Dothistroma Needle Blight on High Altitude Pine Forests in Montenegro

Jelena Lazarević[1]*, Kateryna Davydenko[2] and Hanna Millberg[3]

[1] University of Montenegro Biotechnical Faculty, Mihaila Lalića 1, 81 000 Podgorica, Montenegro,

[2]Ukrainian Research Institute of Forestry & Forest Melioration, Pushkinska street, 86 Kharkov 61024, Ukraine

[3]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Almas allé 5, 756 51 Uppsala Sweden

** Corresponding author: ena.lazarevic@gmail.com, tel. +382 20 268 434*

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Abstract

Dothistroma needle blight (DNB) is one of the most serious and widespread needle diseases of pines. Its current distribution was investigated in high altitude native and planted pine forests in Montenegro. The suitability for the disease under different climatic conditions is discussed. Using molecular methods, polymerase chain reaction (PCR) with species specific primers, *Dothistroma septosporum* (Dorog) M. Morelet was detected from needles of *Pinus nigra* Arn., *Pinus nigra* 'Dalmatica' (Visiani) Franco, *Pinus sylvestris* L., *Pinus mugo* Turra, *Pinus heldreichii* H. Christ, *Pinus peuce* Griseb. and *Picea abies* Karst. in different parts of Montenegro, at altitudes between 800 and 2150 m. *Dothistroma pini* Hulbary was detected from *P. sylvestris*, *P. nigra* and *P. mugo* from restricted area in Northwestern part of Montenegro at altitudes between 800 and 1850 m. This is the first report of DNB on *P. mugo* and *P. abies* in Montenegro, and the first time that *D. septosporum* was detected from *P.nigra* 'Dalmatica'. Also, *D. septosporum* was detected through native forests of *P. heldreichii* and *P. peuce*. The climatic conditions and altitudes where DNB was detected were different from what has previously been reported for this region. From this study, we can conclude that DNB is present in high-altitude pine forests throughout Montenegro. However, the intensity of the disease is low due to prevailing environmental conditions, which are probably not favorable for DNB development.

Keywords: *Dothistroma septosporum*, *Dothistroma pini*, *Pinus heldreichii*, *Pinus peuce*, *Pinus mugo*, *Pinus nigra* 'Dalmatica', climate

Introduction

Dothistroma needle blight (DNB), also known as red band needle blight, is one of the most serious and widespread needle diseases of pines (Karadžić 1988, 2004; Barnes et al. 2008, Barnes et al. 2014). DNB is caused by two fungal species, *Dothistroma septosporum* (Dorog) M. Morelet (teleomorph *Mycosphaerella pini* Rostr.), which is considered a cosmopolitan species, and *Dothistroma pini* Hulbary (teleomorph unknown), which has more restricted distribution. Distinguishing the two species from each other based on the morphology of their conidia and microscopic characteristics is almost impossible (Barnes et al. 2004, Ioos et al. 2010).

Dothistroma septosporum is by far the most important invasive pathogen of non-native pine species (Ivory 1994, Karadžić 2004, Barnes et al. 2014). It has been observed on more than 42 pine taxa worldwide and also on other conifers from genera *Abies*, *Cedrus*, *Larix*, *Picea* and *Pseudotsuga* (Drenkhan et al. 2016). During the past 50 years, *D. septosporum* has been reported from an increasing number of countries, in Europe as well as in the other continents. The known range of disease has continuously been expanded (Jankovsky et al. 2009, Drenkhan et al. 2014, Drenkhan et al. 2016) and serious epidemics have emerged around the world (Barnes et al. 2014). Climate change has been proposed as the one of reasons for the increased severity of the disease (Woods et al. 2005, Woods et al. 2016).

Dothistroma pini has a restricted distribution. It has been detected only in the Northern Hemisphere, in North America and Europe. It was found in USA, Russia, Ukraine, Hungary, France, and recently in eight more European countries on different pine species (Barnes et al. 2004, Barnes et al. 2008, Drenkhan et al. 2016). However, distribution of *D. pini* could be much more extensive in Europe (Jankovsky et al. 2009), as the recent research from Balkan Peninsula shows (Lazarević et al. 2014, Drenkhan et al. 2016).

The disease infects the needles and can result in needle defoliation, reduction in stem diameter increment and height growth and, in severe cases the death of tree (Gibson et al. 1964, Karadžić 2004).

