BOTANICA LITHUANICA 2011, 17(1): 29–37

OCCURRENCE OF NEW ALIEN PATHOGENIC FUNGUS *MYCOSPHAERELLA DEARNESSII* IN LITHUANIA

Svetlana Markovskaja, Audrius Kačergius, Aušra Treigienė

Institute of Botany of Nature Research Centre, Laboratory of Mycology, Žaliųjų Ežerų Str. 49, LT-08406 Vilnius, Lithuania, e-mail: svetlana.markovskaja@botanika.lt; audrius.kacergius@botanika.lt; ausra.treigiene@botanika.lt

Abstract

Markovskaja S., Kačergius A., Treigienė A., 2011: Occurrence of new alien pathogenic fungus *Mycosphaerella dearnessii* in Lithuania [Naujas svetimžemis patogeninis grybas *Mycosphaerella dearnessii* Lietuvoje]. – Bot. Lith., 17(1): 29–37.

In 2009, *Lecanosticta acicola*, an anamorphic stage of pathogenic ascomycetous fungus *Mycosphaerella dearnessii*, causing foliage disease of pines known as brown spot needle blight has been for the first time reported in Lithuania. This alien fungus was found in *Pinus mugo* plantations in the Curonian Spit (western Lithuania). During detailed investigations in 2009 and 2010, only anamorphic stage was found on heavily damaged needles; teleomorph was not observed. The fungus was identified by both morphological characters and molecular PCR-based methods. Information on the distribution, taxonomy, morphology and ecology of this needle pathogen is provided.

Keywords: brown spot needle blight, distribution, invasive fungal pathogens, needle diseases, *Pinus*.

INTRODUCTION

The ascomycetous fungus *Mycosphaerella dearnessii* M. E. Barr (*Ascomycota, Dothideomycetes, Capnodiales, Mycosphaerellaceae*) is a dangerous pine pathogen, causal agent of so-called brown spot needle blight (EPPO/CABI, 1997), included into the A2 list of Quarantine Pests of European and Mediterranean Plant Protection Organization (EPPO, 2009) and into the list of Quarantine Pests of Lithuania (Lietuvos Respublikos žemės ūkio ministro įsakymas Nr. 3D-264, ... 2010).

This fungus has two development stages (morphs) ascribed to two genera: ascomycetous genus *Mycosphaerella* Johanson and anamorphic genus *Lecanosticta* Syd. (*Coelomycetes*). The fact that they undergone several taxonomic and nomenclatural changes adds to complications in common understanding of the species. First the anamorphic stage was found and originally described as *Cryptosporium acicola* Thüm. in 1878, later, in 1926, the teleomorphic stage was found and described as *Oligostoma acicola* Dearn. After that taxonomic position of both morphs has been changed on several occasions and currently the fungus is recognized as *Mycosphaerella dearnessii* M. E. Barr with anamorph *Lecanosticta acicola* (Thüm.) Syd. (Barr, 1972). Among its numerous synonyms (Index Fungorum, 2011), *Scirrhia acicola* (Dearn.) Sigg. is one of the most commonly used, but it is not correct as the species does not belong to the genus *Scirrhia* (Barr, 1972). As a rule, *Mycosphaerella dearnessii* is found in its anamorphic (conidial) stage and sometimes according to its anamorph name the disease is also referred to as Lecanosticta needle blight (Pehl, 1995; Brown Spot Needle Blight of Pines..., 2010).

All fungi belonging to the anamorphic genus *Lecanosticta* are known as specific pine pathogens

infecting needles (Evans, 1984; Marmolejo, 2000), meantime the ascomycetous genus *Mycosphaerella* is characterized by wide ecological plasticity and only *M. dearnessii* (anam. *L. acicola*), *M. pini* E. Rostr. ex Munk (anam. *Dothistroma septosporum* (Dorog.) M. Morelet) and *M. gibsonii* H. C. Evans (anam. *Pseudocercospora pini-densiflorae* (Hori et Nambu) Deighton) are specific for pines; other species of this genus occur on various herbal and woody plants (Crous, 2009).

