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Divergence in culture morphology between two related species, *Dothistroma septosporum* and *D. pini*

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Dothistroma needle blight (DNB) is one of the most important pine needle diseases worldwide. The disease is caused by two fungal species, *Dothistroma septosporum* and *D. pini*, which are similar not only in terms of their ecology, causing similar symptoms in hosts of the genus *Pinus*, but also in terms of their morphological characteristics. The morphologies of 353 cultured *Dothistroma* isolates from 11 different pine species in Slovakia and their relation to *Dothistroma* species and/or fungus origin (host) were studied and evaluated by means of the Chi-square test. The cultures were classified into eight categories according to pigmentation. *Dothistroma pini* isolates were assigned to 4 of the 8 categories, and *D. septosporum* isolates were assigned to all 8 categories with varying frequencies. The Chi-square test revealed that the culture morphology categories of *D. pini* and *D. septosporum* differed significantly. Interactions between the evaluated factors (culture morphology, *Dothistroma* and host species) were analysed and showed significant differences.

Key words: Dothistroma needle blight, *Pinus* host, culture pigmentation.

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Adamčíková K., Ondrušková E., Pažitný J., Jánošíková Z. (2021): Rozdiel v morfológii kultúr dvoch príbuzných druhov, *Dothistroma septosporum* a *D. pini*. – Czech Mycol. 73(1): 109–119.

Dothistroma needle blight (DNB) je jedno z najvýznamnejších ochorení ihlíc borovíc vo svete. Ochorenie je spôsobené dvoma druhmi húb, *Dothistroma septosporum* a *D. pini*, ktoré sú podobné nielen z pohľadu ich ekológie, spôsobujú zhodné symptómy na hostiteľoch z rodu *Pinus*, ale aj z hľadiska ich morfologických charakteristík. Zhodnotili sme morfológiu 353 kultúr týchto dvoch druhov hubových patogénov izolovaných z 11 rôznych hostiteľov na Slovensku. Kultúry boli zatriedené do 8 kategórií podľa ich sfarbenia. Izoláty *D. pini* boli zatriedené do štyroch rôznych kategórií a *D. septosporum* do všetkých ôsmich kategórií s rôznou frekvenciou. Pomocou chí-kvadrát testu sme analyzovali vzájomné vzťahy medzi morfológiou kultúr, oboma druhmi rodu *Dothistroma*, ale aj hostiteľskými druhmi borovíc, z ktorých boli dané patogény odobraté. Test potvrdil, že morfológia kultúr *D. pini* a *D. septosporum* sa odlišuje signifikantne. Interakcie medzi hodnotenými faktormi (morfológia kultúr, druhy rodu *Dothistroma* a hostiteľské dreviny) vykazovali významné rozdiely.

INTRODUCTION

Dothistroma needle blight (DNB) is one of the most serious foliar diseases of *Pinus* spp. worldwide, continuously widening its range of new hosts and geographical regions (Watt et al. 2009).

Comparison of DNA sequence data from four regions (rDNA ITS, β-tubulin 1, β-tubulin 2 and the translation elongation factor) of the pathogen genome has clearly shown that this disease is caused by two distinct fungus species: *Dothistroma septosporum* (Dorog.) Morelet (teleomorph: *Mycosphaerella pini* Rostr.) and *Dothistroma pini* Hulbary (telemorph: unknown) (Barnes et al. 2004).

The fungi are similar in terms of their ecology and cause similar symptoms in their hosts in the genus *Pinus* (Barnes et al. 2004). *Dothistroma septosporum* is the pathogen responsible for the epidemics in the Southern and Northern Hemisphere and has a worldwide distribution, while *D. pini* was initially only known from the north central United States (Barnes et al. 2004). Originally, the geographical ranges of *D. pini* and *D. septosporum* did not appear to overlap (Barnes et al. 2004, 2008), but more recent records of *D. pini* across Europe (Drenkhan et al. 2016, Ondrušková et al. 2017, Ortíz de Urbina et al. 2017, Jánošíková-Hečková et al. 2018, Matsiakh et al. 2018, Mullett et al. 2018) suggest a wider distribution than initially thought.

The host range of *D. septosporum* is extremely wide, comprising 55 taxa (52 species + 3 varieties) in six genera (*Abies*, *Cedrus*, *Larix*, *Picea*, *Pinus* and *Pseudotsuga*; Drenkhan et al. 2016, Jánošíková-Hečková et al. 2018, Matsiakh et al. 2018). In contrast, the known host range of *D. pini* is much narrower, comprising only 20 taxa in two genera (*Pinus*, *Picea*; Drenkhan et al. 2016, Jánošíková-Hečková et al. 2018, Matsiakh et al. 2018, Mullett et al. 2018).