Dothistroma needle blight has been known in Montenegro since the late 1980ies (Karadžić 1990, 2004) while in Serbia, the disease has been known since 1955 (Krstić 1958). *M. pini* (teleomorph of *D. septosporum*) was observed there for the first time in 1979 (Karadžić 1986). The disease was, together with other needle pathogens of pine, intensively investigated in the region of Serbia and Montenegro from 1975 to 2005. *D. septosporum*, together with *Sphaeropsis sapinea* (Dyko) Sutton, was recognized then as the main causal agent of decline of *P. nigra* in forest plantations (Karadžić 1986, 1987, 1990, 2004, 2010, Milijašević 1994, 2002). The disease also appeared in forest nurseries and in natural forests. In 1988, there was a severe epidemic of DNB in *P. nigra* plantations near Pljevlja in northern Montenegro (Karadžić 1990, 2004). The disease was also observed on *Pinus sylvestris* L. in the northern part of the country (Karadžić 2010), on *Pinus halepensis* Mill. (Karadžić and Vujanović 1992, Karadžić 2004) and *Pinus pinea* L. (Karadžić 2010) near the Adriatic coast. DNB has only been identified based on morphological characteristics (Karadžić 1990, 2004, 2010, Karadžić and Vujanović 1992) and, hence, it was unknown if DNB in Montenegro was caused by *D. septosporum* or *D. pini*. In recent studies molecular methods, such as polymerase chain reaction (PCR) with species specific primers were used to identify *Dothistroma* spp. directly from the needles, and *D. septosporum* was detected from needles of *P. nigra*, *P. sylvestris*, *P. halepensis*, *Pinus heldreichii* H. Christ. and *Pinus peuce* Griseb., while *D. pini* was detected from *P. sylvestris* (Lazarević et al. 2014).

In the Balkan region, DNB has a one-year life cycle (Karadžić 2004). Conidia are dispersed by rain splash and their dissemination takes place from the beginning of April until the end of October, while the critical period for infection ranges from the beginning of May until the end of June. The length of the incubation period depends on climatic conditions, and in natural conditions it normally ranges from 4 to 6 months (Karadžić 2004). Under controlled conditions, the conidia germinate in temperatures between 5-30°C, with an optimum at 22°C (Karadžić 2004) and germination favors the high humidity (Karadžić 1988). The fungal mycelium develops in temperatures from 3 to 29°C (Karadžić 2004). According to Watt et al. (2009), the upper temperature threshold for growth is 31°C. With sufficient moisture and if the temperature is above 5°C, the fungus can infect needles throughout the year (Sinclair et. al. 1987). Currently, DNB is present in Montenegro, but the severity of the disease does not appear to be high (Lazarević et al. 2014.)

It has been suggested that, in the region of western Balkan, the climatic conditions at altitudes higher than 900 m will be unfavorable for DNB (Karadžić 1986, 1997, 2004), but considering the current world distribution of the disease (Watt et al. 2009. Drenkhan et al. 2016), it is likely though, that DNB could appear at these higher altitudes in the northern parts of Montenegro. Besides, DNB has already been recorded at few localities in high elevation forests of *P. heldreichii* and *P. peuce* in southern part of the country.

The aim of this study was to i) evaluate the current situation of DNB in high mountain forests, primarily in *P. heldreichii*, *P. peuce* and *P. mugo* forests; ii) using PCR with species specific primers, to determine which species of *Dothistroma* is present there iii) to evaluate possible risks posed by the disease on the base of prevailing environmental conditions in different high altitude pine forests.

Materials and methods

Field investigation and sampling

Different types of pine forests in Montenegro, spread in mountain areas, were examined for DNB. There were analyzed the needles of *P. nigra*, *P. sylvestris*, *P. mugo*, *P. heldreichii* and *P. peuce*, from altitudes between 800 - 2150 m above the sea level. *P. nigra* was examined at Mt. Durmitor (1 locality/3 samples), Mt. Prokletije (1 /3), Mt. Orjen (2/6) and Mt. Lovćen (1/5) in native forests or plantations as well as near the city of Pljevlja (2/14), where it was planted as protective or the part of urban greeneries. Forests of *P. sylvestris* L. were examined in Durmitor (3/20) and Prokletije (1/4) mountain massifs, and also near the cities of Pljevlja (2/11) and Rožaje (1/3;). *P. mugo* was sampled on few localities and altitudes throughout mountain massifs of Durmitor (NW MNE; 5/25) and Bjelasica (Central MNE; 1/4), as well as in planted forest at Mt. Lovćen (SW MNE; 1/8). In the case of *P. heldreichii*, it relates to Mt. Orjen (5/17), Mt. Prekornica (2/32), Mt. Žijevo (3/46) and Prokletije massif (2/16), and *P. peuce* at Prokletije massif at Mt. Bogićevica (2/11), Mt. Visitor (1/4) and Mt. Zeletin (1/4). There were also examinet the needles of *P. nigra* ssp. *dalmatica* (Visiani) Franco growing in forest plantation, near Adriatic Sea, on ca 500 m of altitude (1/8). Additionally, needles of *Picea abies* Karst., from Mt. Durmitor (1/4) were analyzed. In total, there were 44 localities, 248 needle samples, six different species and one subspecies of conifers investigated (Table 1).

