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LECANICILLIUM APHANOCLADII – A NEW SPECIES TO THE MYCOFLORA OF LITHUANIA AND A NEW PATHOGEN OF TREE LEAVES MINING INSECTS

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Abstract

Pečiulytė D., Kačergius A., 2012: *Lecanicillium aphanocladii* – a new species to the mycoflora of Lithuania and a new pathogen of tree leaves mining insects [*Lecanicillium aphanocladii* – nauja Lietuvos mikofloros rūšis ir naujas medžių lapus minuojančių vabzdžių patogenas pasaulyje]. – Bot. Lith., 18(2); 133–146.

Popular park and city trees horse chestnut, *Aesculus hippocastanum* L. and small-leaved linden, *Tilia cordata* Miller suffer from damages of leaf mining moths, *Cameraria ohridella* Deschka & Dimic 1968 and *Lithocolletis issikii* Kumata 1825 (= *Phyllonorycter issikii* Humber 1822 (both *Lepidoptera*, *Gracillariidae*), respectively. In 2010–2011, fungi causing moth caterpillar mortality under field conditions were investigated in Vilnius city, Lithuania. Statistical analyses showed high recovery percentage (30.36 % and 31.92 % from *C. ohridella* and *L. issikii* caterpillar cadavers, respectively) of a new fungus, which was thereafter identified as *Lecanicillium aphanocladii* Zare & W. Gams 2001 based on a morphological characteristics and ITS rDNR sequence, which showed 99 % identity with *Aphanocladium aranearum* (Petch) W. Gams 1971 GenBank accessions No. AF455489 and AF455405 of ribosomal RNA gene (now renamed as *L. aphanockladii*).

Keywords: entomopathogens, *Lecanicillium aphanocladii*, leaf miners, *Cameraria ohridella*, *Phyllonorycter issikii*, horse chestnut, small-leaved linden.

INTRODUCTION

The genus *Phyllonorycter* Humber 1822 (= *Lithocolletis* Humber 1825) belongs to the subfamily *Lithocolletinae* (*Lepidoptera*, *Gracillariidae*) (Lopez-Vaamonde et al., 2003). It is one of the most species-rich genera of all *Lepidoptera* with the majority of species found in the temperate regions (Kuznetsov, 1981; Davis & Deschka, 2001). Morphological studies suggest that genus *Phyllonorycter* Hübner is most similar and is monophyletic with the genus *Cameraria* Chapm. as its sister group (Lopez-Vaamonde et al., 2003; Buszko, 2006). Two morphologicaly comparable invasive moths, *Lithocolletis issikii* Kumata 1825 (= *Phyllonorycter issikii* Kumata 1822) and *Cameraria ohridella* Deschka and Dimic 1986, ravage leaves of popular park and city trees – small-leaved linden (*Tilia cordata* Miller) (*Tiliaceae*) and horse chestnut (*Aesculus hippocastanum* L.) (*Hippocastanaceae*).

Cameraria ohridella was described in former Jugoslavia (Deschka & Dimic, 1986). During the last 30 years, the invader spread over Europe, reached Russia and Turkey (Lees et al., 2011). In Lithuania, *C. ohridella* was first registered in 2002 in the suburbs of Klaipėda, western Lithuania (Ivinskis & Rimšaitė, 2006). *Lithocolletis issikii* was described in Japan (Kumata, 1963). Later it was found in Korea (Kumata et al., 1983), Russia, in Prioksko-Terraskii Biospheric Reservation near Moscow (Osipova, 1990) and Primorskij Kraj (Ermolaev & Zorin, 2011b). In Lithuania, *L. issikii* was first recovered in 1997 during faunistic investigations of *Tilia cordata* in Pagėgiai Park (Šilutė district) and Vilnius city (Noreika, 1998). The species widens its range very rapidly towards the west. In 1996, this pest

reached southeastern Poland, in 1999 – the surroundings of Katowice, and in the same year, first mines of *Tilia* in Czech Republic were observed near Brno (Šefrová, 2002).

Cameraria ohridella and *Lithocolletis issikii* females lay eggs on underside of the leaves on the host plant, catterpillars penetrate into the leaves and develop up to 5–6th sinstars (Noreika, 1998; Syeryebrennikov, 2008). *L. issikii* prefer the trees in undergrowth; imago hibernate (Noreika, 1998). Two or three generations of *Cameraria ohridella* can be recorded during a vegetation period; caterpillars of the last generation pupate within the mines and hibernate in the fallen leaves (Syeryebrennikov, 2008). The common feature of all leaf miners is that the caterpillars feed within leaves for at least some caterpillar stages (Faeth, 1991). Caterpillars destroy parenchyma of leaves, forming large mines, in which feed up to the last instars and pupate. Plant tissues contain toxic secondary compounds, which function as defence against consumers. However, insects can protect themselves from such compounds by detoxification and sequestration, and a few insects use symbiotic microorganisms in detoxification (Douglas, 2009). On the other hand, each individual plant hosts one or more endophytes, that implicates a rich reservoir of microorganisms, some of which can infect leaf miners (Petrini, 1986). The aim of this investigation was to isolate and identify fungi associated with the mortality of two invasive moths, *C. ohridella* and *L. issikii*, which are characterized by very comparable morphology, but feed and develop on foliages of different trees, *Aesculus hippocastanum* and *Tilia cordata*, respectively.