The presence of both *Dothistroma* species on the same tree and in some cases on the same needle has been confirmed (Barnes et al. 2011, Piškur et al. 2013, Queloz et al. 2014, Ondrušková et al. 2017).

The morphological characteristics of the two DNB fungi are very similar: the only credible difference is the width of the conidia (Barnes et al. 2004, 2008). On both infected needles and in culture, the conidia of *D. pini* are on average slightly wider than those of *D. septosporum* (Barnes et al. 2008). According to Barnes et al. (2008), species identification based on conidial morphology alone is ambiguous and may lead to misidentification. Due to the nearly identical morphologies of these two fungi and their ability to cause the same disease, DNA-based methods are the only unambiguous methods of distinguishing *D. septosporum* and *D. pini* (Barnes et al. 2011).

Barnes et al. (2004) also investigated differences in growth rate and culture morphology in an attempt to find morphological differences between these *Dothistroma* species. They observed substantial variability in the culture morphology

of *Dothistroma* species among isolates (32 in total) from different countries, isolates obtained within a single country and even subcultures of the same isolate inoculated onto replica plates.

The aim of the present study was to investigate the relation between culture morphology and the two *Dothistroma* species and to determine if cultures of both species are sufficiently different to permit preliminary species identification based on culture morphology. In addition, variation within samples originating from different hosts was evaluated.

MATERIAL AND METHODS

Dothistroma cultures. The *Dothistroma* culture collection of the Institute of Forest Ecology SAS (IFE SAS), Department of Plant Pathology and Mycology, Nitra, was used as the source of *Dothistroma* cultures for analyses. The cultures were isolated, according to the method described by Ondrušková et al. (2017), from 11 different pine species (*Pinus aristata*, *P. cembra*, *P. coulterii*, *P. densiflora*, *P. flexilis*, *P. jeffreyi*, *P. mugo*, *P. nigra*, *P. ponderosa*, *P.* ×*schwerinii* and *P. sylvestris*) collected from 39 localities in Slovakia (see Electronic supplement). Conidiomata from needles were excised under a binocular microscope using a scalpel, placed in sterile distilled water on a glass slide, gently crushed with a scalpel and transferred in water onto the surface of water agar (15 g/l) supplemented with an antibiotic (100 mg/l streptomycin sulphate) in Petri plates using a micropipette. Blocks of agar were cut from the plates in areas where germinating conidia occurred and then transferred to new water agar plates and subsequently sub-cultured. In total, 353 cultures were purified and analysed. Cultures were incubated on 3% malt extract agar (MEA; Carl Roth, Karlsruhe, Germany) at 20 °C in the dark until colonies formed. Before *Dothistroma* species were identified and confirmed with molecular methods, the morphology of each culture (form, elevation, margin, surface, opacity and pigmentation) was documented using a digital camera (Sony Alpha A200 with a Minolta AF 50-mm F2.8 macro lens).

Each culture (2–3 weeks old) was visually and macroscopically evaluated for its morphology, culture pigmentation being the main characteristic assessed. Eight categories were defined based on culture pigmentation (Tab. 1, Fig. 1).

All cultures were identified to species by means of molecular analysis. DNA was extracted using the E.Z.N.A. Fungal DNA Mini-Kit (Omega Bio-Tek Inc., Norcross, GA, USA), following the manufacturer's instructions. Conventional PCR conditions using species-specific primers described by Groenewald et al. (2007) were used for DNA amplification as described by Ondrušková et al. (2017) and Jánošíková-Hečková et al. (2018).

Statistical analyses. Culture morphology is a categorical variable, therefore the Chi-square test in the STATISTICA 10 software was used to analyse differences in the morphology of *Dothistroma* species, by comparing the frequencies of culture morphology categories. We tested the null hypothesis that the frequencies of the culture morphology categories do not differ between *Dothistroma* species. In order to evaluate the influence of host trees on the culture morphology categories of individual *Dothistroma* species, we analysed the frequencies of culture morphology categories only on those host species where both *Dothistroma* species were identified. Data with a low sample size ($N \leq 12$ per host species) were excluded from the analysis.