The purpose of the field investigation was to examine whether the disease was present in high mountain pine forests, on the different pine species and in the different environmental conditions. The goal also was to confirm using molecular methods that suspected symptoms were caused by *D. septoposrum/D. pini*. Based on previous experience of the disease (our personal observations), it was not always evenly distributed in the forest stand. Furthermore, all the needles on a specific tree are not infected. Therefore, we did not apply a specific sampling schedule and the needles with suspected symptoms of DNB were collected. Such suspected symptoms were red bands, dying needle tips and/or subepidermal acervuli (Karadžić 2004). By chosen localities, the native range of distribution for *P. heldreichii*, *P. peuce* and *P. mugo* in Montenegro is well covered, and it enables an evidence of presence of disease, as well as evaluation of disease intensity on stand level. On the other hand, *P. nigra* and *P. sylvestris* were not being observed in bigger populations or in forest plantations, with pathogens detected from the single trees only.

Needle samples were stored in -20 °C until further molecular analysis.

Climates on researched localities

Montenegro is predominantly mountainous country in South Eastern Europe. It is located in the central part of moderately warm zone in Northern Hemisphere (41º52' and 43º32' latitude North and 18º26' and 19º22 longitude East). Owing to its geographical position, relief dissection, atmospheric circulation and vicinity of the Adriatic Sea, there are big differences within a small area between the climates in costal and high mountains regions. Warm temperature climate (C) is found in lower parts of the country, while the cold climate type (D) is found in higher inland mountain regions, above 1000-1200 m (according to Köppen climate classification, Burić et al. 2014). The warm climate (C) is represented by two climate types, Cs and Cf. Two subtypes: Csa and Csb can be distinguished within the Cs (Mediterranean) climate (Burić et al. 2014). For the purpose of this study, we were especially interested in climates in the mountains near the Adriatic Sea, previously defined as perhumid Mediterranean-submediterranean mountain climate (Stevanović and Stevanović 1995, Walter and Breckle 1985). According to Burić et al. (2014), it was positioned inside Cs/s''/b subtype. Further, humid warm temperate climate type (Cf) is found with subtype Cfb. The cold climate (D) is found in higher regions with Df climate types and two subtypes- Dfb mainly on altitudes up to 1500-1600 m a.s.l. and Dfc, on altitudes above 1600 m a.s.l. Climates for each locality are given in Table 1. In the text that follows, in combination with prevailing forests for the area, the main characteristics of dominant mountain climates in Motenegro are given in short.

The *P. heldreichii* forests grow in high mountain regions (1200 – 2000 m alt.) exposed to the Mediterranean climate (perhumid Mediterranean-submediterranean mountain climate) (Stevanović et al. 1994). The area is characterised by subalpine climate, cold winters and chilly summers, with average annual temperature of ca 3°C. In the area, winter minimum is below -30°C and summer maximum is above 35°C. The mean annual precipitation is about 2500 mm, but rainfalls during the vegetation season make only ca. 8% of total. During the vegetation season, the rainfall is often followed by 40-70 days-long periods of drought. The rainfall is maximal in late autumn and early winter, while minimal during the summer months, when the drought occurs (Hydrological and Meteorological Service of Montenegro). The *P. heldreichii* and *P. peuce* forests in the Mt. Prokletije (eastern Montenegro) grow under the similar, but colder environmental conditions. During the summer, the forests in this area also sustain the drought, but the temperature extremes are not as high.

