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Natural infection of *Fraxinus angustifolia* by *Hymenoscyphus fraxineus* in Slovakia

MIRIAM KÁDASI-HORÁKOVÁ[1]*, KATARÍNA ADAMČÍKOVÁ[1], KATARÍNA PASTIRČÁKOVÁ[1], VALÉRIA LONGAUEROVÁ[2] AND MIRIAM MAĽOVÁ[2]

[1] *Branch for Woody Plant Biology, Institute of Forest Ecology, Slovak Academy of Sciences, Akademicka 2, SK-94901 Nitra, Slovakia*

[2] *Department of Forest Protection and Game Management, National Forest Centre - Forest Research Institute, T. G. Masaryka 22, SK-96092 Zvolen, Slovakia*

**Corresponding author; e-mail: kadasi@savzv.sk, tel. +42 137 6943339*

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Abstract

The fungus *Hymenoscyphus fraxineus* is responsible for dieback of common ash (*Fraxinus excelsior*) and in some parts of Europe also of narrow-leaved ash (*F. angustifolia*). The first symptoms of ash dieback have been recorded on *F. excelsior* in Slovakia since 2004. This study reports about the first natural occurrence of *H. fraxineus* on *F. angustifolia* in Slovakia. The field investigation was carried out in 2014. The segments of diseased shoots and last year's petioles were collected in clonal seed orchard situated in southwest part of the country. The fungus was isolated from infected host tissue and identified using molecular techniques (DNA extraction from pure cultures and apothecia, conventional PCR).

Key words: *Fraxinus angustifolia*, *Hymenoscyphus fraxineus*, molecular techniques, ash dieback.

Introduction

The fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya is the sexual stage of *Chalara fraxinea* T. Kowalski. *H. fraxineus*, the causal agent of ash dieback, attack mainly common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl). The susceptible hosts have also been recorded black (*F. nigra* Marshall), green (*F. pennsylvanica* Marshall), white (*F. americana* L.), Manchurian (*F. mandshurica* Rupr.) and manna (*F. ornus* L.) ash trees (Drenkhan and Hanso 2010, Kirisits and Schwanda 2015). Symptoms of ash dieback are variously coloured (brownish to orange) bark necroses and cankers without exudates on stems and branches, leading to dieback of trees (Kowalski 2006, Schumacher et al. 2010). Ash dieback was identified for the first time in Poland (Kowalski 2006).

Ash dieback caused by *H. fraxineus* has been reported in many countries in Europe, for example Austria (Cech 2006, Halmschlager and Kirisits 2008), Czech Republic (Jankovský and Holdenrieder 2009), Hungary (Szabo 2009), Latvia, Estonia, Romania and Russia (Kirisits et al. 2009) and others. Since 2004, based on the presence of the symptoms, ash dieback has been recorded also in Slovakia (Kunca 2006). The first report of the *H. fraxineus* occurrence in Slovakia was described only on *F. excelsior* using molecular techniques (Adamčíková et al. 2015).

Development of molecular tools designed in the Internal Transcribed Spacer sequences (ITS) of nuclear ribosomal DNA cistrons for detecting *Chalara fraxinea* was recently performed (Chandelier et al. 2010, Ioos et al. 2009, Johansson et al. 2010). Another molecular technique is identification to species level by conventional PCR according to Johansson et al. (2010), where the targeted gene is the 18S gene and the ITS-2 region of rDNA operon.

The aim of this work is to identify *H. fraxineus* on narrow-leaved ash (*F. angustifolia*) for the first time in Slovakia by molecular tools.

Material and Methods

Sampling

In March 2014, symptomatic twigs of *F. angustifolia* (stem sections 10-20 cm long containing the lesion) were collected. The total number of collected twig or stem samples was 14. Also last year's petioles from leaf litter with apothecia were collected during July 2014. The investigation was carried out in clonal seed orchard of ash species in Trstice, Galanta District, Slovakia (48°24´32"N, 17°47´39"E). The total number of collected apothecia was 8. The samples were placed in paper bags and 1.5-ml Eppendorf sterile tubes and stored at 4 °C before further transferred to laboratory for analysis. Voucher specimens are deposited in the Plant Pathology Herbarium NR (Institute of Forest Ecology of Slovak Academy of Sciences, Nitra, Slovakia).