RESULTS

Culture morphology

All evaluated *Dothistroma* cultures had irregular forms, raised elevation, curled margins, and were opaque. The culture surfaces were dull and wrinkled. The pigmentation varied, encompassing shades of grey, black, brown, orange and pink (Fig. 1).

Of the 353 *Dothistroma* cultures, 35.1% (124 isolates) were assigned to culture morphology category 5, with orange-brown-grey pigmentation. Categories 4 (orange-grey culture with white margins), 1 (dark grey-black) and 6 (peach orange) were similarly abundant, representing 15.6% (55 isolates), 15.3% (54 isolates) and 11% (39 isolates), respectively. Less than 30 isolates were assigned to categories 2 (6.2%, grey-black culture with white margins), 3 (6.2%, white-grey), 7 (7.6%, pale pink) and 8 (2.8%, pale pink with dark margins) (Tab. 1).

Dothistroma species

Molecular tools were used to determine that 70 of the 353 studied *Dothistroma* isolates were *D. pini*, and 283 were *D. septosporum*.

All *D. pini* isolates were assigned to 4 of the 8 categories – specifically categories 1, 2, 3 and 5 (Fig. 2). More than 95% of *D. pini* isolates were assigned to categories 1 (65.7%, 46 isolates) and 2 (28.6%, 20 isolates). Only three isolates were assigned to category 3 and one isolate to category 5.

Dothistroma septosporum isolates were represented in each category with varying frequencies (Fig. 2). The most frequently identified category was 5 (43.5%), followed by categories 4, 6 and 7, with 19.4%, 13.8% and 9.5% of cultures, respectively. Some isolates of *D. septosporum* (10 isolates, 3.5%) were assigned to category 8. In categories 1 and 2, in which *D. pini* cultures were abundant, *D. septosporum* cultures represented 2.8% and 0.7% of cultures (Tab. 1).