At the mountains in northwestern and central Montenegro (Mt. Durmitor and Mt. Bjelasica) coniferous forests dominated with *P. abies*, *A. alba* and *P. sylvestris* (All. *Picetum excelsa montanum*) are present at altitudes of ca 1400-1500 m. Above the 1700-2300 m., high mountain shrubbery vegetation of *P. mugo* appears (Stevanović et al. 1994). The area is characterized by humid boreal climate (Df), cold, long lasting and snowy winters and short and chilly summers. At 1450 m average annual temperature is ca. 4.6°C, air temperature in the coldest month is below -30°C and the temperature of the warmest month goes to 14-15 °C. Mean annual precipitation is between 1250 and 2000 mm. The altitude has the primary influence on temperature (inversely) and also on the occurrence of wet periods throughout the year. The primary precipitation maximum is in the autumn and the secondary is in the spring. Yet the summer precipitation sum is smaller than the winter one (Hydrological and Meteorological Service of Montenegro, Burić et al. 2014).

DNA extraction and PCR

In order to only detect infections while not detecting spores on the needle surface, needle samples were washed prior to DNA extraction. Needles were first washed in 96% ethanol for 30 seconds, followed by 1.5 minutes in 2% sodium hypochlorite and then rinsed twice in 96% ethanol. Each needles' sample was placed in a screw cap tube with a screw and two nuts, freeze dried and then homogenized

Table 1. Occurrence of *Dothistroma* needle blight in Montenegrin mountains

No	Host	Location	Alt.	Latitude	Longi-tude	V	Primeval vegeta-tion	Cli-mate	SP	Ac	PCR	DNB
1	*P. heldreichii*	Ostrog monastry	900	42,669	19,030	UG	*Seslerio-Ostryetum-carpinetum*	Csa	II	-	+	*D. sept*
2	*P. heldreichii*	Mt. Orjen (1)	1050	42,5736	18,6383	F	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
3	*P. heldreichii*	Mt. Orjen (2)	1600	42,5222	18,5397	F	*Pinetum heldreichii*	Csbx*	II	-	+	*D. sept*
4	*P. heldreichii*	Mt. Orjen (3)	1800	42,5775	18,5858	F	*Pinetum heldreichii*	Csbx*	II	-	+	*D. sept*
5	*P. heldreichii*	Mt. Orjen (4)	1000	42.5072	18,5572	F	*Fagion moesiacae*	Csbx*	II	-	+	*D. sept*
6	*P. heldreichii*	Mt. Orjen (5)	1150	42,5436	18.5240	F	*Pinetum heldreichii*	Csbx*	II	-	+	DNB
7	*P. heldreichii*	Mt. Prekornica (1)	1250	42, 6241	19.1995	F	*Pinetum heldreichii*	Csbx*	III II I	- - -	+ + +	*D. sept*
8	*P. heldreichii*	Mt. Prekornica (2)	1700	42, 6589	19,1853	F	*Pinetum heldreichii*	Csbx*	III II	- -	+ +	*D. sept*
9	*P. heldreichii*	Mt. Žijevo (1)	1300	42,4908	19,5472	F	*Pinetum heldreichii*	Dfc	II I	- -	+ +	*D. sept*
10	*P. heldreichii*	Mt. Žijevo (2)	1950	42,5753	19,5592	F	*Pinetum heldreichii*	Dfc	II I	- -	+ +	*D. sept*
11	*P. heldreichii*	Mt. Žijevo (3)	1750	42,5986	19,5383	F	*Pinetum heldreichii*	Dfc	III II	- -	+ +	*D. sept*
12	*P. heldreichii*	Mt. Prokletije (1)	1300	42,4414	19,8081	F	*Pinetum heldreichii*	Dfwbx’’	III II	+	+	*D. sept*
13	*P. heldreichii*	Mt. Prokletije (4)	2150	42,6145	19,8802	F	*Pinetum heldreichii*	Dfc	II	-	+	*D. sept*
14	*P. mugo*	Mt. Durmitor (4)	1850	43,1719	19,0797	F	*Pinetum mughii*	Dfc	I II	- -	+ +	*D. sept* *D. pini*
15	*P. mugo*	Mt. Durmitor (5)	1750	43,1681	19,0592	F	*Pinetum mughii*	Dfc	II I	- -	+ +	*D. sept*
16	*P. mugo*	Mt. Durmitor (6)	1650	43,0953	19,0208	F	*Pinetum mughii*	Dfc	II	-	+	*D. sept*
17	*P. mugo*	Mt. Durmitor (3)	1600	43,1730	19,0839	F	*Picetum excelsa montanum*	Dfc	II	-	+	*D. sept*
18	*P. mugo*	Mt. Durmitor (1)	1450	43,1489	19,0942	F	*Picetum excelsa montanum*	Dfs’’bx	II	-	+	*D. pini*
19	*P. mugo*	Mt. Bjelasica	1850	42,8753	19,6985	F	*Pinetum mughii*	Dfc	II	-	+	DNB
20	*P. mugo*	Mt. Lovćen	1100	42,3813	18,8344	FP	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
21	*P. nigra*	Pljevlja (1)	820	43,3616	19,3616	UG	*Querco carinetum betuli*	Cfwbx	II I	- -	+ +	*D. sept*
22	*P. nigra*	Mt. Durmitor (1)	1450	43,1489	19,0900	F	*Picetum excelsa montanum*	Dfs’’bx	II	-	+	*D. sept*
23	*P. nigra*	Mt. Durmitor (1)	1450	43,1489	19,0900	F	*Picetum excelsa montanum*	Dfs’’bx	II	-	+	*D. pini*