Sample preparation

Isolation of the pathogen was done from cankers. The outer bark was carefully removed and small sections (0.5 × 0.5 cm) from the canker margin were cut out. After surface sterilization (1 min ethanol 96%, 1 min NaClO 4%, 30 s ethanol 96%), the samples were washed in distilled water, dried on sterile paper and placed in Petri dishes on the surface of 2 % ash leaf malt extract agar (AMEA; 20 g/l malt extract Roth, 15 g/l agar, amended with 50 g fresh *F. excelsior* leaflets removed after autoclaving, Kirisits et al. 2013). AMEA was supplemented with antibiotic (100 mg/l streptomycin sulphate) added before effusing the media in Petri dishes. The Petri dishes were incubated at 20 °C in the dark and subcultures were made on AMEA without antibiotics. The cultures were used for DNA extraction.

DNA extraction

The samples (apothecia, mycelium) were homogenized in the presence of liquid nitrogen using a mortar and pestle. Total DNA of each sample was extracted from 100 mg of homogenized apothecia and mycelium using the EZ-10 Column Plant Genomic DNA Purification Kit (Bio Basic Canada Inc.) following the manufacturer's instructions.

PCR amplification and gel analysis

For analysis of DNA directly from apothecia and mycelium, a conventional PCR approach was used. PCR amplification was performed using the fungal-universal primers ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993). DNA sequencing of ITS rDNA operon was done according to Husson et al. (2011). The amplification was carried out in a 20 µl reaction mix containing 2 ng of genomic DNA. The PCR was performed using T1 Thermocycler 96. PCRs were initialized with a denaturation step at 95 °C for 14 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s and extension at 72 °C for 80 s. The thermal cycling ended with a final extension step at 72 °C for 10 min. The PCR products were size separated by gel electrophoresis on 1% agarose gel, stained with an ethidium bromide and visualized under UV light. Prior to sequencing, target fragments were directly purified using a PCR Purification Kit (Macherey-Nagel, Germany). Each amplified product was diluted with 30 µL H_2O and visualized with an ABI3130xl sequencer from Applied Biosystems. The retrieved sequences were compared by BLAST (Basic Local Alignment Search Tool, available at http://www.ncbi.nlm.nih.gov/BLAST/) against DNA sequences deposited in GenBank for *H. fraxineus* (accession number HM193468).

For identification to species level was performed a conventional PCR using specific primers 18S gene and the ITS-2 region of rDNA operon and the expected size of amplicon was 456 bp (Johansson et al. 2010). The amplification of DNA was performed in 20 µL reaction volumes using approximately 2 ng of template DNA. After an initial denaturation step for 15 min at 95 °C, 35 cycles were performed each comprising a denaturation step at 95 °C for 20 s, an annealing step at 62 °C for 30 s and an extension step at 72 °C for 1 min followed by a final extension step for 8 min at 72 °C. Molecular-grade water was used as negative amplification control during preparation of reaction mix.

Results and Discussion

The health state of ash trees in clonal seed orchard situated in Trstice village in Slovakia was investigated and sampled in the period from March to July 2014. The orchard consists of 2 ash species: common ash (*F. excelsior*) and narrow-leaved ash (*F. angustifolia*). The symptoms typical for ash decline and ash dieback were present in the evaluated locality. Ash dieback is characterized by a remarkably wide range of symptoms (Kirisits et al. 2009, Kowalski et al. 2010). Disease symptoms range from necrotic leaf spots to bark cankers associated with xylem necroses and wilting, eventually leading to tree death. The dieback starts with ascospore infection of leaves. Ascospores are produced during summer in apothecia on leaf remnants from the previous year on the ground and dispersed by wind (Kowalski and Holdenrieder 2009, Timmermann et al. 2011, Gross et al. 2012, Kirisits et al. 2012). Leaf infection is followed by necrotic lesions spreading along the rachis into the shoot, and wood discoloration (Kowalski and Holdenrieder 2008, Bakys et al. 2009, Kräutler and Kirisits 2012). The above symptoms were recorded on investigated locality on *F. angustifolia* host tree. Ash dieback was recorded for the first time in the eastern part of Slovakia in 2004 (Kunca 2006). Later, symptoms were observed in other localities (Leontovyč and Kunca 2009) and now ash dieback has spread throughout Slovakia without any natural limits (Kunca et al. 2011). *H. fraxineus* was confirmed in several

localities in different types of vegetation over Slovakia (Adamčíková et al. 2015). All these records originated from Slovakia referred to the ash dieback only on *F. excelsior* host tree.