Most likely *M. dearnessii* is of American origin, mainly because this fungus for the first time was found in North America more than 130 years ago (USA, South Carolina) and now is widely spread throughout South and North Americas in native pine forests where it causes serious problems (Gibson, 1979; Evans, 1984). In Europe and other regions (Africa, Asia, Australia, New Zealand) the disease appeared later; it usually occurs on exotic pines grown in parks and plantations (EPPO, 2005; 2009). According to the EPPO data (*Mycosphaerella dearnessii* and *Mycosphaerella pini*, ...2010) the first report of brown spot needle blight in the EPPO region was in 1978, although some authors have claimed that in Europe the disease appeared earlier, already in about 1940-ies: in Bulgaria (the first known record in Europe) and Austria on *Pinus nigra* Arn. (Petrak, 1961), and in Spain on *P. radiata* D. Don. (Martinez, 1942). During the last decades *Mycosphaerella dearnessii* has more widely spread in Austria (Brandstetter & Cech, 1999, 2003; Kirisits & Cech, 2006) and has been reported from many other European countries: Germany (Karadzic, 1989; Peil, 1995), Serbia and Croatia (Karadzic, 1989; Novak-Agbaba & Halambek, 1997), France (Chandalier et al., 1994), Switzerland (Holdenrieder & Sieber, 1995), Austria, Italy (La Porta & Capretti, 2000), Estonia (Hanso & Drenkhan, 2008, 2009), Sweden (Barklund, 2008), Czech Republic (Jankovský et al., 2009) commonly occurring on *Pinus mugo* Turra, *P. nigra*, *P. uncinata* Mill. ex Mirb., *P. halepensis* Mill., *P. radiata* and on some other pine species. Recently, the fungus has been recorded also on *P. sylvestris* L. in Slovenia (Jurc & Jurc, 2009). Occurrence of *Mycosphaerella dearnessii* on a wide spectrum of introduced and native pines in Europe suggests that many species within the genus *Pinus* L. are potential hosts for this pathogen.

The main aim of this study was to document the occurrence and expansion of *Mycosphaerella dearnessii* in Lithuania, to discuss its distribution, morphological and ecological peculiarities, and to evaluate invasiveness of this pathogen.

MATERIAL AND METHODS

The specimens of infected needles of 20–60-year-old *Pinus mugo* Turra trees were collected in Lithuanian coastal zone (Curonian Spit, Smiltynė Forest District, N 55°38′ E 21°06′ and Juodkrantė Forest District, N 55°31′ E 21°06′). The collected specimens bearing typical symptoms – brown spots (Sinclair et al., 1987) were as soon as possible transported to the laboratory and examined using a stereomicroscope Nikon SMZ 745T at the magnifications up to x40 and a light microscope Olympus CX 41 at x400 and x1000 magnification. Descriptions and photomicrographs were made from fresh preparations in distilled water and in lactic acid. The disease-causing fungus was identified using morphological characters and conidiogenesis of its anamorphic stage by direct examination of infected needles and pure cultures isolated on malt extract agar (MEA) and on carrot-glucose agar (CGA) (Sutton, 1980; OEPP/EPPO diagnostic protocols, 2005, 2008). The pure cultures were examined after 2–4 weeks of cultivation at 25 °C, under photoperiod 12/12 h (daylight/dark). Specimens of dried needles and culture isolates LA733A and LA733B are deposited in Herbarium of the Institute of Botany of the Nature Research Centre (BILAS).

Additionally, the identity of the isolates was confirmed by PCR-based methods: restriction fragment analysis (ITS-RFLP) and sequencing of rDNA ITS (internal transcribed spacer) region. Genomic DNA was extracted from two pure culture isolates of *Mycosphaerella dearnessii*: LA733A and LA733B with NucleoSpin® Plant II Kit (Macherey-Nagel GmbH & Co. KG, Germany) following manufacturer's instructions and using approximately 100 mg of mycelium (wet weight). The ITS region was amplified in 25 µl reactions on TProfessional 96 Gradient Thermocycler (Biometra GmbH, Germany) in the following mixture: ~ 25 ng of template, 0.25 units of *AmpliTaq* Gold 360 Polymerase (Applied Biosystems, USA), 2.5 µl 10× *AmpliTaq* Gold 360 buffer, 2 µl $MgCl_2$, 0.2 mM of each dNTP, 10 µM of primers ITS5 and ITS4 (White et al., 1990). PCR conditions were the following: initial denaturation for 10 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, with final extension for 10 min at 72 °C.

