First Report of the Invasive Ash Dieback Pathogen *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *F. angustifolia* in Serbia

NENAD KEČA[1]*, THOMAS KIRISITS[2] AND AUDRIUS MENKIS[3]

[1] *University of Belgrade, Faculty of Forestry, Belgrade, Department of Forestry, 1, Kneza Višeslava street, 11030 Belgrade, Serbia*

[2] *Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria*

[3] *Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, Uppsala, Sweden*

**Corresponding author: nenad.keca@sfb.bg.ac.rs, tel. +381 63 580499*

Keča, N., Kirisits, T. and Menkis, A. 2017. First Report of the Invasive Ash Dieback Pathogen *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *F. angustifolia* in Serbia. *Baltic Forestry* 23(1): 56–59.

Abstract

In Serbia, unambiguous symptoms of ash dieback disease were for the first time observed in September 2015. Symptoms included dead shoots and occasionally small necrotic lesions in the bark accompanied by characteristic wood discoloration. Isolation of fungal cultures from symptomatic tissues of *F. excelsior* and *F. angustifolia* and their sequencing using the internal transcribed spacer of the rDNA (ITS rDNA) as a marker confirmed the presence of the ash dieback pathogen, *Hymenoscyphus fraxineus.*

Keywords: *Chalara fraxinea*, emerging forest disease, *Fraxinus* spp., new disease report.

Introduction

Ash dieback has been for the first time observed in the early 1990's in north-eastern Poland and has since then spread into large parts of Europe causing decline and mortality in natural stands and plantations of European ash, *Fraxinus excelsior* L. (Timmermann et al. 2011, McKinney et al. 2014). The causal agent of the disease is the ascomycete fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, which was first found and described in its asexual form as *Chalara fraxinea* T. Kowalski in Poland (Kowalski 2006, Baral and Bemmann 2014, Gross et al. 2014). To date, *H. fraxineus* has been reported from the majority of the distribution range of *F. excelsior* in Europe, excluding, among other areas, Serbia and other parts of the eastern Balkans. *H. fraxineus* is responsible for high mortality rates on *F. excelsior*, but can also cause severe disease on narrow-leaved ash, *F. angustifolia* Vahl (Kirisits et al. 2010, McKinney et al. 2014). In contrast, on flowering or manna ash (*F. ornus* L.) the fungus inflicts only inconspicuous and negligible leaf symptoms, does not progress into woody parts and is therefore not a serious pathogen (Kirisits and Schwanda 2015).

According to the national forest inventory, *F. angustifolia* and *F. excelsior* cover 1.1% and 0.6% of the forest area, respectively, and together constitute 2% of the total standing wood volume in Serbia (Banković et al. 2009). Despite being rare in forest stands, *Fraxinus* spp. are of particular value because they create unique ecological niches in floodplain forests and mixed beech forests and are important as ornamental trees in city parks and greeneries.

In Serbia, the occurrence of *H. fraxineus* has been monitored since ash dieback was reported in neighboring Croatia (Barić and Diminić 2010) and Bosnia and Herzegovina (Treštić and Mujezinović 2013, T. Treštić, pers. comm., the earlier record in this country from 2009 was reported later, in 2014, by Stanivuković et al. 2014). Although symptoms resembling the disease have been observed from 2010 to 2014, *H. fraxineus* was not confirmed, and the observed damages were attributed to other causal agents, such as drought, *Phytophthora* spp. and attacks by the ash weevil (*Stereonychus fraxini* De Geer 1775).

Material and Methods

Sampling was carried out in forests at three localities in September 2015. At each of the localities between 3 and 8 stands or groups of trees were surveyed for the presence of ash dieback symptoms. The focus of the survey was on younger trees (1-3 years old) for the presence of necrotic lesions in the bark, but older trees were also studied for the presence of defoliation and epicormic shoots in the crown. Two localities were in south-western Serbia along the border with Bosnia and Herzegovina, at Tara (N43°56'56'', E19°24'01'', 1000 m a.s.l.) and Debelo Brdo (N44°04'01'', E19°38'16'', 800 m a.s.l.), and one locality was in north-western Serbia at the border with Croatia, at Molovin (N45°11'17'', E19°19'35'', 200 m a.s.l.). The distance between the localities Tara and Debelo Brdo was ca. 40 km, and the distance from these to the locality Molovin was ca. 120 km.