MATERIALS AND METHODS

Sampling, isolation and microscopic analysis. *Aesculus hippocastanum* and *Tilia cordata* leaves containing mines were collected in Vilnius city, Lithuania in 2010–2011. Mines with the caterpillars at 5–6th instars were selected for choice; last instar caterpillar did not feed. Leaves with the mines were incubated in wet chambers at 20 ± 2 ºC in a room. Development of the caterpillars and emergence of adult moths was revised daily. A total of 1264 *Cameraria ohridella* and 756 *Lithocolletis issikii* caterpillars within the mines were incubated aiming to determine caterpillar mortality and mycose frequency. After two weeks (a time needed to adult moth emergence; Syeryebrennikov, 2008), numbers of diseased caterpillars were determined. Caterpillars, from which adults did not emerge, were considered diseased.

Segments containing mines (2.5–3 cm^2, due to mine area) with diseased caterpillars were cut out from the leaves. Surface sterilization was done by 0.5 % (v/v) sodium hypochlorite (NaOCl). Each segment was treated with 75 % ethanol for 1 minute followed by immersion for 2 min in sodium hypochlorite and again for 30 sec in 75 % ethanol. Then, mines were dissected aseptically to take out the caterpillar cadavers, which were placed on the moistened paper in separate sterile Petri dishes. Dishes were sealed, incubated in the room at 20 ± 2 ºC and revised daily for mycosis symptoms. Cadavers of both moth adults that were found within the first two days after their emergence were also incubated and analysed. The fungi were isolated and identified according to their morphology using low magnification stereomicroscope (×40 magnification) of cadavers (or colonies) and by preparing slides for a light microscopy (×400 and ×1000 magnifications). When fungi mycelium, covering the cadaver, did not produce conidia (or spores) small segments of the mycelium were dissected and plated on a half-strength potato dextrose agar (PDA), supplemented with chloramphenicol (0.1 g L^{-1} w/v) and sub-cultured on three media: PDA, Sabouraud dextrose agar (SDA), and Czapek's Dox agar (CDA) (all three from Liofilchem-Italy). Identification of fungi followed standard methods based on macro- and micro-morphological features (Samson, 1974; Domsch et al., 2007; Kiffer & Morelet, 2000; Pitt, 1985; Sung et al., 2007; Gams, 1971; Zare & Gams, 2001). Olympus CX41 microscope was used for the microscopic diagnoses, and Q-Imaging MicroPublisher 5.0 RTV microscope camera was used for the microscopic pictures.

DNA extraction, amplification, sequencing and analysis. Genomic DNA was extracted with NucleoSpin® Plant II Kit (Macherey–Nagel GmbH & Co. KG, Germany) according to manufacturer's instruction using approximately 100 mg wet weight of mycelium. The internal transcribed spacers 1 and 2, including the 5.8S rDNA, were amplified in 25 µl reactions on TProfessional 96 Gradient Thermocy-

cler (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 MgCl2, 0.2 mM of each dNTP, 10 µM of primers ITS5 and ITS4 (White et al., 1990). The PCR conditions were as follows: 10 min at 95 °C as initial denaturation, followed 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, with final extension of 10 min at 72 °C. For sequencing, the PCR products in 620 bp size were purified according to the Protocol for PCR Product Clean-up with Exonuclease I and FastAP™ Thermosensitive Alkaline Phosphatase (Ltd. Fermentas, Lithuania). Purified PCR products were sequenced using Macrogen (Macrogen Europe, Amsterdam, and the Netherlands) on an ABI 3730XL DNA sequencer. Four different PCR products from specimen and a total of four repeats for each sequence from both ends (5' and 3') were sequenced. The rDNA homology searches were performed through the Internet at the server of National Centre for Biotechnology Information (National Institutes of Health, Bethesda, USA).

Statistical analyses. Diversity of fungi species isolated from the moth cadavers was calculated by the Shannon-Wiener index (*H'*) according to each species frequency, using equation:

$$H' = -\sum_{i=1}^{S} (p_i \ln p_i),$$

where S is the total number of species and p_i is the frequency of the i_{th} species (Krebs, 1989). To compare fungal communities associated with two months mortality, Sörensen's similarity index (C_s) was calculated using equation: (C_s = 2c/a + b) (Sörensen, 1948), where a and b are the numbers of fungi isolated from *Cameraria ohridella* and *Lithocolletis issikii* caterpillars or adults, respectively, and c is the number of shared species.

RESULTS

One thousand two hundred and sixty four *Cameraria ohridella* caterpillars within the mines were incubated in the laboratory. Caterpillar mortality reached 9.2 % in 6 days, 11.18 % in 9 days, and 45.2 % (572 out of 1264 investigated) in two weeks of incubation in wet chambers (Table 1).

Mortality of the 19.58 % caterpillars was induced by fungi, which were detected in 112 caterpillar cadavers out of the 572 examined during the investigation. Thirty five *C. ohridella* adults (out of 692 emerged, comprising 5.06 %) were diseased in two days after they had emerged from the caterpillars (Table 1). Twelve adult moths (34.29 % of the diseased) showed mycoses symptoms.

Three hundred and seventy eight *Lithocolletis issikii* caterpillars were incubated within the mines *in vitro*; 53.97 % (204 out of 378) of them diseased in two weeks rearing period (Table 1). Cadavers' viability analyses revealed high abundance of the caterpillar mortality caused by the fungi; 71.57 % (292 out of 408 diseased) diseased caterpillars showed mycoses symptoms. Three hundred and forty eight adult moths emerged from 756 incubated *L. issikii* caterpillars and 48 adults diseased in two days after their emergence. Eighteen cadavers (39.58 %) showed mycoses symptoms.