An ITS-RFLP technique was applied for preliminary identification of the fungus and differentiation of *M. dearnessii* from its closest relative – *Mycosphaerella pini* Rostr. ex Munk according to Peil et al. (2004) and OEPP/EPPO protocol (2008). For the digestion,

restriction endonucleases *Hinf I*, *Hae III*, *Hpa I* and *Hpa II* were used.

For sequencing, the PCR products were purified according to the Protocol for PCR Product Clean-up with Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas UAB, Lithuania). Purified PCR products were sequenced by Macrogen company (Macrogen Inc., Seoul, Korea) using ABI 3730XL DNA sequencer. The rDNA homology searches were performed through the internet using the BLAST (Basic Local Alignment Search Tool) database from the National Center for Biotechnology Information (NCBI), Bethesda, USA (Altschul et al., 1997). Neighbour-joining analysis and sequence pair distances among different fungal isolates were performed using Clustal W program under DNASTAR Lasergene 8.1.4 software package (DNASTAR, Inc., Madison, USA). Stability of clades was tested using a total of 1000 neighbor-joining bootstrap replications.

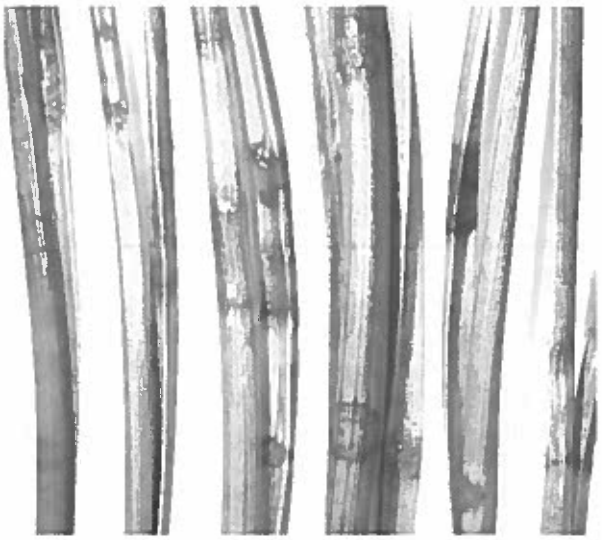

Fig. 1. Symptoms of brown spot needle blight on needles of *Pinus mugo*: typical brown spots with the yellowish border

RESULTS AND DISCUSSION

Results of our study showed that needles of mountain pine in the Curonian Spit are infected by an alien pathogenic fungus *Mycosphaerella dearnessii*.

The symptoms typical of brown spot needle blight for the first time were observed in 2009 in Smiltynė Forest District, forest compartment No 15 on three heavily diseased 30–40-year-old *Pinus mugo* trees. In 2010, more diseased trees (20–60-year-old) were found in some new localities in Smiltynė and also in Juodkrantė Forest Districts (approx. within the area of 20 km^2).

Symptoms

The first disease symptoms appeared on the 2-year-old or on the current-season needles in July as yellow spots, later becoming brown with the yellowish border and usually enlarging to bands and causing premature needle death (Fig. 1). Typically, the infected needles start to die from the top; spots and bands mainly occur in the central zone, and living (green) tissues sometimes remain at the base (Sinclar et al., 1987). During our observation in September–October 2009 we noted a rather severe infection of *Pinus mugo* needles by *Mycosphaerella dearnesii*. Virtually all heavily infected 2- and 3-year-old needles were brown with visible parallel grayish-green elongate fruiting bodies of the pathogen. According to our observations, the fruiting bodies (conidiomata, Fig. 2) of *Lecanosticta acicola* (anamorphic stage of *M. dearnessii*) developed

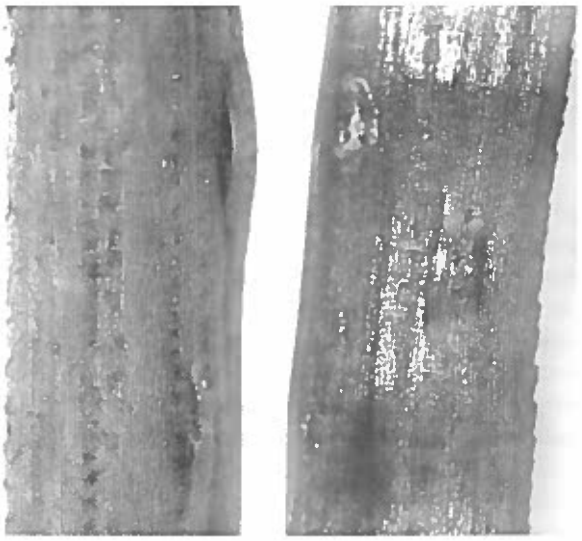

Fig. 2. Acervular conidiomata of *Lecanosticta acicola*, an anamorphic stage of *Mycosphaerella dearnessii* on infected needles of *Pinus mugo*: acervuli developed under the epidermis along dead (brown) parts of the needles