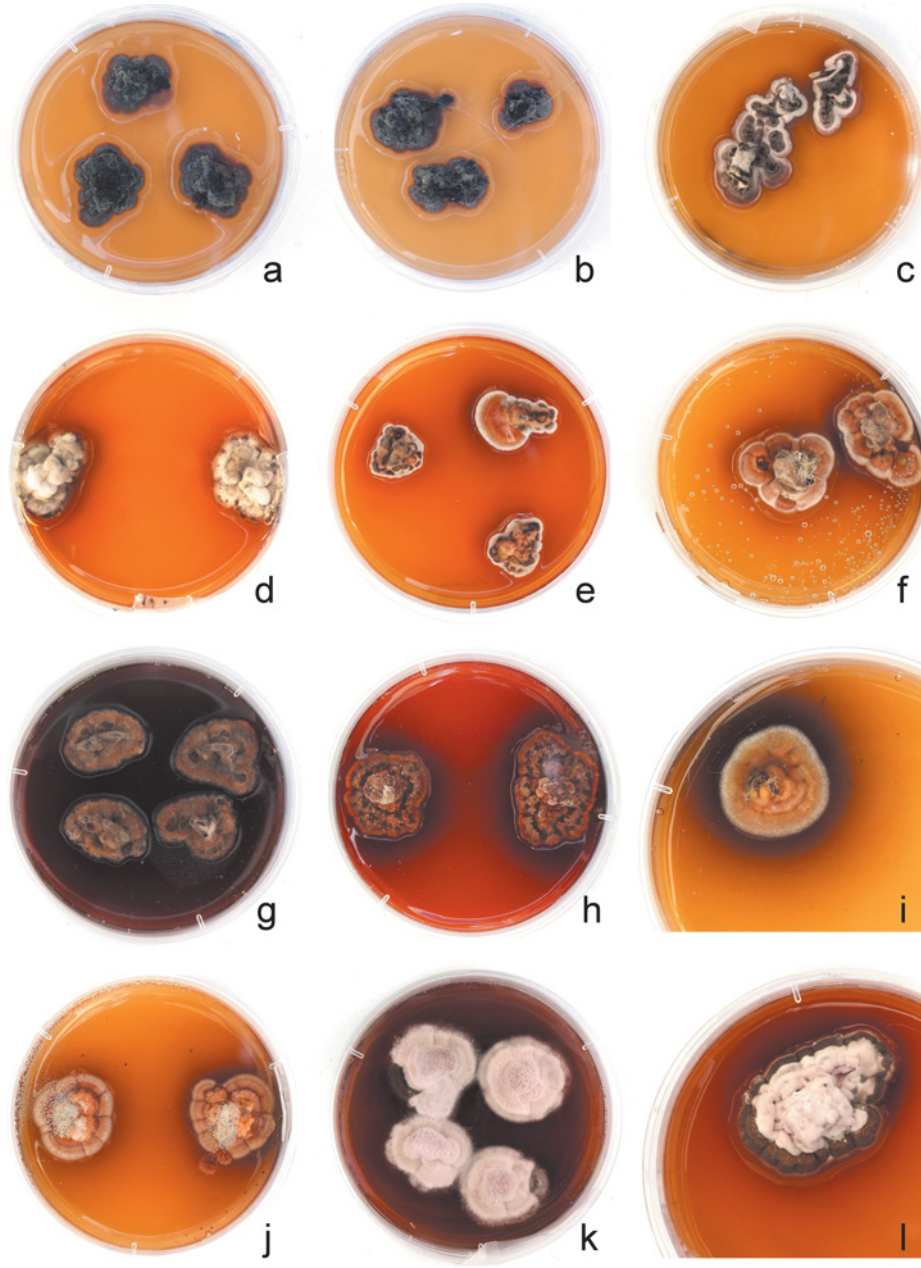

Fig. 1. Eight culture morphology categories of Slovak *Dothistroma* isolates from different pine host trees according to the pigmentation defined in this study: **a**, **b** – category 1; **c** – category 2; **d** – category 3; **e**, **f** – category 4; **g**, **h** – category 5; **i**, **j** – category 6; **k** – category 7; **l** – category 8. A description of each culture morphology category is given in Tab. 1; **a**–**c** = *D. pini* isolates, **d**–**l** = *D. septosporum* isolates. Photos: J. Pažitný.

Tab. 1. Assignment of *Dothistroma* spp. isolates to culture morphology categories according to their pigmentation.
Abbreviations: DP – *Dothistroma pini*, DS – *D. septosporum*.

Category	Description of culture morphology	Fig. 1*	Number of isolates		
			DP	DS	Total
1	Dark grey-black culture	a, b	46	8	54
2	Grey-black culture with white margins	c	20	2	22
3	White-grey culture	d	3	19	22
4	Orange-grey culture with white margins	e, f	0	55	55
5	Orange-brown-grey culture	g, h	1	123	124
6	Peach orange culture	i, j	0	39	39
7	Pale pink culture	k	0	27	27
8	Pale pink culture with dark margins	l	0	10	10

* visualisation of individual culture morphology categories in Fig. 1, letters correspond to labelling in Fig. 1

Relation of culture morphology to the studied factors

The Chi-square test confirmed that there were significant differences in the frequencies of culture morphology categories between *Dothistroma* species (Chi-square: 276.160, df = 7, $p < 0.00001$), therefore the null hypothesis was rejected.

Fig. 3 shows frequencies of culture morphology categories of both *Dothistroma* species on pine hosts with sample size $N > 4$. Among the studied cultures, both *Dothistroma* species were identified on 4 host species: *P. mugo*, *P. nigra*, *P. ponderosa* and *P. sylvestris*.

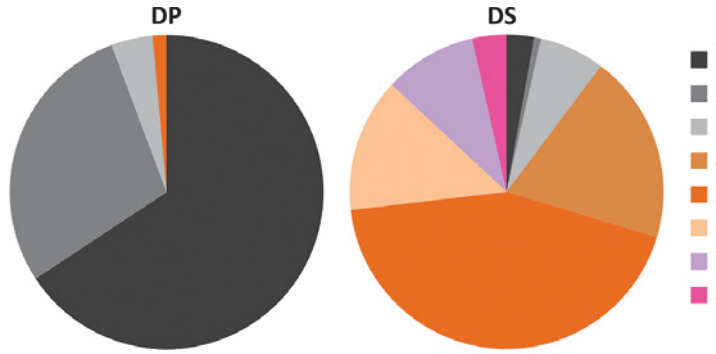

Fig. 2. Frequency of culture morphology categories of *Dothistroma* species. Each pie chart represents a single *Dothistroma* species, the sectors in the pie charts represent individual culture morphology categories according to Tab. 1.
Abbreviations: DP – *Dothistroma pini*, DS – *Dothistroma septosporum*.