Twenty three fungus species belonging to 17 genera were isolated from both moth caterpillar cadavers (Table 2). Seventeen species (from 15 genera) and 15 species (from 11 genera) were isolated from *Cameraria ohridella* and *Lithocolletis issikii* caterpillar ca-

Table 1. Ratio of the *Cameraria ohridella* and *Lithocolletis issikii* (=*Phyllonorycter issikii*) *Lithocolletis* caterpillars, which diseased and showed mycoses symptoms after two weeks incubation within the mines in wet chambers

	C. ohridella (n = 1264)*	*L. issikii* (n = 756)
Number and percentage (in brackets) of diseased caterpillars	572 (45.2 %)	408 (53.97 %)
Number and percentage (in brackets) of caterpillars with mycoses symptoms	112 (5.59 %)	292 (71.57 %)
Number and percentage (in brackets) of diseased adults in two days after their emergence from the viable caterpillars	35 (5.06 %)	48 (13.79 %)
Number and percentage (in brackets) of diseased moth adults with mycoses symptoms	12 (34.29 %)	18 (39.58 %)

*Number of caterpillars incubated within the mines in laboratory at 25 ºC and 95 % RH.

davers, respectively. *Lecanicillium* W. Gams & Zare was the most common and abundant genus isolated from the cadavers with mycoses symptoms. *Lecanicillium aphanocladii* Zare & W. Gams dominated comprising 30.36 % and 21.92 % occurrence in the *Cameraria ohridella* and *Lithocolletis issikii* caterpillars' cadavers, respectively. *Trichothecium roseum* (Pers.) Link was the second fungus more frequently isolated from the diseased caterpillars (Table 2), particularly common in the *L. issikii* caterpillars with 20.21 % occurrence. *Isaria fumosorosea* Wize and *T. roseum* were isolated with the same (8.93 %) occurrence. Other known fungal entomopathogens, *Beauveria bassiana* (Blas.-Criv.) Vuill., *L. lecanii* (Zimm.) Zare & W. Gams and *Metarhizium anisopliae* (Metschn.) Sorokin, were obtained from 2.74–5.48 % caterpillar cadavers. Fungus *M. anisopliae* was isolated only from *L. isikii* caterpillar cadavers with mycoses symptoms. Fungi *Alternaria alternata* (Fr.) Keissl., *Cladosporium cladosporioides* (Fresen.) G.A. de Vries and *Penicillium chrysogenum* Thom were isolated from the caterpillar cadavers of both moth species. Thus, the composition of fungi recovered from different hosts mining moths and species abundance did not differ; this was confirmed by the Shannon-Wiener diversity indices H′ = 2.4539 and 2.3602 for *Cameraria ohridella* and *Lithocolletis issikii*, respectively (Table 2).

The results of the mycological analyses of moth adult cadavers collected during the cadavers rearing

Table 2. Distribution of fungal species recovered from the cadavers of *Cameraria ohridella* and *Lithocolletis issikii* (= *Phyllonorycter issikii*) caterpillars collected in field and incubated within the mines in the laboratory

Fungal species	Number and percentage (in brackets) of isolated fungi occurrence*	
	C. ohridella caterpillars	*L. issikii* caterpillars
Acremonium strictum W. Gams	8 (7.14)	
Alternaria alternata (Fr.) Keissl.	4 (3.57)	9 (3.08)
Aspergillus parasiticus Speare	4 (3.57)	
Beauveria bassiana (Bals.-Criv.) Vuill.	6 (5.36)	16 (5.48)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	6 (5.36)	41 (14.04)
Colletotrichum sp.	4 (3.57)	5 (1.71)
Erysiphe flexuosa (Peck) U. Braun *et* S. Takamatsu	2 (1.79)	
Fusarium proliferatum (Matsush.) Nirenberg		13 (4.45)
F. oxysporum Schltdl.		11 (3.77)
F. subglutinans (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas	2 (1.79)	
Harzia acremonioides (Harz) Costantin	4 (3.57)	
Isaria fumosorosea Wize	10 (8.92)	7 (2.4)
Lecanicillium aphanocladii Zare & W. Gams	34 (30.36)	64 (21.92)
L. lecanii (Zimm.) Zare & W. Gams	4 (3.57)	8 (2.74)
L. psalliotae (Treschew) Zare & W. Gams		14 (4.79)
Metarhizium anisopliae (Metschn.) Sorokin		11 (3.77)
Oedocephalum glomerulosum (Bull.) Sacc.	6 (5.36)	
Penicillium citrinum Thom	2 (1.79)	
P. chrysogenum Thom	2 (1.79)	9 (3.08)
P. oxalicum Currie & Thom		16 (5.48)
Scopulariopsis bevicaulis (Sacc.) Bainier		9 (3.08)
Trichoderma harzianum Rifai	4 (3.57)	
Trichothecium roseum (Pers.) Link	10 (8.92)	59 (20.21)
Total number of caterpillars with mycoses symptoms	**112**	**292**
Total number of isolated fungi species	**17**	**15**
Total number of isolated fungi genera	**15**	**11**
Shannon-Wiener diversity index (*H′*)	**2.4539**	**2.3602**