under the epidermis along the dead brown parts of the needles in September–October. As a rule, needle shedding started from the lower branches. The fungus overwinters as conidiomata or as vegetative mycelium in infected needles; conidia are discharged during wet weather in spring and spread by splashing rain, wind, animals or man and infect new needles until late summer (OEPP/EPPO, 2005). Overall, ascomata of the

teleomorph stage of the pathogen are rarely found and difficult to identify (Peihl, 1995; Brandstetter & Cech, 2003); the ascomata of *M. dearnessii* have not been found in Lithuania either.

The macroscopic symptoms of the infected pine needles were very similar to those caused by some other needle pathogens such as *Mycosphaerella pini, M. gibsonii* H.C. Evans, *Sphaerospsis sapinea* (Fr.) Dyko et Sutton and *Lophodermium* spp. Some of them, especially *Lophodermium seditiosum* Minter, Staley et Millar, *L. pinastri* (Schard.) Chevall. and *Sphaeropsis sapinea* were frequently found on *Pinus mugo* and *P. sylvestris* in the Curonian Spit (our personal unpublished data). In some occasions the fruit bodies of those fungi occurred on the same needles as *Mycosphaerella dearnesii*.

M. dearnessii can credibly be identified by detailed microscopic examination of its anamorphic stage – mature conidiomata (acervuli) bearing conidia, which clearly distinguish this pathogen from the above mentioned fungi by color, shape, size of its conidia and conidial wall ornamentation, or applying molecular techniques such as RFLP-ITS or DNA sequencing.

Morphological description of the examined specimens

Conidiomata acervular. Acervuli olive-brown or dark green, subepidermal, elongate, arranged parallel along the needle, composed of thick-walled textura angularis, 600–800 x 80–100 µm. Dehiscence irregular, by longitudinal split pushing back a flap of epidermis that remains attached (Fig. 2). Conidiophores semimacronematous, simple or branched, 1–2(3) septate, sometimes anastomosing, 25–72 × 3–3.5 µm, subhyaline to pale brown, smooth. Conidiogenous cells holoblastic, annellidic, integrated or discrete, indeterminate, cylindrical, 11.5–18.5 × 2.5–3 µm, hyaline or subhyaline, smooth, with 1–3 often widely spaced percurrent proliferations. Conidia elongate to cylindrical, with rounded apex and truncate base, straight or lightly curved, often irregular, 1–3(5) septate, 20–48(50) × 3–6 µm, thick-walled, rough or verrucose, subhyaline or olivaceous to pale brown or brown, guttulate (Fig. 3).

Culture on MEA was characterized by slow growth (2–3 mm a week), colonies stromatic, grayish to olive-green with white edge, after a month of cultivation producing a yellow diffusate and reaching 14 mm in diam. Culture on CGA was slow-growing (1–2 mm a week), colonies stromatic, olive-black, after a month of cultivation reaching 7 mm in diam. On both media the fungus produced a slimy olive conidial mass.

Specimens examined: Curonian Spit, Smiltynė Forest District, forest compartment No 15, September 8, 2009, needles of *Pinus mugo* Turra, S. Markovskaja, BILAS 48733; Smiltynė Forest District, forest compartment No 17, August 26, 2010, needles of *Pinus mugo* Turra, S. Markovskaja, BILAS 48800; Smiltynė Forest District, forest compartment No 4, August 26, 2010, needles of *Pinus mugo* Turra, S. Markovskaja, BILAS 48801; Juodkrantė Forest District, environs of Juodkrantė, September 29, 2010, needles of *Pinus mugo* Turra, S. Markovskaja, BILAS 48799.

Distribution in Lithuania: The Curonian Spit is the only area of *Mycosphaerella dearnessii* occurrence in Lithuania so far.