Collected samples included shoots showing symptoms of dieback and necrotic lesions. Disease symptoms on leaves or petioles were not observed during the surveys. In Tara and Debelo Brdo, *F. excelsior* was sampled, while in Molovin shoots were collected from *F. angustifolia*. Fungal isolation followed largely the procedures described by Kirisits et al. (2012). Symptomatic shoots were cut into 5-8 cm long segments containing the zone between necrotic and healthy tissues. Shoot segments were surface sterilized in 96% ethanol for one min, followed by three min sterilization in 4% NaOCl and then for 30 s in 96% ethanol. Subsequently, the outer bark was carefully peeled off and shoot pieces containing phloem and wood were cut off and placed onto 2% malt extract agar (MEA) amended with 100 mg/l of streptomycin sulphate. Petri dishes with shoot pieces were incubated at temperatures between 4 and 10°C in the dark and checked regularly during a period of 6 weeks.

Outgrowing fungal cultures were grouped and preliminary identified based on colony morphology and micromorphological characteristics using microscopy. Molecular identification of fungal cultures was carried out as described by Menkis and Vasaitis (2011). The internal transcribed spacer of the fungal ribosomal DNA (ITS rDNA) was sequenced for representative cultures using primers ITS1F and ITS4 (White et al. 1990). Isolation of DNA was done using the CTAB method, and ITS rDNA was amplified by PCR (Menkis and Vasaitis 2011). Sanger sequencing in both directions was performed by Macrogen Inc. (Seoul, South Korea). Raw sequence data were analyzed using the SeqMan Pro version 12.0 software from the DNASTAR package (DNASTAR, Madison, WI, USA). GenBank database and BLASTn analysis were used to determine the identity of the sequences (Altschul et al. 1997).

Results

The number of trees surveyed during this study was around 1000. At the three study sites, no clearly discernable symptoms of ash dieback were present in the crown of older trees, on which crown thinning and dieback of annual shoots were very similar to the drought driven decline of various other tree species. One- to three-year-old saplings also showed most often only dieback of leading shoots or of smaller lateral shoots, while small lesions in the bark accompanied by wood discoloration, which are typical for ash dieback caused by *H. fraxineus*, occurred sparsely.

During the field survey at three locations, 1-3 symptomatic samples from 45 trees were collected and used for fungal isolation. In total, 10 isolates resembling the ash dieback pathogen were cultured from sampled shoots of *F. excelsior* (7 isolates, 3 from Tara and 4 from Debelo Brdo) and *F. angustifolia* (3 isolates from Molovin). Based on morphological examination, all of them were preliminary identified as *H. fraxineus*. Phialoconidia in short chains were 3.3-4.0 x 2.0 µm in size, while phialides were 15-25 x 3-5 µm in size.

Sequencing of ITS rDNA from four cultures, three from *F. excelsior* and one from *F. angustifolia* resulted in high-quality sequences between 879 and 880 bp in length. Sequence alignment and intraspecific comparisons showed that all of them were identical and therefore belonged to the same fungal species. BLASTn analysis revealed that the closest match of the sequences was to different sequences of *H. fraxineus*, with sequence similarity between 99% and 100% (HM193468, Husson et al. 2011) between them. Sequence similarity to the related and native European species *Hymenoscyphus albidus* (Roberge ex Gillet) W. Phillips was 98% (HM193455, Husson et al. 2011) or lower. The ITS rDNA sequence of one representative *H. fraxineus* isolate from Serbia was deposited at GenBank (accession no. KX255648, isolate NK2-DB 22/3-215 from *F. excelsior*).

Discussion

The results demonstrated that the ash dieback pathogen *H. fraxineus* has spread to Serbia after about 25 years since its first European observation (based on symptoms) in north-eastern Poland. *H. fraxineus* is a wind-dispersed pathogen that spreads with ascospores, which are produced in apothecia on ash leaf rachises and petioles in the leaf litter (Timmermann et al. 2011, Gross et al. 2014). Movement of infected nursery seedlings also played a role in the spread of the fungus across Europe (Timmermann et al. 2011, Kirisits et al. 2012), but this was likely not important in Croatia, Bosnia and Herzegovina, and Serbia, where ash is rarely planted in forests.