*Data are presented as total number of cadavers from which fungus was isolated and percentage (in brackets) from all cadavers with mycoses symptoms

in the laboratory are presented in Table 3. In two days after the emergence, 12 cadavers of *Cameraria ohridella* and 18 cadavers of *Lithocolletis issikii* adults were found with the mycoses symptoms. Six fungal species (Table 3) were isolated from adult cadavers; all of them belong to the same group of fungi species, which caused both moths caterpillar mortality (Table 2). *Lecanicillium aphanocladii* was the most commonly isolated from the diseased adult moths; the fungus was detected in 41.67 % and 38.89 % of *Cameraria ohridella* and *Lithocolletis issikii* cadavers, respectively. Three of the six species were recovered from one of two moth adults: *Acremonium strictum* W. Gams – from *Cameraria ohridella* adults, while *Aspergillus parasiticus* Speare and *Fusarium oxysporum* Schltdl. – from *Lithocolletis issikii* adults. *Cladosporium cladosporioides*, *Isaria fumosorosea* and *Lecanicillium aphanocladii* were isolated from the diseased adults belonging to both moth species.

In this investigation, the complexes of fungi isolated from diseased *Cameraria ohridella* caterpillars and adults as well as the complexes of fungi recovered from *Lithocolletis issikii* caterpillars and adults differed: calculated Sörensen's similarity indices (C_s) were 0.38 and 0.40, respectively. Analysis of fungus species diversity for both species of the moth caterpillars showed that *C. ohridella* and *L. issikii* caterpillar mortality was caused by comparable fungal species assemblages ($C_s = 0.68$). Less similarity ($C_s = 0.50$) was determined for the fungi isolated from the diseased *C. ohridella* and *L. issikii* adults.

In the present investigation, two morphotypes belonging to *Lecanicillium* genus were more frequently recovered from *Cameraria ohridella* and *Lithocolletis issikii* caterpillar cadavers (30.36 % and 21.92 % recovery; Table 1), thus, strains of these two morphotypes were examined not only morphologically, but sequences of the ITS regions of rDNR were also analysed. Based on the morphological characteristics, both morphotypes were assigned to the large clade of *Verticillium* Nees (= *Lecanicillium* W. Gams & Zare) species with white fluffy colonies lacking any resting structures. *Lecanicillium aphanocladii* was identified based on morphological description of *Acremonium aranearum* Petch (Petch, 1932), micro- and macro-morphological despription of *Aphanocladium aranearum* (Petch) W. Gams (Gams, 1971) and morpho-icones of *Aphanocladium aranearum* (Petch) W. Gams (Matsushima, 1975). *Aphanocladium arenearum* (Petch) W. Gams 1971 (= *Acremonium aranearum* Petch 1932) now is renamed as *Lecanicillium aphanocladii* Zare & W. Gams 2001 and assigned to *Cordycipitaceae*. This was the first record of *L. aphanocladii* for Lithuanian mycobiota. Fungus grows similarly on four cultivation media (PDA, MEA, CA and SA) (Fig. 1) forming white and deeply woolly colonies with some differences in the colony reverse pigmentation starting from the 7th day of cultivation at 25 ºC in the dark due to the medium composition. The culture medium influences also an intensity of conidia formation (data not presented). Fungus forms short, basally swollen, with narrow tip, rapidly collapsing into inconspicuous denticles conidiophores ('aphanophialides' in the sense of Gams, 1971 after Zare & Gams, 2004), which bear single

Table 3. Distribution of fungal species recovered from the cadavers of *Cameraria ohridella* and *Lithocolletis issikii* (= *Phyllonorycter issikii*) adult moths emerged from the caterpillars reared within the mines in the laboratory

Fungal species	Number and percentage (in brackets) of fungal isolates occurrence*	
	C. ohridella adult moths (n = 12**)	*L. issikii* adult moths (n = 18)
Acremonium strictum W. Gams	1 (8.33)	
Aspergillus parasiticus Speare		1 (5.56)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	3 (25.0)	5 (27.77)
Fusarium oxysporum Schltdl.		1 (5.56)
Isaria fumosorosea Wize	3 (25.0)	4 (22.22)
Lecanicillium aphanocladii Zare & W. Gams	5 (41.67)	7 (38.89)
Total number of moth adults with mycoses symptoms	**12**	**18**

* Data are presented as number of adult moth cadavers from which fungus was isolated and percentage (in brackets) from all cadavers with mycoses symptoms. **Total number of the analysed adult moth cadavers.

ellipsoidal conidia; aphanophialides scatter laterally along the procumbent or prostrate aerial hyphae (Fig. 2, A). Growing the moth caterpillar cadavers, *L. aphanocladii* form comparable mycelium, aphanophialides and conidia starting from the 3rd day of incubation in wet chamber (Fig. 2, A and B).

Other fungus, *Lecanicillium psalliotae,* forms comparable white, fluffy colonies as *L. aphanocladii* do, except that red pigment was intensively secreted into growth medium, particularly into MEA and CA media. To address the molecular taxonomy and relationships, the ITS rDNAs of the isolate were amplified using the sets of ITS primers. According to rDNA homology searches performed in NCBI GenBank, the closest related taxa based on ITS sequences are: *Aphanocladium aranearum* (GenBank AF455489; Identities = 611/613 (99 %), Gaps = 1/613 (0 %)); *A. aranearum* (GenBank AF455405; Identities = 611/613 (99 %), Gaps = 1/613 (0 %)); *Lecanicillium psalliotae* (GenBank AB160994; Identities = 609/611 (99 %), Gaps = 0/611 (0 %)); *L. saksenae* (GenBank AB360362; Identities = 609/619 (98 %), Gaps = 1/619 (0 %)); *L. psalliotae* (GenBank AB360367; Identities = 598/619 (97 %), Gaps = 4/619 (1 %)). *Aphanocladium aranearum* has transferred to *Lecanicillium* genus and has been renamed as *L. aphanocladii*.