Microscopic examination of the infected needles and fungal cultures revealed a remarkable variability in the conidiogenesis of the fungus. As a rule, on MEA and CGA media the fungus produced branched, sometimes anostomosing conidiophores with numerous proliferations and relatively large conidia (up to 50 µm long and 4.0–6.0 µm wide); while on the needles simple and branchless conidiophores with smaller conidia (20–36-µm-long and 3.0–5.0-µm-wide) predominated. We suspect that size of conidia may also vary depending on host and climate and on different fungal populations or races; for example, according to Sutton (1980) and Marmolejo (2000) conidia of American specimens are 15–35 × 3.0–4.0 µm in size, while in European material they are slightly larger: 15–55 × 2.5–5.0 µm (Chandelier et al., 1994), 10–55 × 2.0–4.5 µm (Mel'nik, 1997), 16–42 × (2)4.0–5.0 µm (Jurc & Jurc, 2009) or 21–44 × 3.0–5.0 µm (Jankovský et al., 2009). Conidia of the Lithuanian specimens seem to be among the larger ones: 20–48(50) × 3.0–6.0 µm.

Identification by molecular methods

Using RFLP of the ITS region of rDNA with four restriction endonucleases we carried out express confirmation of species identity of our *M. dearnessii* strains as described by Peihl et al. (2004). PCR products were successfully amplified (Fig. 4), restriction endonucleases *Hinf I, Hae III, Hha I* and *Hpa II* produced unique restriction fragment patterns that help distinguishing *M. dearnessii* from other species occurring on *Pinus* (Peihl et al., 2004). The restriction patterns were compared with those of *Mycosphaerella dearnessii* published in EPPO Protocol (OEPP/EPPO, 2008) and provided by Peihl et al. (2004). To address the molecular taxonomy and genetic relationships, the ITS region of rDNA of our isolates were sequenced and aligned with known *Mycosphaerella* nucleotide sequences. The results of neighbour-joining analysis