The fungus isolation from symptomatic *F. angustifolia* host tissues was successful in 3 samples from 14 collected. Colonies were cottony, white, orange-brown or fulvous brown, slow growing as typical for *H. fraxineus*. The obtained pure cultures were used for DNA extraction.

DNA was successfully extracted from 11 samples (3 isolates, 8 apothecia) collected in Trstice. The PCR-based test using species specific primers according to Johansson et al. (2010), forward primer for 18S gene, reverse primer for ITS2 region of the DNA operon produced an amplicon of 456 bp. The 18S ribosomal PCR to test the integrity of the DNA showed positive results for all samples. The confirmation of results, due to possible cross-reaction with *H. albidus* (Gillet) W. Phillips, was done by DNA sequencing of ITS rDNA operon for all samples (DNA extracted from 3 isolates also 8 from apothecia). The ITS sequences obtained with primers ITS1/ITS4 for all samples were identical and showed a high degree of similarity to DNA sequence deposited in GenBank for *H. fraxineus* with accession number HM193468 (Husson et al. 2011). Sequence identity of the rDNA ITS1/ITS4 locus was 100% among the 11 samples from diseased *F. angustifolia*. The ITS sequences obtained in this study were also deposited to GenBank (for GenBank accession numbers see Table 1).

This study reports about the first occurrence of ash dieback and *H. fraxineus* on *F. angustifolia* in Slovakia. Until now it has been confirmed in Slovakia only on *F. excelsior* (Kunca 2006, Adamčíková et al. 2015).

The occurrence of the disease on *F. angustifolia* is not unexpected, but the impact of the disease on this tree species is less documented than that on *F. excelsior* (Kirisits et al. 2010, Hauptman et al. 2012, McKinney et al. 2014). It has also been reported from surrounding countries: Czech Republic (Jankovský and Holdenrieder 2009), Austria and Hungary (Kirisits et al. 2010).

In conclusion, this study demonstrated that the ash dieback pathogen attacks not only *F. excelsior*, but also *F. angustifolia* as in many European countries also in Slovakia.

Table 1. List of samples of *Hymenoscyphus fraxineus* collected from *Fraxinus angustifolia* in clonal seed orchard of ash species in Trstice used for molecular studies

Sample designation	Date of sampling	Fungal tissue* used for DNA extraction	Herbarium specimen no.**	GenBank accession no.
CH_30	26 Mar 2014	M	-	KU736880
CH_31	26 Mar 2014	M	-	KU736881
CH_32	26 Mar 2014	M	-	KU736882
H_61	16 Jul 2014	A	NR 5321	KU736883
H_63	16 Jul 2014	A	NR 5322	KU736884
H_64	16 Jul 2014	A	NR 5320	KU736885
H_71	16 Jul 2014	A	NR 5319	KU736886
H_72	16 Jul 2014	A	-	KU736887
H_73	16 Jul 2014	A	-	KU736888
H_74	16 Jul 2014	A	NR 5317	KU736889
H_75	16 Jul 2014	A	NR 5318	KU736890

*M = mycelium, A = apothecia

** NR = Plant Pathology Herbarium of the Institute of Forest Ecology, Slovak Acad. Sci., Nitra, Slovakia

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References

Adamčíková, K., Kádasi Horáková, M., Jankovský, L. and Havrdová, L. 2015. Identification of *Hymenoscyphus fraxineus*, the causal agent of ash dieback in Slovakia. *Biologia* 70: 559-564.

Bakys, R., Vasaitis, R., Barklund, P., Ihrmark, K. and Stenlid, J. 2009. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology* 58: 284-292.

Cech, T. 2006. Eschenschäden in Österreich [Ash dieback and premature leaf shedding in Austria]. *Forstschutz Aktuell* 37: 18-20.

Chandelier, A., André, F. and Laurent, F. 2010. Detection of *Chalara fraxinea* in common ash (*Fraxinus excelsior*) using real time PCR. *Forest Pathology* 40: 87-95.