DISCUSSION

Invasive tree pests, *Cameraria ohridella* and *Lithocolletis issikii* cause serious horse chestnut and small-leaved linden leaf damages in Lithuania (Noreika, 1998; Ivinskis & Rimšaitė, 2006; Snieškienė et al., 2011). The present investigation was undertaken to document the spectrum of fungi, causing these leaf miners mortality under field conditions. Strains recovered from the insects, developing on the tree foliage, usually became relevant host-hyperparasites. Entomopathogenic fungi are promising alternatives to chemical insecticides and can be registered as plant protection agents (Strasser et al., 2010). In the present study, 23 morphospecies of fungi were identified from both moth diseased caterpillars and six morphospecies – from the diseased moth adults. General fungal entomopathogens (*Beauveria bassiana*, *Isaria fumosorosea*, *Lecanicillium lecanii* and *Metarhizium anisopliae*) comprised 15.85 % of all fungi recovered from diseased *Cameraria ohridella* caterpillars and 14.39 % of the fungi recovered from *Lithocolletis issikii* caterpillar cadavers. None of them dominated as agents causing leaf miner mycoses. The most commonly recovered fungi were *Lecanicillium aphanocladii* and *Trichothecium roseum*. *L. aphanocladii* comprised 30.36 % and 9.92 % of all fungi

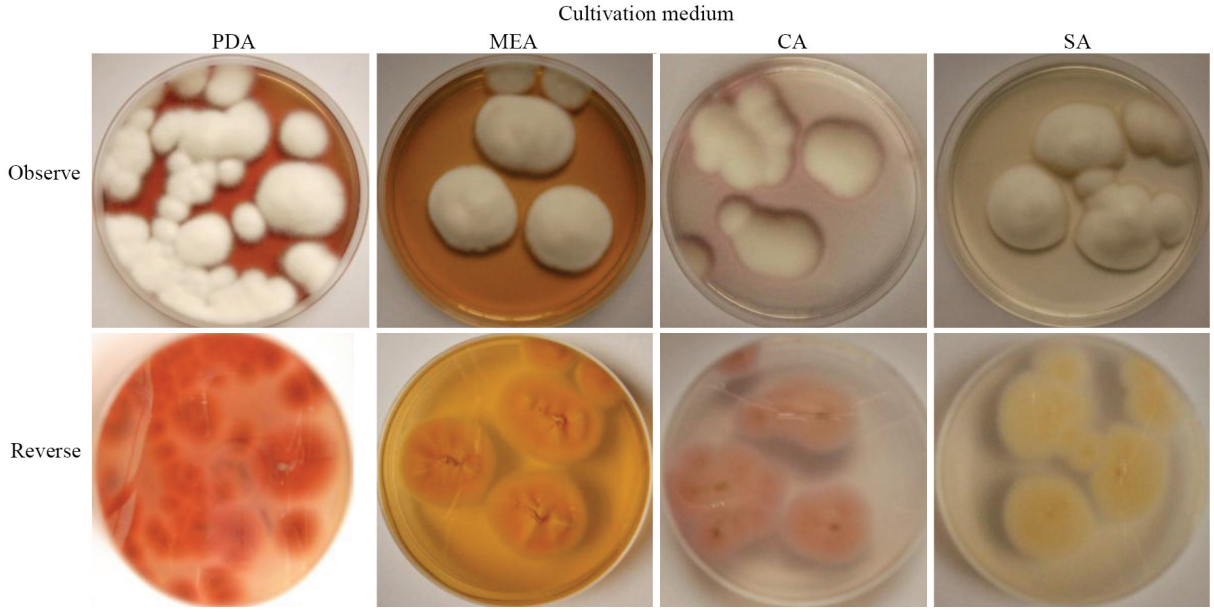

Fig. 1. Appearance of *Lecanicillium aphanocladii* colonies after seven days growth on the potato dextrose agar (PDA), malt extract agar (MEA), Czapek's Dox agar (CDA), and Sabouraud agar (SA) at 25 ºC in the dark