Alt-Altitude (m above sea level); Latitude & Longitude – geographical coordinates in decimal degree (WGS84); V-type of vegetation (F-natural forest, P-forest plantation; UG-Urban greenery/shelterbelts; Primeval vegetation (according to Stevanović et al. 1995); Climate-Climate type according Kőppen system; SP-symptoms present: age of processed needles; Ac-Acervuli present; PCR- PCR applied; DNB – *Dothistroma* species detected by PCR (*Dothistroma septosporum- D. sept.* and *Dothistroma pini- D. pini)*

Table 1. Continued

No	Host	Location	Alt.	Latitude	Longi-tude	V	Primeval vegeta-tion	Climate	SP	Ac	PCR	DNB
24	*P. nigra*	Mt. Orjen (1)	1050	42,5736	18,6383	P	*Fagion moesiacae*	Csbx	II I	- -	+ +	*D. sept*
25	*P. nigra*	Mt. Orjen (6)	820	42,4942	18,5486	P	*Orno-Quercetum ilicis*	Csbx	II	-	+	DNB
26	*P. nigra*	Mt. Lovćen	1100	42,3814	18,8344	P	*Fagion moesiacae*	Csbx	II	-	+	*D. sept*
27	*P. nigra*	Pljevlja (2)	820	43,3530	19,3558	UG	*Querco carpinetum betuli*	Cfwbx	II	-	+	*D. pini*
28	*P. nigra*	Pljevlja (2)	820	43,3530	19,3558	UG	*Querco carinetum betuli*	Cfwbx	II	-	+	*D. sept*
29	*P. nigra*	Mt. Prokletije (1)	1300	42,4967	19,8169	P	*Fagion moesiacae*	Dfwbx''	II	-	+	*D. sept*
30	*P. nigra 'Dalmatica'*	Mt. Vrmac	520	42,4250	18,7490	P	*Orno-Quercetum ilicis pinetosum*	Csa	II I	- -	+ +	*D. sept*
31	*P. peuce*	Mt. Prokletije (2)	1850	42,5828	20,0311	F	*Pinetum peucis*	Dfc	III II I	- - -	+ + +	*D. sept*
32	*P. peuce*	Mt. Prokletije (3)	2000	42,5650	20,0332	F	*Pinetum peucis*	Dfc	II I	- -	+ +	*D. sept*
33	*P. peuce*	Mt. Prokletije (4)	2150	42,6146	19,8821	F	*Pinetum peucis*	Dfc	II I	- -	+ +	*D. sept*
34	*P. peuce*	Mt. Prokletije (4)	1800	42,6330	19,8359	F	*Pinetum peucis, Abietum, Fagetum*	Dfc	II	-	+	DNB
35	*P. sylvestris*	Pljevlja (1)	820	43,3617	19,3617	UG	*Querco-carpinetum betuli*	Cfwbx	II	- -	+ +	*D. sept*
36	*P. sylvestris*	Pljevlja (1)	820	43,3617	19,3617	UG	*Querco carinetum betuli*	Cfwbx	II	- -	+ +	*D. pini*
37	*P. sylvestris*	Mt. Durmitor (2)	1300	43,2250	19,1386	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*
38	*P. sylvestris*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*
39	*P. sylvestris*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. pini*
40	*P. sylvestris*	Mt. Durmitor (4)	1850	43,1697	19,0786	F	*Pinetum mughii*	Dfc	II I	- -	+	*D. sept*
41	*P. sylvestris*	Mt. Durmitor (4)	1850	43,1697	19,0786	F	*Pinetum mughii*	Dfc	II I	-	+	*D. pini*
42	*P. sylvestris*	Rožaje	1110	42,8244	20,1711	UG	*Picetum excelsae submontanum*	Dfwbx''	II	-	+	*D. sept*
43	*P. sylvestris*	Mt. Prokletije (1)	1300	42.4414	19.8080	P	*Fagion moesiacae*	Dfwbx''	II I	- -	+ +	*D. sept*
44	*Picea abies*	Mt. Durmitor (1)	1450	43,1455	19,0981	F	*Picetum excelsa montanum*	Dfs''bx	II	-	+	*D. sept*