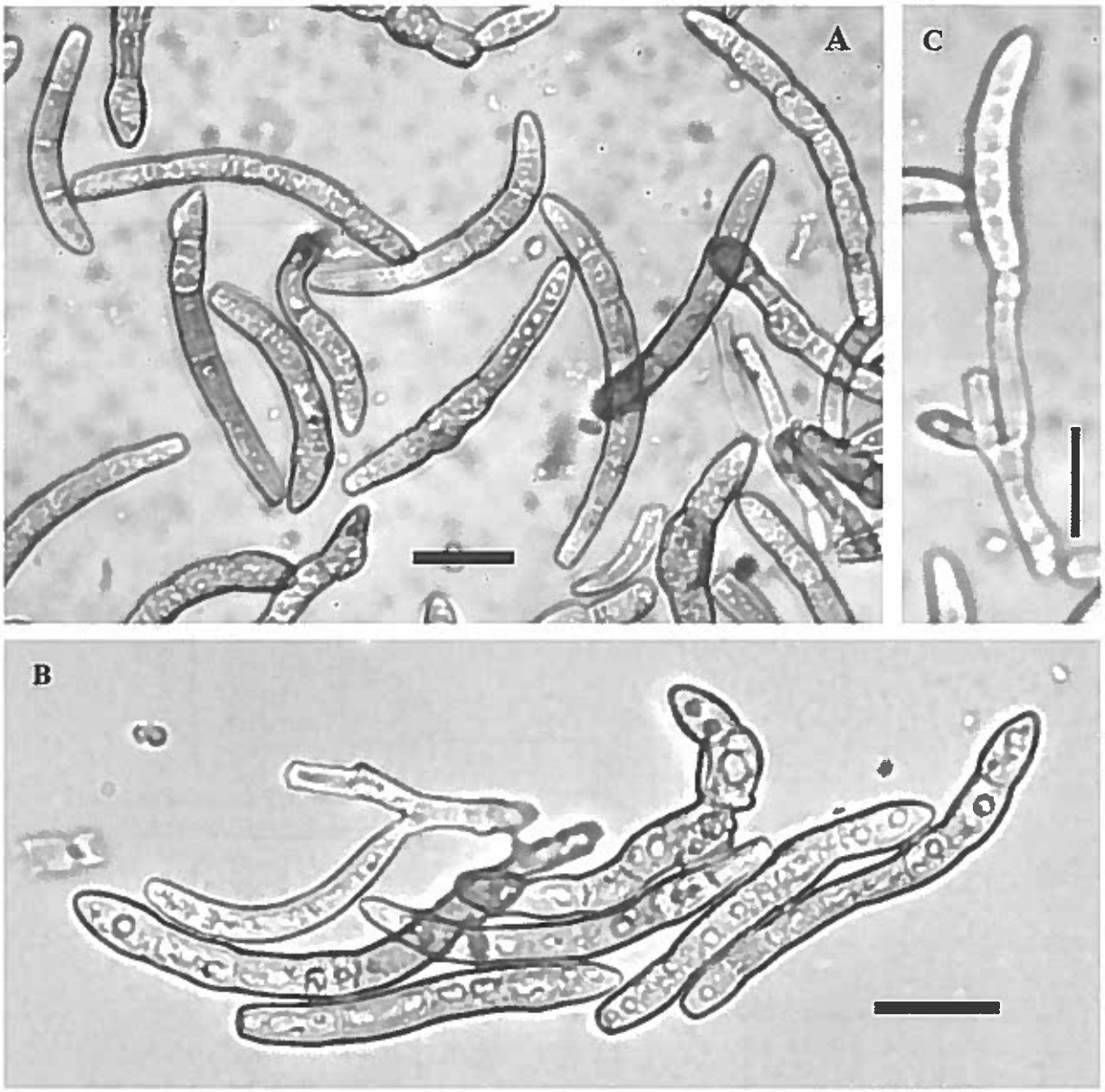

Fig. 3. Micrographs of reproductive structures of *Lecanosticta acicola*, an anamorphic stage of *Mycosphaerella dearnessii*: conidia (A) and conidia with conidiophores (B, C). Scale bars = 10 μm

are presented in Fig. 5. As expected, all strains of *M. dearnessii* were clustered in highly supported clade in the bootstrap analysis. High bootstrap values were found within *M. pini* clade, too. Our strains LA733A and LA733B (respective GenBank accession no. HM367707 and HM367708) formed a separate subclade together with *M. dearnessii* strains originating from USA (GenBank accession no. AF211195) and France (GU214663). ITS sequence pair similarities between our *M. dearnessii* strain (HM367707) and other *Mycosphaerella* species randomly selected from the Gen Bank (Table 1) ranged from 71.9 % (with *M. endophytica* Crous et H. Smith., DQ267580) to 100 % (with several *M. dearnessii* sequences deposited in the GenBank; accession no. DQ019333–DQ019335, GU214663, EU117117 and AF211194–AF211196). The lowest similarity (95.1 %) within group of *M. dearnessii* sequences deposited in the GenBank

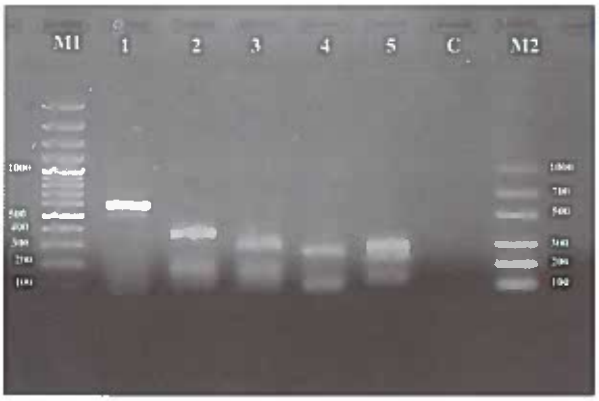

Fig. 4. ITS-RFLP patterns of *Mycosphaerella dearnessii* isolate LA733A. M1 – 100 bp marker, 1 – ITS amplicon, 2 – *Hha I*, 3 – *Hpa II*, 4 – *Hae III*, 5 – *Hinf I*, C – control, M2 – MassRuler express marker

was found as sequence of our strain (HM367707) was compared to that of Japanese origin (AF362070).

Apparently we are dealing with an initial stage of *M. dearnessii* invasion into Lithuanian pine stands (arrival and naturalization, establishment and beginning of spread into new areas). Specificity of the region (the Curonian Spit) with its changeable climate and poor soil conditions, and presence of non-native host tree species, *Pinus mugo*, have likely facilitated the invasion and establishment of this alien fungus. Moreover, air conditions during the summer of 2010 (high air humidity and air temperature) were highly favourable for pathogen spread and successful infection of pine