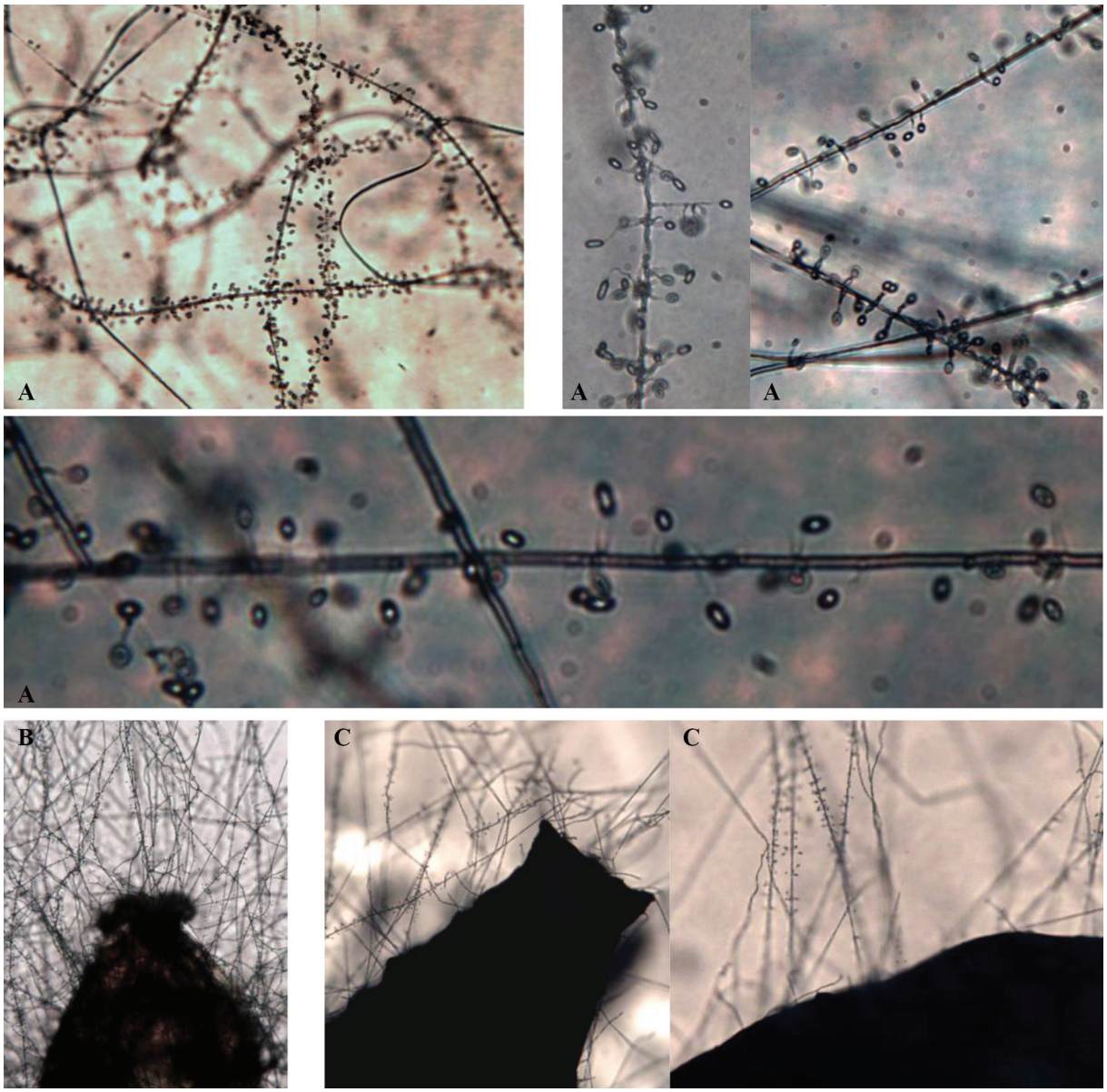

Fig. 2. Hyphae and aphanophialides bearing on the narrow tips solitary elipsoidal conidia in culture on potato dextrose agar (A) and on the cadavers of *L. issikii* (B) and *C. ohridella* (C) caterpillar cadavers

isolated from *Cameraria ohridella* and *Lithocolletis issikii*, respectively; *T. roseum* comprised 21.92 % and 20.21 % of all fungi isolated from *Cameraria ohridella* and *Lithocolletis issikii*, respectively. These findings were unexpected. However, both moths are invasive to Lithuania: *C. ohridella* was firstly recorded in 2004 (Ivinskis & Rimšaitė, 2006), *L. issikii* – in 1997 (Noreika, 1998). Native antagonists must adapt to new hosts. During the present investigation, three fungi species (*Lecanicillium aphanocladii*, *L. lecanii* and *L. psalliotae*) belonging to *Verticillium* sect. *Prostrata* were commonly isolated from the diseased individuals. *Verticillium* from section *Prostrata*, now *Lecanicillium* (Gams, 1971; Zare et al., 2000; Zare & Gams, 2001), is comprised of species some of which exhibit high levels of chitinase activity, nematophagous (mainly parasitizing eggs) and fungicolous biocontrol agents (Gams et al., 2004). *Lecanicillium* isolates comprised 33.93 % and 29.45 % of all fungi isolated from *Cameraria ohridella* and *Lithocol-*