Alt-Altitude (m above sea level); Latitude & Longitude – geographical coordinates in decimal degree (WGS84); V-type of vegetation (F-natural forest, P-forest plantation; UG-Urban greenery/shelterbelts; Primeval vegetation (according to Stevanović et al. 1995); Climate-Climate type according Kőppen system; SP-symptoms present: age of processed needles; Ac-Acervuli present; PCR- PCR applied; DNB – *Dothistroma* species detected by PCR (*Dothistroma septosporum- D. sept.* and *Dothistroma pini- D. pini)*

in a fast prep shaker (Precellys 24 Bertin Technologies). About 100-300 µl of the homogenized needle material per sample were used for DNA extraction where 1 ml of CTAB-buffer (3% cetyltrimethylammonium bromide, 2 mM EDTA, 150 mM Tris-HCl, 2.6 M NaCl, pH 8) was added to each sample, which was then incubated for 1 h at 65° C. After centrifugation, the supernatant was transferred to a new tube and mixed with an equal volume of chloroform and centrifuged. The supernatant was then transferred to a new tube, precipitated on ice with 1.5 volumes of isopropanol, washed with 70% ethanol and redissolved in 50 µl milliQ water. The extracted DNA was then purified using JetQuick DNA purification kit (Genomed GmbH), according to manufacturer's instructions. DNA concentrations were measured using NanoDrop (Thermo Scientific) and samples were diluted to 10-50 ng/ul.

Dothistroma septosporum specific primers Dstub2-F (5'-CGAACATGGACTGAGCAAAAC- 3') and Dstub2-R (5'-GCACGGCTCTTTCAAATGAC-3') and *Dothistroma pini* specific primers DPtef-F (5'-ATTTTTCGCTGCTC GTCACT-3') and Dptef-R (5'- CAATGTGAGATGT TCGTCGTC-3') were used for amplification with conventional PCR (Ioos et al. 2010). The thermal cycling was carried out in an Applied Biosystem Gene Amp PCR System 2700 thermal cycler. An initial denaturation step at 95° C for 3 min was followed by 35 amplification cycles of denaturation at 95° C for 30 s, annealing at 60 ° C for 30 s, and extension at 72° C for 60 s. The thermal cycling was ended by a final extension step at 72° C for 10 min (Ioos et al. 2010). To confirm the presence of *D. septopsporum* and *D. pini* in the samples, PCR products were visualized on a 1% agarose gel and compared to a positive control that was included in each PCR run. For *D. septosporum*, the PCR product was about 230 base pairs and for *D. pini* the PCR product was about 190 base pairs (Ioos et al. 2010).

Results

In all, DNB was confirmed at 44 spots throughout high mountain forests in Montenegro. In total, 6 different plant species and one subspecies were investigated (Table 1). Symptoms of DNB were found in 9 mountain massifs and on 28 localities ranging from 820-2150 m of altitude, as well as on one hill near Adriatic coast at 520 m of altitude. DNB was found on: *P. nigra*, *P. nigra* ssp. *dalmatica*, *P. sylvestris*, *P. mugo*, *P. heldreichii* and *P. peuce,* and also on *Picea abies. D. septosporum* was identified using species specific primers in needles from all investigated pine species and also from *P. abies*. *D. pini* was identified with species specific primers in restricted area on northwestern part of Montenegro on *P. sylvestris, P. mugo* and *P. nigra*.

The disease intensity on stand level is currently low, but variable between different pine species. The infection level could also vary between different climates and also local environmental conditions, even in different years.

The climatic conditions in mountain regions of Montenegro may be less favorable for DNB development. It seems that *P. heldreichii*, *P. sylvestris* and *P. mugo* sustain it well in their native habitats, while the *P. peuce* is might be a more susceptible and more endangered species regarding DNB.

Acervuli were only found in one site, on *P. heldreichii* needles in Mt. Prokletije, where *D. septosporum* was later confirmed by molecular analysis. On the other localities and pine species, acervuli and conidia were not recorded.