On the Balkans and Dinaric Alps, the first report of *H. fraxineus* was from Slovenia in 2007 (Hauptman et al. 2012) and only two years later, in 2009, from Croatia (Zalesina-Gorski kotar, Barić et al. 2012, Županić et al. 2012), from an area which is approximately 400 km from the border with Serbia. Also in 2009, the disease was discovered in the western part of Bosnia and Herzegovina, at Jelašinovci (Stanivuković et al. 2014), and about four years later, in 2013, it was found in a nursery in the central part of Bosnia and Herzegovina (Busovača, Treštić and Mujezinović 2013, T. Treštić, pers. comm.). In 2014, symptoms of ash dieback were observed in eastern Bosnia and Herzegovina, at Vlasenica (N. Keča, unpubl. data), which is located just about 20 km from the border with Serbia. Based on these reports and the results of the present study, it can be estimated that the disease front was moving forward at an average rate of approximately 60 km per year, which is within the range (30-75 km) reported for various parts of Europe, as reviewed by Gross et al. (2014). Natural spread of *H. fraxineus* from Hungary into Serbia is unlikely because there are few ash forests in the northern province of Vojvodina, where the climate is also warm and dry, and thus less suitable for the development of the ash dieback pathogen. According to the most recent reports from Romania (Chira et al. 2016), the pathogen is present in the Carpathian Mountains about 60 km from the Serbian border, but until the end of 2015 symptoms of ash dieback have not been observed on the Serbian side (N. Keča, unpubl. data). Disease incidence at the three localities in Serbia where *H. fraxineus* was confirmed was low, which suggests that *H. fraxineus* was discovered in an early phase of the epidemic.

In the present study, all findings of *H. fraxineus* on *F. excelsior* and *F. angustifolia* were from trees growing in natural forests. Field surveys and sampling in September 2015 showed that the most frequently observed symptom was dead annual shoots, while small necrotic lesions in the bark were encountered only occasionally. Symptoms of decline were observed only on young, 1-3 m high trees in the understory. Fungal culturing and subsequent morphological and molecular identification of isolates confirmed the presence of *H. fraxineus.* Symptoms on *F. angustifolia* were not typical, but the ash dieback pathogen was isolated from totally necrotic 1- to 2-year-old and up to 1 m high understory seedlings which did not show distinct shoot lesions. In 2012 and 2013, such symptoms were connected with extreme drought in the area, but no isolations were attempted. It can therefore not be unambiguously ruled out that trees may already at that time have been damaged by *H. fraxineus*.

It is possible that abundant precipitation in 2014 has stimulated the production of abundant inoculum of *H. fraxineus*, facilitating the pathogen's further spread from Bosnia and Herzegovina through the Dinaric Alps, towards eastern parts of the Balkan Peninsula. Spread towards northern Serbia, i.e. to the region bordering Croatia, was probably along the Sava River where the disease was confirmed in low intensity in 2011 on *F. angustifolia* (Županić et al. 2012).

Further studies are needed in order to evaluate the present distribution and future spread of the ash dieback pathogen into other regions of Serbia and the eastern Balkans as well as its virulence to and impact on different *Fraxinus* species in this part of Europe. It is well documented that the severity of the disease is higher on sites with high relative air humidity, lower temperatures and mesophilic environmental conditions (Keßler et al. 2012, Hauptman et al. 2013, Gross et al. 2014). The coming years will show if warm and dry continental climate will be a limitation for the spread and development of *H. fraxineus* in Serbia and other parts of the eastern Balkans.

Acknowledgements

This work has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 635646, POnTE (Pest Organisms Threatening Europe). We also acknowledge the COST Action FP1103 FRAXBACK, "Fraxinus dieback in Europe: elaborating guidelines and strategies for sustainable management" (2012-2016).

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

Banković, S., Medarević, M., Pantić, D., Petrović, N., Šljukić, B. and Obradović, S. 2009. The growing stock of the Republic of Serbia - State and problems. *Bulletin of the Faculty of Forestry* 100: 7-30, (in Serbian with English summary).

Baral, H.-O. and Bemmann, M. 2014. *Hymenoscyphus fraxineus* vs. *Hymenoscyphus albidus* – A comparative light microscopic study on the causal agent of European ash

dieback and related foliicolous, stroma-forming species. *Mycology* 5: 228-90.

