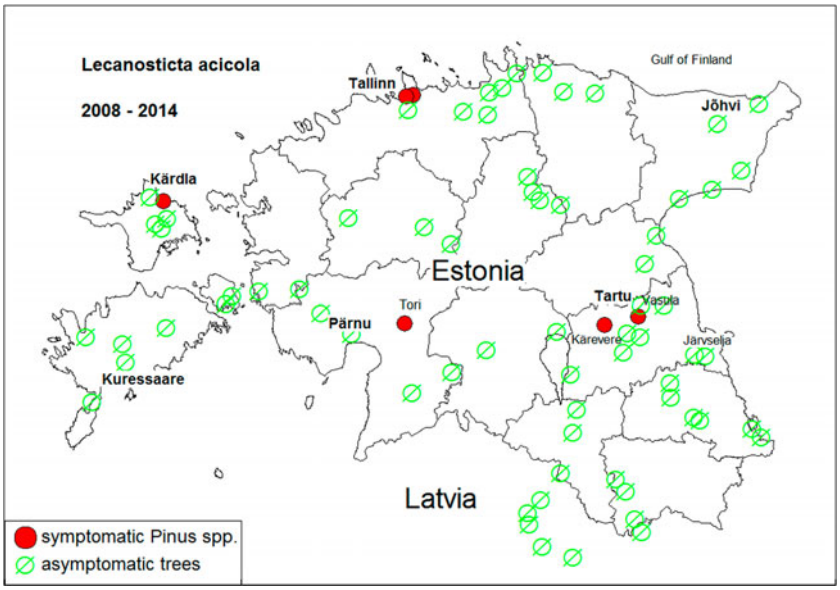

Figure 1. Location of the monitoring plots investigated on the occurrence of *Lecanosticta acicola* in Estonia and northern Latvia.

and were standing with their canopies closely connected. In an urban green area of Kärdla (western Estonia, Hiiumaa Island), a group of *P. mugo* trees was found infected by *L. acicola*. Also in Vasula (east-central Estonia) two *P. mugo* trees were found infected. The distance between these trees in Vasula was approximately 6–8 meters. The elder tree was about 35 and younger was about 10 years old. In Kärevere (east-central Estonia) about 20 *P. mugo* trees of ca 30 years old, growing in a hedge side by side were found to be infected. In the new locations (Tori, Kärdla, Vasula, and Kärevere) the infection severity was high; only the current year needles were found to be still attached to branches but already infected. In Pirita (northern Estonia, close to Tallinn) about 25-year-old *P. mugo* trees, growing as a dense stand, were found to be infected. In the TBG, infections were detected in *P. ponderosa*, *P. uncinata*, *P. mugo,* and *P. mugo* var. *pumilio.* The observation of *L. acicola* on *Pinus mugo* var. *pumilio* in 2011 in TBG is the first record of BSNB on this host variety.

Conidial dimensions of *L. acicola* in Estonia (on *P. mugo* in Tori, July 2013, $n = 30$) were 19.1–41.4 × 3.4–4.8 µm, with the average being 30.4 × 4.0 µm. Fruit bodies of *L. acicola* were found to contain conidia all year (Table 1, cf. conidia vs. sampling dates).

Until 2014, the mating type analyses of the first six Estonian isolates of *L. acicola* revealed only the presence of one mating type, MAT1-1. In July 2014, the second mating type (MAT1-2) was also found for the first time in Estonia, on the Island Hiiumaa (western Estonia) and later in October of 2014 in Kärevere (east-central Estonia).

No sexual stage of *L. acicola* was observed during the monitoring years.

Discussion

Until 2008, the closest point to Estonia where *L. acicola* was documented was in Germany, about 1000 km southwest from Estonia (Drenkhan & Hanso 2009). A year later, the pathogen was found in the Curonian Spit of Lithuania (Markovskaja et al. 2011), over 500 km south from Tallinn. Until today, there were no known records of BSNB in Latvia nor in southern Estonia (Figure 1). Thus, a steady gradual dispersal of the fungus from south to northern Estonia seems to be doubtful. An unintentional introduction through the botanical garden seems more likely. The northward spreading of *L. acicola* may have been supported by the climatic conditions (especially warmer winters; see Hanso & Drenkhan 2013), and by the introduction of alien conifer species into the green areas, botanical gardens, or arboretums. Transport of living plants and soil has increased worldwide, and invasive pathogens have been introduced mainly through living plants and wood (Santini et al. 2013). For instance, in China *L. acicola* is thought to have become common after the large-scale import of slash pine seeds from USA in the 1970s (Harrington & Wingfield 1998).

The two diseases, DNB (Hanso & Drenkhan 2008) and BSNB (Drenkhan & Hanso 2009), were observed for the first time in Estonia with only two years between the observations. DNB and BSNB are such similar forest diseases that they are, as a pair, even discussed in the same EPPO document (Anonymous 2008). These two diseases are highly similar in their etiology, but our results indicate some major differences between them; we found that while DNB has rapidly colonized all of Estonia (Drenkhan & Hanso 2009), the spread of BSNB has been much slower. Moreover, while DNB was found

colonizing the native Scots pine just a year after its first observation on an exotic species, six years has passed from the first observation of BSNB, but without appearing on Scots pine. Also, this pathogen was not found on some other exotic conifers investigated (*Picea* and *Abies*, Table 1).

Because *L. acicola* is considered to be a new colonizer in our environment, the mating types of the fungus present here are also important to consider. Mating types may indicate the occurrence or absence of the fungal sexual reproduction, as the presence of only one mating type gene explains the absence or rarity of the sexual stage (Groenewald et al. 2007). Recently, the existence of only one mating type of *D. septosporum* was confirmed in South America and Australasia (Barnes et al. 2014), regardless of the considerable efforts in research of this needle pathogen. Existence in a population of only one mating type has an important practical relevance; an occasional introduction of the second type of the fungus will strongly increase the genetic diversity and therefore often also the virulence of the fungus (Smart & Fry 2001; Paoletti et al. 2006). Existing of both mating types may indicate somewhat longer existence of the fungus in Estonia. However, the discovery of a second mating type (MAT1-2) later may also indicate recent arrivals of new genets of the fungus in Estonia.

Future investigations of *L. acicola* are needed for a better understanding of the population structure of the pathogen, the pathways of its arrival and dissemination inside Estonia and, possibly, into and inside other Baltic and Nordic countries. An intriguing question is also whether BSNB will colonize or not Scots pine so far in the north.

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Disclosure statement

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