Discussion and Conclusions

The results demonstrate that DNB is widespread across the high altitude pine forest in Montenegro (Table 1). Range of *P. heldreichii*, *P. peuce* and *P. mugo* in Montenegro is well covered in here presented research, enabling evidence of presence of disease and evaluation of disease intensity on stand level. *P. nigra* and *P. sylvestris* were observed in urban greeneries or as single trees inside polidominant coniferous forests with pathogens being detected from just the single trees. Throughout the earlier researches in *P.nigra* plantations (Lazarević et al. 2014), no symptoms of DNB were observed at lower altitudes.

Dothistroma septosporum was reported for the first time on *P. heldreichii* and on *P. peuce* from native forest stands recently (Lazarević et al. 2014). During this study, the presence of DNB has been confirmed using species specific primers on *P. heldreichii* on many new localities, also at higher altitudes than before. It was detected in five different mountain massifs at the altitudes between 1300 - 1850 m (Table 1). Typically, the symptoms of DNB were not present on the current year needles, but they were visible on 2, 3 and 4 years old needles. Infected needles had red bands, dying needle tips and/or necrotic, pale green or orange-yellow tissue around or between the red bands. Needles were retained on the twig, and did not become fully necrotic. Fruit bodies were found on rare occasions (Lazarević et al. 2014). The disease intensity on stand level is currently low, but could vary depending on microclimatic conditions. It is sometimes also increased on skeletal soils or on tree line habitats that are nutrient poor, dry and exposed.

The *P. heldreichii* forests grow in high mountain regions exposed to the Mediterranean climate, which is probably limiting for DNB. It could be considered that average daily temperatures, air humidity and precipitation conditions are likely to be favorable for DNB infection and disease development during late spring and early in summer (May, June and the beginning of July). Later in summer, the climate conditions are probably less favorable for spore dispersal and infection. The dry period is coming, with only sporadic rain, and with the maximum day temperatures reaching 40 °C in open forest stands. Moreover, *P. heldreichii* usually grows in open and exposed positions on shallow limestone soils and, characteristically, the trees are sparsely distributed. The relative humidity inside this forest is thus assumed to be low, which probably further limits the development of fruit bodies, conidia dissemination and new infections (Gadgil 1977). From previous studies, *P. heldreichii* has been reported as slightly susceptible to DNB (Bendarova et al. 2006), which is consistent with our observations.

The occurrence of DNB was observed on *P. peuce* forests on eastern Montenegrin border. For the first time, symptomatic needles were collected from the plants from natural regeneration on ca 1850 m altitude (Lazarević et al. 2014). Later observations showed that symptoms were present across *P. peuce* forests in the region, at altitudes ca 1900-2100 m on natural regeneration and on older trees. On *P. peuce*, the symptoms of DNB were visible on the current year needles as sporadic red dots and bends. Two years old needles become fully necrotic at the end of vegetation period (September) and falling down from the twigs. Intensity of disease is abundant on natural regeneration and on the young trees, and could be even locally increased in vicinity of glacial lake (loc. Bogićevica). The intensity of disease is weaker at lower altitudes in mixed forests, where symptoms of disease are sporadic.

P. peuce forests appears to be more favoring for DNB than the *P. helderichii* sites, despite they grow under similar climate conditions, since the humidity inside the forest stand is higher. *P. peuce* grows predominantly on silicate soils, in pure or mixed forest stands. It forms dense forests, where the humidity is likely to be higher, especially near the forest floor and where temperature extremes are mitigated by the dense forest cover. There are also other factors that may influence the disease, such as host susceptibility. There no previous reports about *P. peuce* susceptibility to disease (Watt et al. 2009), except that Barnes et al. (2009) reported the untypical symptoms and usually brownish needles.

On *P. mugo,* DNB was detected at mountain massifs in Northern and central Montenegro, where it appears as native, on southern border of its range, and also near Adriatic coast, where a small planted *P. mugo* forest exists at 1100 m alt.

On native habitats, at altitudes between 1450 and 1850 m, the symptoms of DNB on *P. mugo* were sporadic on the current year needles, but more visible on 2, 3 and older needles. Infected needles had red bands and dying needle tips. Needles were retained on the twig and examples of defoliation (needle loss) were not observed. Needles did not become fully necrotic except rarely, and then mainly due to presence of other pathogens or insect attack. In many fragmented populations DNB symptoms were even more sporadic, but evident.

Further, *P. mugo* was sampled in Mt. Lovćen, near Adriatic coast. A small *P. mugo* forest is planted here in Mediterranean mountain climate and in micro depression. On this locality, *P. mugo* was heavily infested by *D. septosporum* and other needle pathogens (mainly by *Cyclaneusma* sp.). Needles from the last vegetation had intensively developed DNB symptoms. Needles from one year before were heavily infested and necrotic, in many cases fallen from twigs. On Mt. Lovćen, there is much more rainfalls and site is much warmer during the whole year, than across native *Pinetum mughii* on the North of the country (humid boreal climate). There are no data on the origin of *P. mugo,* planted in early 1960. Hence, host susceptibility could vary and influence a big difference in health status of *P. mugo* on two localities.