Barić, L. and Diminić, D. 2010. First report of the pathogenic fungus *Chalara fraxinea* Kowalski on common ash (*Fraxinus excelsior* L.) in Gorski Kotar. Glasilo Biljne zaštite 10: 33-34, (in Croatian with English summary).

Chira, D., Chira, F., Taut, I., Popovici, O., Blada, I., Donita, N., Bandiu, C., Gancz, V., Biris, I.A.,Popescu, F., Tanasie, S. and Dinu C. 2016. Evolution of ash dieback in Romania. In: R. Vasaitis, R. Enderle (Editors), Dieback of European Ash (Fraxinus spp.): Consequences and Guidelines for Sustainable Management. Uppsala, Swedish University of Agricultural Sciences, p. 185-194.

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V. and Sieber, T.N. 2014. *Hymenoscyphus pseudoalbidus*, the causal agent of European ash dieback. *Molecular Plant Pathology* 15: 5-21.

Hauptman, T., Ogris, N. and Jurc, D. 2012. Ash dieback in Slovenia. *Forstschutz Aktuell* 55: 62-63.

Hauptman, T., Piškur, B., de Groot, M., Ogris, N., Ferlan, M. and Jurc, D. 2013. Temperature effect on *Chalara fraxinea*: heat treatment of saplings as a possible disease control method. *Forest Pathology* 43: 360-370.

Husson, C., Scala, B., Caël, O., Frey, P., Feau, N., Ioos, R. and Marçais B. 2011. *Chalara fraxinea* is an invasive pathogen in France. *European Journal of Plant Pathology* 130: 311-324.

Keßler, M., Cech, T.L., Brandstetter, M. and Kirisits, T. 2012. Dieback of ash (*Fraxinus excelsior* and *Fraxinus angustifolia*) in Eastern Austria: disease development on monitoring plots from 2007 to 2010. *Journal of Agricultural Extension and Rural Development* 4: 223-226.

Kirisits, T. and Schwanda, K. 2015. First definite report of natural infection of *Fraxinus ornus* by *Hymenoscyphus fraxineus*. *Forest Pathology* 45: 430-432.

Kirisits, T., Matlakova, M., Mottinger-Kroupa, S., Halmschlager, E. and Lakatos, F. 2010. *Chalara fraxinea* associated with dieback of narrow-leafed ash (*Fraxinus angustifolia*). *Plant Pathology* 59: 411 (*New Disease Reports* [2009], 19, 43).

Kirisits, T., Kritsch, P., Kräutler, K., Matlakova, M. and Halmschlager, E. 2012. Ash dieback associated with *Hymenoscyphus pseudoalbidus* in forest nurseries in Austria. *Journal of Agricultural Extension and Rural Development* 4: 230-235.

Kowalski, T. 2006. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathology* 36: 264-270.

McKinney, L.V., Nielsen, L.R., Collinge, D.B., Thomsen, I.M., Hansen, J.K. and Kjær, E.D. 2014. The ash dieback crisis: genetic variation in resistance can prove a long-term solution. *Plant Pathology* 63: 485-499.

Menkis, A. and Vasaitis, R. 2011. Fungi in roots of nursery grown *Pinus sylvestris*: ectomycorrhizal colonization, genetic diversity and spatial distribution. *Microbial Ecology* 61: 52-63.

Stanivuković, Z., Karadžić, D. and Milenković, I. 2014. The first record of the parasitic fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya on common ash in Bosnia and Herzegovina. *Forestry - Belgrade* 3-4: 19-33, (in Serbian with English summary).

Timmermann, V., Børja, I., Hietala, A.M., Kirisits, T. and Solheim H. 2011. Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *EPPO Bulletin* 40: 14-20.

Treštić, T. and Mujezinović, O. 2013. Ash dieback in Bosnia and Herzegovina?! In: Program and abstracts, COST Action FP1103 FRAXBACK, 4th Management Committee Meeting and Workshop "Frontiers in ash dieback research", Malmö, Sweden, September 4-6, 2013: 30-31.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (Editors): PCR protocols: A guide to methods and applications. San Diego, California Academic Press, p. 315-322.

Županić, M., Barić, L., Pernek, M. and Diminić, D. 2012: Distribution of *Chalara fraxinea* in Croatia. *Radovi* (Hrvatski šumarski institut) 44: 125-134, (in Croatian with English summary)