letis issikii, respectively. Some anamorphic genera, including *Aphanocladium* and *Acremonium* (fungi were recovered from moth caterpillars), are morphologically similar to *Verticillium* sect. *Prostrata* and are hypothesized to be closely related (Zare et al., 2000). *Verticillium* spp. belongs to the groups of fungicolous, entomopathogenous and nematophagous fungal pathogens (Zaayen van & Gams, 1982). Hosts of the entomopathogenous species include species of *Arachnida*, *Coleoptera*, *Hymoptera* and *Lepidoptera* (Gams, 1971). Hosts of fungicolous species comprise a variety of *Ascomycota* and *Basidiomycota*, including fleshy fungi of *Hymenomycetes*, rusts of the *Urediniomyces* (Zaayen van & Gams, 1982; Gams & Zare, 2001) and powdery mildew of the *Leotiomycetes* (Kiss, 2003). Nematophagous *Verticillium* species from *Prostrata* sect. also have a broad host range (Gams, 1988). As the host range of the mentioned section is quite diverse, it is hardly surprising that *Verticillium* species, now renamed as *Lecanicillium,* recovery from leaf miners percentage (varying from 2.74 % to 30.36 %) and the total percent from all isolated fungi (33.93 % and 29.45 % for *Cameraria ohridella* and *Lithocolletis issikii*, respectively) are high. Belonging to the genus *Verticillium*, sect. *Prostrata* entomopathogenic fungi species have recently been reclassified using morphological characteristics and Internal Transcribed Spacer region (ITS) sequences as genus *Lecanicillium* (Zare & Gams, 2001). *Lecanicillium* species have a wide host range and have been isolated from variety of insect orders (after Zare & Gams, 2001). Strains of *L. muscarium* and *L. lecanii* have been isolated from aphids, scales, whiteflies, trips and other insects in various regions of the world (Hall, 1981; Askary et al., 1998; Cuthbertson et al. 2005; Wang et al., 2005; Askary & Yarmand, 2007; Goettel et al., 2008; Anand & Tiwary, 2009; Ren et al., 2010). Identification of two fungus species *L. aphanocladii* and *L. psalliotae* from leaf miners during this investigation was unexpected. *L. psalliotae* with falcate conidia is known as fungicolous (Zare et al., 2000) and nematophagous (Yang et al., 2005; Gan et al., 2007) fungus. Little is known about *L. aphanocladii* (= *Aphanocladium aranearum*) characteristics and pathogenicity. Therefore, to confirm morphological identification, sequences of the ITS regions of fungal rDNR were analysed using BLAST programme through the Internet server at the National Centre for Biotechnology Information (National Institutes of Health, Bethesda, USA). The ITS region of our strain showed 99 % identity with *Aphanocladium aranearum* (Petch) W. Gams 1971 GenBank accessions No. AF455489 and AF455405. *Aphanocladium aranearum* has been renamed as *L. aphanocladii*.

Sequences of the ITS regions of rDNR were analysed phylogenetically (Zare et al., 2000) and the most important members, the aggregates of entomophagous *Lecanicillium lecanii*, fungicolous *L. psalliotae* and *L. aphanocladii* (= *Aphanocladium aranearum*) and *Aphanocladium dimorphum* belong to the same large clade, thus, probably, possessing comparable physiological peculiarities. Recently, *L. aphanocladii* has been isolated and proved as potential biological control agent against aphids (Zare & Mohammadi, 2006). *Aphanocladium aranearum* (formerly *A. aranearum* (Petch) W. Gams var. *simense*) has been identified as fungicolous fungus parasitic on *Agaricus bisporus* (Lange) Sing. (Chen et al., 1985).

The origin of *Lecanicillium aphanocladii* as well as of *L. psalliotae* on *Aesculus hippocastanum* and *Tilia cordata* foliage is not clear. *Lecanicillium* species being entomopathogenic can be transmitted by insects horizontally from plant to plant; *Lecanicillium aphanocladii* and *Lecanicillium psalliotae* were isolated not only from the moth caterpillars, but also from the moth adults. Moreover, there is information about dual activity of *Verticillium* isolates against powdery mildew and insects: Kim et al. (2007) demonstrated dual activity of two species *Lecanicillium attenuatum* and *L. longisporum* against aphids and powdery mildew, Askary et al. (1998) described dual activity of one *L. lecanii* strain against powdery mildew and aphids. Other authors (Biali et al., 1972; Spencer & Atkey, 1981; Kiss, 2003) also detected relationship between the *Verticillium* species and powdery mildew. In Lithuania, incidence of powdery mildew on horse chestnut and small-leaved linden foliage has been well documented (Grigaliūnaitė et al., 2005; Stankevičienė et al., 2010). It is fair to say that powdery mildew disease agent *Erysiphe flexuosa* (Peck) U. Braun & S. Takamatsu with unusual chasmothecial appendages was observed on the *Cameraria ohridella* caterpillar cadavers within the mines. Thus, further investigations are necessary to define

the origin of *Lecanicillium aphanocladii* on the horse chestnut and linden foliages, and species association with other fungi (endophytic and epiphytic) as well as with pests, leaf miners *Cameraria ohridella* and *Lithocolletis issikii*.

In this investigation, *Trichothecium roseum* was second frequently isolated fungus after *Lecanicillium aphanocladii*. This species is epiphytic fungus on the foliage of many trees in Lithuania. *T. roseum* is important mycoparasite with significant antifungal activity (Jayprakashvel et al., 2010; Zhang et al., 2010). Recently, *T. roseum* has been discussed as scale insect pathogen (Xie et al., 2012) and isolated from *Aphis pomi* de Geer (*Homoptera*, *Aphididae*) and *Tetranychus articae* Koch (*Acari*, *Tetranychoidae*) (Draganova & Simova, 2001). *T. roseum*, a typical saprobe reported also as phytopathogen on apples, tomatoes, nectarines, plums and prunes, produces several potentially toxic secondary metabolites, particularly trichothecenes and roseotoxins (Springer et al., 1984). The activity of roseotoxin B is known to be similar to the activity of destruxins of entomopathogenic fungi and phytotoxins of *Alternaria brassicae* (Berk.) Sacc. (Žabka et al., 2006). Hydrolytic enzymes like chitinase are key factors in entomopathogenic fungi infections of insects (Mayorga-Reyes et al., 2012). Ability to produce chitinase integrate general entomopathogens, *Beauveria bassia*, *Lecanicillium lecanii*, *Metarhizium anisopliae* as well as fungicolous and nematophagous fungi, *Lecanicillium psalliotae*, *Trichothecium roseum* and some strains of *Aphanocladium* genus (Blaiseau et al., 1992; Bidochka et al., 1993; Bing-Lan et al., 2003; Barreto et al., 2004; Li et al., 2004; Yang et al., 2005).