Dothistroma needle blight has been observed for the first time in Montenegro in *P. nigra* plantations in the city of Pljevlja (foothills of Mt. Durmitor) in 1979 (Karadžić 1986, 2004). This area with continental climate has been considered to be a typical habitat for DNB (Karadžić 1986, 2004, Watt et al. 2009, Lazarević et al. 2014). During the last decades, DNB has been present here and in wider region particularly in 5 to 25 year old *P. nigra* plantations, established at altitudes up to 900 m (Karadžić 1986, 1997, 2004). During recent research on 50 years old forest plantations of *P. nigra* in northern Montenegro, no symptoms of DNB were observed (Lazarević et al. 2014). Presence of *D. septosporum* was detected by species specific primers on needles of *P. nigra* and *P. sylvestris* from urban greeneries in Pljevlja (Lazarević et al. 2014). It was also detected on planted *P. nigra* on Mt Lovćen, as well as in 10 years old plantation in the eastern part of Montenegro (Table 1).

During here presented investigations, *D. septosporum* was detected on *P. sylvestris* and *P. nigra* on Mt. Durmitor at 1450 m alt. and also on needles of *P. abies*, at the same locality. *P. abies,* which dominated in this forest area, is new DNB host for Montenegro. Needles of *P. abies* from previous vegetation were symptomatic, with red bends, but intensity of infection was low.

Dothistroma septosporum was further detected on *P. sylvestris* on few more localities at altitudes up to 1850 m on Mt. Durmitor, then in eastern part of Montenegro on planted *P. sylvestris* at 1200 – 1300 m asl.

After the first finding of *D. pini* on *P. sylvestris* in city of Pljevlja (Lazarević et al. 2014), researches have continued with more samples from the same region. Consequently, *D. pini* was identified in needle samples collected at altitudes 1300 – 1850 m on *P. nigra*, *P. sylvestris* and also on *P. mugo* on the Mt. Durmitor. Much more DNA samples from different pines and regions in Montenegro were tested with *D. pini* specific primers, but without positive result. Hence, we can consider that *D. pini* is present in restricted area in northern part of Montenegro, on Mt. Durmitor and its surroundings, between 820 and 1850 m of altitude. Despite the presence of both *D. septosporum* and *D. pini*, the level of infection on *P. mugo* and *P. sylvestris* on Mt. Durmitor is sporadic and of low intensity. Both *D. septosporum* and *D. pini* were sometimes recorded on the same host tree.

Finally *D. septosporum* was detected on *P. nigra* ssp. *dalmatica* on the Adriatic coast (Vrmac peninsula in Boka bay, Mt. Vrmac), on altitude of ca 500 m. *P. nigra* ssp. *dalmatica* grows here in even-aged forest plantation of ca 50 years old trees, in Mediterranean climate (Csa). The plantation is declared as seed stand (Isajev et al. 2011). *D. septosporum* was detected from the shoots from the top of the crowns, and also from lowest branches, from current and previous year needles. Symptoms were more obvious on lower branches, but also mixed with other needle pathogens. According to our knowledge, this is the first record of *D. septosporum* on *P. nigra* ssp. *dalmatica*. In the nearness, clear DNB symptoms were observed on *Pinus pinaster* Aiton, what has not been confirmed by molecular methods until now.

According to here presented results, DNB is widely distributed in different mountain pine forests across Montenegro. The disease intensity is currently low. It has been suggested that, in the region of western Balkan, the climatic conditions at altitudes higher than 900m will be unfavorable for DNB (Karadžić 1986, 1997, 2004), and the disease was not recorded higher during the intense investigations in 1975-2005 (Karadžić 1986, 1997, 2004). The fact that the characteristic symptoms with red bands and fruit bodies are rare in these sites makes it difficult to determine if DNB is a new disease in these forests. It is likely that the disease may be overlooked or mistaken for other diseases. It seems that *P. heldreichii*, *P. sylvestris* and *P. mugo* sustain well on their native habitats, while the *P. peuce* might be more susceptible and more endangered species regarding DNB. Also, it can be expected that with the change of environmental conditions, sensitivity of pine species according DNB would be different, what was evident on more stressful habitats.

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