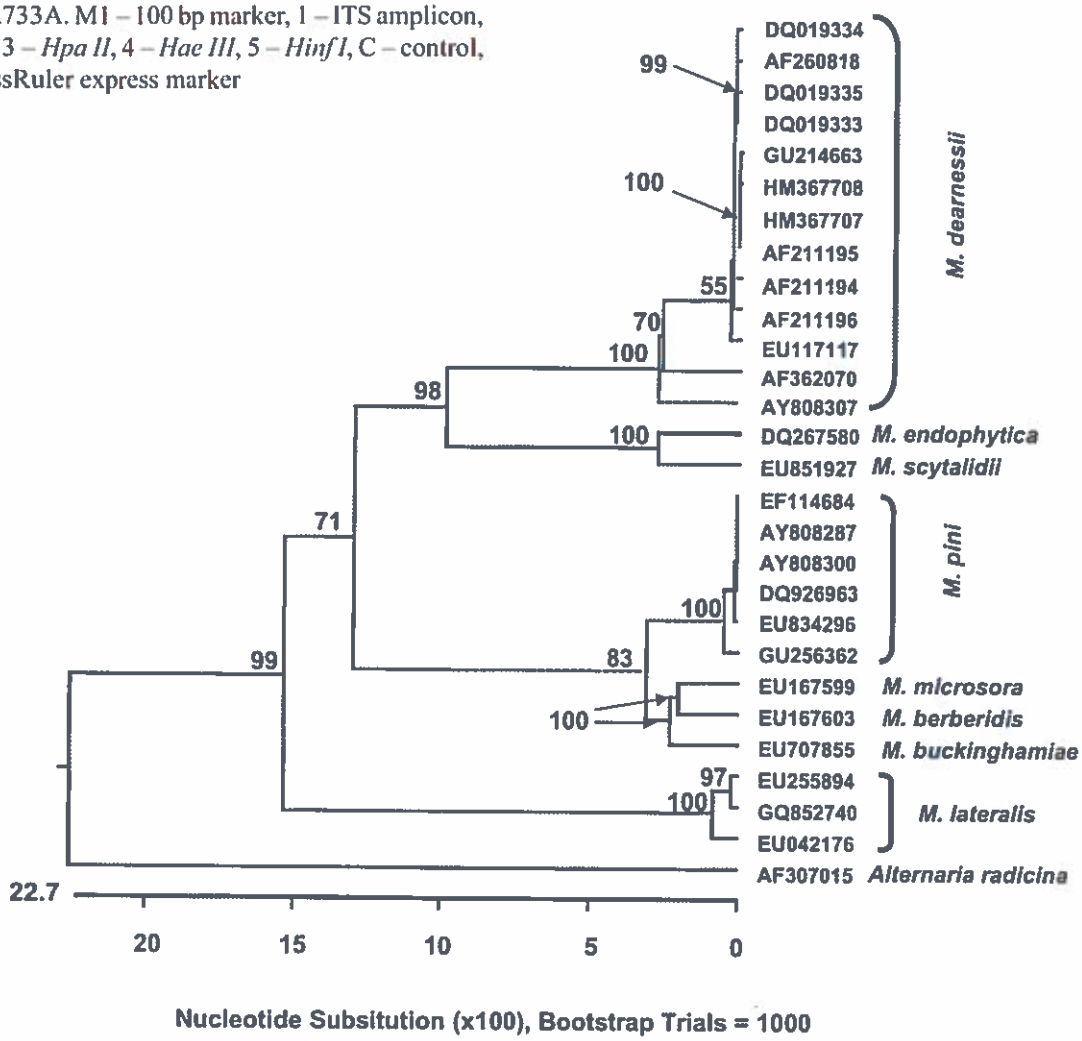

Fig. 5. Neighbor-joining tree of ITS1-5.8S-ITS2 rRNA gene of various *Mycosphaerella* species, calculated by Lasergene 8.1.4 (DNASTAR, Inc., Madison, USA) without pairwise correction. Numbers on the branches show bootstrap values obtained from 1000 replications and rounded to the nearest integer. The values are presented only for branches supported by more than 50 %. Length of the branches is proportionate to numbers of changes. *Alternaria radicina* Meier, Drechsler et E. D. Eddy (AF307015) was used as an outgroup species to root the tree

Table 1. List of percent similarity of twenty six strains of the genus *Mycosphaerella* compared to ITS nucleotide sequences of *Mycosphaerella dearnessii* with *Alternaria radicina* (AF307015) used as outgroup