It is difficult to control populations of leaf miners. Contact insecticides can affect only eggs and adult moths. Moreover, *Aesculus hippocastanum* and *Tilia cordata* grow in parks and other green environments, so insecticide use is restricted. Caterpillars and pupae are protected inside the plant leaves and only systemic insecticides, which usually are injected into a trunk of a tree, can suppress moth development – insects ingest the insecticide while feeding on the leaves (Ferracini & Alma, 2007). However, plant pathogens can infect the plants through wounds. Ambivalent situation must be mentioned: caterpillars are protected from predators and other phylloplane microorganisms within the mines, however, inside the mines, humidity is higher and temperature is more stable; thus, conditions for development of microorganisms are more favourable than on leaf surface. Endophytic and epiphytic fungi, which penetrate through the opening made by the caterpillars after their emergence from the eggs, can thrive inside the mines and suppress moth development. Scarce information is available about the relation of leaf miners with fungi and other microorganisms developing on their hosts, horse chestnut and linden foliage. Interactions of the *Lithocolletis issikii* and *Tilia cordata* have been studied (Ermolaev & Motoshkova, 2008; Ermolaev & Zorin, 2011a). Two generations of the moth develop from the end of May to the beginning of August; adults emerge from the caterpillars developing within the mines up to 5th instar (Noreika, 1988). The second generation of *L. issikii* can be reduced by 22 % due to native parasitoids of the family *Eulophidae* (Yefremova & Mishchenko, 2008). The enemy release hypothesis postulates that the invasion success of exotic species is related to the scarcity of natural enemies in the introduction range compared with their native ranges (Colautti et al., 2004). The total mortality of one *Cameraria ohridella* generation can reach 14–99 %, depending on environmental conditions, plant host defence peculiarities, birds and arthropods activity, fungal diseases, etc. (Grabenweger et al., 2005; Girardoz et al., 2007). A hypothesis proposed by Cornell (1989) is that endophagous insects might be more susceptible to pathogens, because the humid internal leaf environment would be conducive to persistence of pathogens. None information is available about pathogenic to *L. issikii* fungi. Most investigations on fungal pathogens associated with *C. ohridella* have been performed in Czech Republic (Richter et al., 2007a, b, c; Kalmus et al., 2008a, b; Lerche et al., 2009). Positive insecticidal results of *Lecanicillium muscarium* (Petch) Zare & W. Gams against *C. ohridella* caterpillars (Kalmus et al., 2008a, b; Lerche et al., 2009) and pupae (Richter et al., 2007c) have been obtained. *Isaria fumosorosea* Wize (= *Paecilomyces fumosoroseus*), *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorokīn have also been shown being pathogenic to insect (Richter et al., 2007a, b; Lerche et al., 2009). Our results partly are comparable: some fungi species such as *Beauveria bassiana*, *Isaria fumosorosea* and *Metarhizium anisolpliae*

were isolated from *Cameraria ohridella* and *Lithocolletis issikii* individuals. The only difference is that we isolated fungi from caterpillars and adult moths, while the mentioned authors isolated fungi from the soil and hibernating pupae. Despite that, it can be suggested that general entomopathogens are pathogenic against invasive moths.

Fungi strains isolated from leaf miners are maintained at the Nature Research Centre, Institute of Botany, Biodeterioration Research Laboratory, (Vilnius, Lithuania). Investigations on the virulence of the isolated fungi strains to leaf miners at different development stages are continued.

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LECANICILLIUM APHANOCLADII, NAUJA LIETUVOS MIKOFLOROS RŪŠIS IR NAUJAS MEDŽIŲ LAPŲ MINAMUSIŲ PATOGENAS

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Santrauka

Populiarius parkuose ir miestuose kaštonus *Aesculus hippocastanum* L. ir smulkialapes liepas *Tilia cordata* Miller puola lapų minamusės – kandelės *Cameraria ohridella* Deschka and Dimic 1968 ir *Lithocolletis issikii* Kumata 1825 (= *Phyllonorycter issikii* Humber 1822). Minėtos rūšys priskirtos *Lepidoptera* būrio *Gracillariidae* šeimai. Abiejų minamusių rūšių vikšrų ir suaugėlių mikozes sukeliančius mikromicetus tyrėme Vilniuje, 2010–2012 metais. Statistinė rezultatų analizė parodė, kad neįprasto morfotipo mikromicetas yra aktyvus *C. ohridella* ir *L. issikii* vikšrų mikozių sukėlėjas (aptinkamumas – atitinkamai 30.36 % ir 31.92 %). Morfologinė charakteristika ir 18S rDNR segmento sekoskaita parodė, kad mikromicetas 99 % atitinka *Aphanocladium aranearum* (Petch) W. Gams 1971 rūšies kamienų Nr. AF455489 ir Nr. AF455405 RNR seką. Dabar ši mikromicetų rūšis pervardinta kitu, *L. aphanockladii* Zare & W. Gams, vardu. Ji Lietuvoje išskirta ir apibūdinta pirmą kartą. Pirmą kartą nustatytas ir šios rūšies patogeniškumas kaštono ir liepos lapų minamusėms.