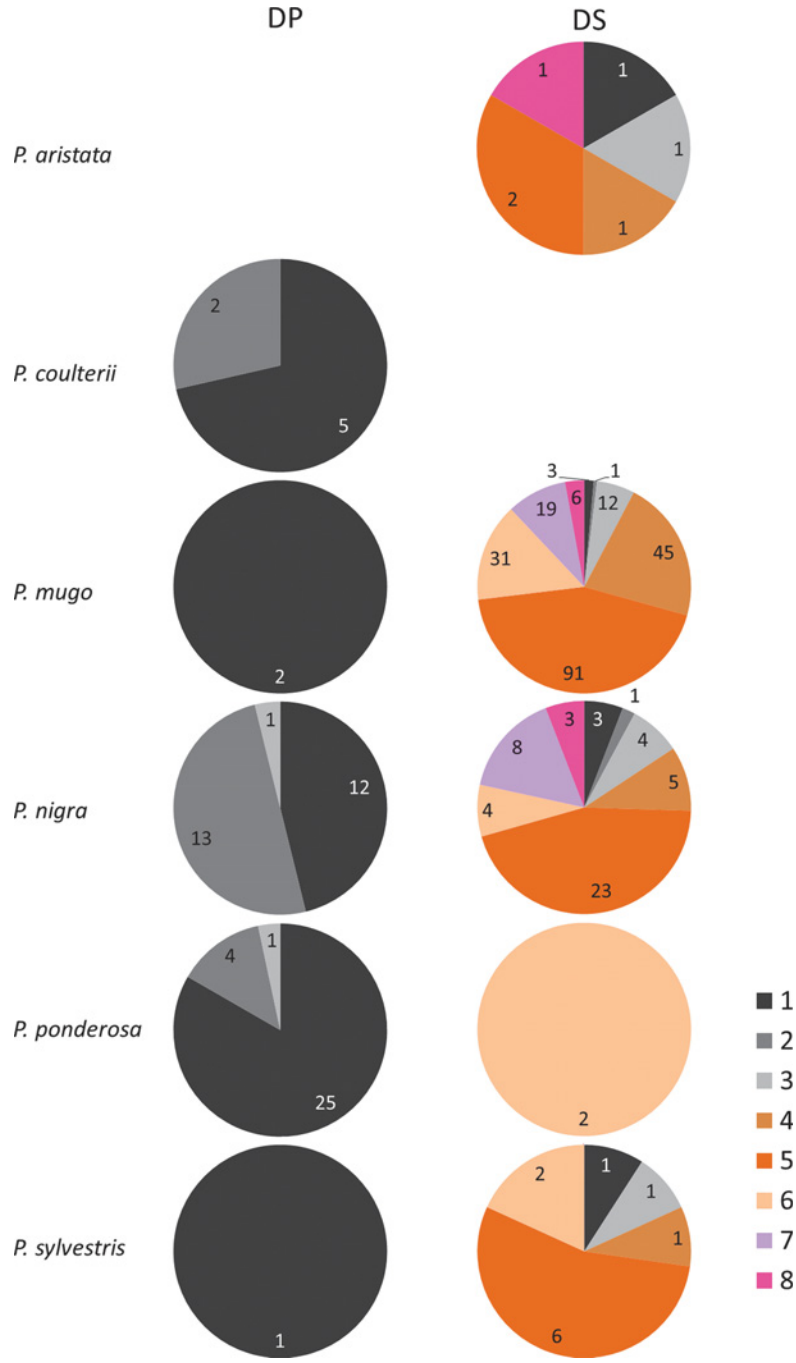

Fig. 3. Frequency of culture morphology categories of *Dothistroma* species arranged by host tree species. Each pie chart represents a single *Dothistroma* species isolated from the same *Pinus* species. The sectors in pie charts represent individual culture morphology categories according to Tab. 1. The numbers in the pie charts correspond to the number of cultures assigned in individual culture morphology categories.
Abbreviations: DP – *Dothistroma pini*, DS – *Dothistroma septosporum*.

Statistical analyses excluded one group with a low sample size from *P. sylvestris*. Chi-square test confirmed significant differences in culture morphology between *D. pini* and *D. septosporum* isolates from *P. mugo* (Chi-square: 82.7885, df = 7, $p < 0.000001$), *P. nigra* (Chi-square: 58.5397, df = 7, $p < 0.000001$) and also *P. ponderosa* (Chi-square: 32, df = 3, $p < 0.000001$). The differences in culture morphology between *D. septosporum* and *D. pini* were significant, independent of host species, which means that the differences were not influenced by host. *Dothistroma pini* isolated from *P. coulteri* and *P. ponderosa* was mostly assigned to culture morphology category 1, while *D. pini* from *P. nigra* was mostly assigned to culture morphology category 2. Larger variance was observed for *D. septosporum* isolates. The prevalence of culture morphology category 5 is evident in almost all isolates of *D. septosporum*, with the exception of *D. septosporum* from *P. ponderosa*, which belongs to culture morphology category 6 (Fig. 3).