No.	Species (GenBank accession No.)	Origin	Sequences pair distances: percent similarity
1	*Mycosphaerella dearnessii* HM367707	Lithuania	This study
2	*Mycosphaerella dearnessii* DQ019333	Canada	100
3	*Mycosphaerella dearnessii* DQ019334	Canada	100
4	*Mycosphaerella dearnessii* DQ019335	Canada	100
5	*Mycosphaerella dearnessii* AY808307	China	99.8
6	*Mycosphaerella dearnessii* AF362070	Japan	95.1
7	*Mycosphaerella dearnessii* GU214663	France	100
8	*Mycosphaerella dearnessii* EU117117	Czech Republic	100
9	*Mycosphaerella dearnessii* AF260818	USA	99.5
10	*Mycosphaerella dearnessii* AF211194	USA	100
11	*Mycosphaerella dearnessii* AF211195	USA	100
12	*Mycosphaerella dearnessii* AF211196	USA	100
13	*Mycosphaerella pini* EF114684	USA	84.5
14	*Mycosphaerella pini* AY808300	USA	85.9
15	*Mycosphaerella pini* AY808287	Chile	85.9
16	*Mycosphaerella pini* GU256362	New Zealand	90.5
17	*Mycosphaerella pini* EU834296	Finland	84.5
18	*Mycosphaerella pini* DQ926963	Bhutan	85.9
19	*Mycosphaerella scytalidii* EU851927	Uruguay	77.4
20	*Mycosphaerella endophytica* DQ267580	South Africa	71.9
21	*Mycosphaerella lateralis* EU255894	Spain	78.7
22	*Mycosphaerella lateralis* GQ852740	Australia	80.1
23	*Mycosphaerella lateralis* EU042176	Australia	80.0
24	*Mycosphaerella microsora* EU167599	Germany	91.2
25	*Mycosphaerella berberidis* EU167603	Germany	89.0
26	*Mycosphaerella buckinghamiae* EU707855	Australia	90.8
27	*Alternaria radicina* AF307015	USA	68.0

needles. Undoubtedly, some risk of infection exists also for *P. sylvestris*, a native pine species in Lithuania that is also very common in the Curonian Spit.

At present, measures aiming to control the disease spread in Lithuania have been initiated by the State Plant Service under the Ministry of Agriculture to prevent potential ecological and economical losses (A. Beniušis, personal communication). From 2011, a broad monitoring and control programme covering Juodkrantė and Kretinga forest nurseries (that cultivate *P. mugo* seedlings of the Curonian Spit's origin) and all coastal pine stands is planned. Currently all *P. mugo* trees heavily infected by *Mycosphaerella dearnessii* in the Smiltynė Forest District have been felled and burned.

Various disease management strategies are to be taken in the future: chemical pathogen control (fungicides), removal of the infected trees and needles, and breeding of resistant pine species or cultivars. Good results of chemical control have been obtained using fungicides benomyl and maneb (Kais et al., 1986), but the most ecological and practical control measure for the majority of pathogenic fungi is to cultivate disease-resistant pine species or cultivars (Gibson, 1979; Crous et al., 1990).

ACKNOWLEDGEMENTS

This study was supported by the priority program of Lithuanian Science and Study Foundation No. C-08031 "Biological invasions in Lithuanian ecosystems under the climate change: causes, impacts and projections" (BINLIT). Anonymous reviewers are thanked for their critical comments and suggestions.

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NAUJAS SVETIMŽEMIS PATOGENINIS GRYBAS *MYCOSPHAERELLA DEARNESSII* LIETUVOJE

Svetlana Markovskaja, Audrius Kačergius, Aušra Treigienė

Santrauka

Lietuvos pamaryje 2009–2010 m. buvo aptiktas naujas patogeninis aukšliagrybis *Mycosphaerella dearnessii*. Kol kas nustatyta tik jo konidijinė stadija – *Lecanosticta acicola*. Šis grybas pažeidė sodintus įvairaus amžiaus kalninės pušies (*Pinus mugo*) miško plotus Kuršių nerijoje, nuo Smiltynės iki Juodkrantės, sukeldamas pavojingą rudąją spyglių dėmėtligę. Grybas buvo identifikuotas pagal anamorfos morfologinius požymius ir patvirtintas molekuliniu PGR metodu. Straipsnyje aptariama jo taksonomija, ekologija, paplitimas ir invazijos grėsmė.