DISCUSSION

Culture morphology describes the characteristics of an individual fungal culture growing on agar in a Petri dish and is useful for preliminary fungus identification. Several terms or markers are used to describe culture morphology, such as form, size, elevation, margin, surface, opacity and pigmentation of fungal colonies. In the *Dothistroma* culture collection analysed here, pigmentation differed across cultures. Therefore, categories for morphology evaluation were defined by culture pigmentation.

Statistical analyses confirmed differences in culture morphology between the two *Dothistroma* species. Thus, *D. septosporum* and *D. pini* isolates had different culture morphologies. These results indicate that culture morphology based on pigmentation is feasible to use as a preliminary identification marker for species determination. *Dothistroma pini* isolates were mostly represented by culture morphology category 1, with dark to medium grey-black pigmentation with or without white margins. The *D. septosporum* isolates dominating culture morphology category 5 were orange-brown-grey. The next three most abundant categories for *D. septosporum* isolates were nos. 4, 6 and 7, characterised by orange, pink and grey colours, respectively, whereas no *D. pini* culture was assigned to any of these categories. There was an absence of orange and pink colours among *D. pini* cultures, but these were the most common colours for *D. septosporum* cultures. Grey and black colonies dominated among *D. pini* cultures.

A comparison of the culture morphologies of these two species with a lower number of isolates was performed previously by Barnes et al. (2004), who found substantial variation in culture morphology among isolates from different countries. In the present study, variation was recorded too, but demonstrated a statistically significant correlation between culture morphology and *Dothistroma* species as well.

Dothistroma septosporum had higher variance in culture morphology, and its isolates were assigned to all 8 categories in different frequencies. Similarly, the highly diverse appearance of *D. septosporum* colonies, regardless of culture conditions, was reported from Poland (Kowalski et al. 2015). That study described reddish brown, grey or grey-beige colours; mucoid, smooth submerged mycelium or aerial mycelium morphologies, and a chamois, powdery or cottony texture. The colonies were sometimes uniform or narrowly zoned, with equal or "sinoatrial" indentations (Kowalski et al. 2015). Such variation has also been reported by other authors (Barnes et al. 2004) and may be attributable to the high genetic diversity found in *D. septosporum* isolates in Europe (Kraj et Kowalski 2013, Barnes et al. 2014). Most *D. septosporum* cultures in the present study had an orange-brown-grey colour (category 5), which was preferentially observed among cultures isolated from *Pinus mugo* and *P. nigra* but also in *P. sylvestris* cultures (Fig. 3). *Dothistroma septosporum* cultures isolated from *P. aristata* were the most variable (in total 6 isolates were analysed and assigned to five different categories: 1, 3, 4, 5 and 8).

On the other hand, Hirst et al. (1999) observed no obvious morphological differences (culture size, growth rate, shape, texture and colour) between *Mycosphaerella pini* isolates[1], including those from different geographic locations.

Isolates of *D. septosporum* show remarkable variations in concentrations of dothistromin production in cultures, affecting the pigmentation of the medium around the culture. In cultures, the most commonly reported pigmentations are red, red-brown and dark reddish brown, while in some isolates, pigment production was not observed at all (Barnes et al. 2004, Kowalski et al. 2015). These medium pigmentations were observed during the present study, but although pigment is not a consistent feature of particular fungal isolates (Kowalski et al. 2015) and its assessment may be interesting, it has not been assessed in the present study, but should be a subject of future research.

Overall, the data of this study showed a relationship between *Dothistroma* culture morphology and *Dothistroma* species, in contrast to previously reported findings (Barnes et al. 2004, Kowalski et al. 2015). This work supports the hypothesis that differences in culture morphology, especially in pigmentation, between different *Dothistroma* species may facilitate preliminary identification of *Dothistroma* pathogens. In addition, the relation of *Dothistroma* culture morphology with host species showed some differences. However, this could be affected by the different (unequal) number of cases for each host. Further related research, such as comparison of microscopic structures of isolates between different culture morphology categories, possible relation between culture morphology and

1 In their paper, the names *Dothistroma pini* and *Mycosphaerella pini* were used as synonyms, as it was published before the separation of the two species.

haplotypes, should be addressed in future investigation of these two *Dothistroma* species.

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