Drenkhan, R. and Hanso, M. 2010. New host species for *Chalara fraxinea*. *New Disease Reports* 22: 16.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identifi-

cation of mycorrhizae and rusts. *Molecular Ecology* 2: 113-118.

Gross, A., Grünig, C.R., Queloz, V. and Holdenrieder, O. 2012. A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Forest Pathology* 42: 252-264.

Halmschlager, E. and Kirisits, T. 2008. First report of the ash dieback pathogen *Chalara fraxinea* on *Fraxinus excelsior* in Austria. *Plant Pathology* 57: 1177.

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

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 Plant Pathology* 130: 311-324.

Ioos, R., Kowalski, T., Husson, C. and Holdenrieder, O. 2009. Rapidin planta detection of *Chalara fraxinea* by a realtime PCR assay using a dual-labelled probe. *European Journal of Plant Pathology* 125: 329-335.

Jankovsky, L. and Holdenrieder, O. 2009. *Chalara fraxinea* – ash dieback in the Czech Republic. *Plant Protection Science* 45: 74-78.

Johansson, S.B.K., Vasaitis, R., Ihrmark, K., Barklund, P. and Stenlid, J. 2010. Detection of *Chalara fraxinea* from tissue of *Fraxinus excelsior* using species-speficic ITS primers. *Forest Pathology* 40: 111-115.

Kirisits, T., Dämpfle, L. and Kräutler, K. 2013. *Hymenoscyphus albidus* is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media. *Forest Pathology* 43: 386-389.

Kirisits, T., Kritsch, P., Kraeutler, 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.

Kirisits, T., Matlakova, M., Mottinger-Kroupa, S., Cech, T.L. and Hlamschlager, E. 2009. The current situation of ash dieback caused by *Chalara fraxinea* in Austria. In: T. Dogmus-Lehtijärvi (Editor), Proceedings of the conference of IUFRO working party 7. 02. 02., Egirdir, Turkey, 11 – 16 May 2009. SDU Faculty Forestry Journal, Serial: A, Special Issue: 97-119.

Kirisits T., Matlakova M., Mottinger-Kroupa S. and Halmschlager E. 2010. *Chalara fraxinea* associated with dieback of narrow-leafed ash (*Fraxinus angustifolia*). *Plant Pathology* 59: 411.

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

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

Kowalski, T. and Holdenrieder, O. 2008. Eine neue Pilzkrankheit an Esche in Europa. *Schweizerische Zeitschrift für Forstwesen* 159: 45-50.

Kowalski, T. and Holdenrieder, O. 2009. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *Forest Pathology* 39: 304-308.

Kowalski, T., Schumacher, J. and Kehr, R. 2010. Das Eschensterben in Europa – Symptome, Erreger und Empfehlungen für die Praxis [Ash dieback in Europe – symptoms, causes and prognosis]. In: D. Dujesiefken (Editor), Jahrbuch der Baumpflege 2010. Haymarket Media, Braunschweig, Germany, p. 184-195.

Kräutler, K. and Kirisits, T. 2012. The ash dieback pathogen *Hymenoscyphus pseudoalbidus* is associated with leaf symptoms on ash species (*Fraxinus* spp.). *Journal of Agricultural Extension and Rural Development* 4(9): 261-265.

Kunca, A. 2006. Occurrence of pest agents in Slovak forests in 2005 and prognosis for 2006. Lesnícky výskumný ústav, Zvolen, 15 pp. (in Slovak)

Kunca, A., Leontovyč, R., Zúbrik. M. and Gubka, A. 2011. Bark beetle outbreak on weakened ash trees and applied control measures. *EPPO Bulletin* 41: 11-13.

Leontovyč, R. and Kunca, A. 2009. Nárast odumierania niektorých listnatých drevín (jaseň, gaštan jedlý, topole) v dôsledku aktivizácie hubových patogénov [Increased dieback of broadleaved trees (ash, sweet chestnut, poplars) due to fungal pest agents]. In: A. Kunca (Editor), Aktuálne problémy v ochrane lesa 2009, Nový Smokovec, p. 105-109. (in Slovak)

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.

Schumacher, J., Kehr, R., and Leonhard, S. 2010. Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. *Forest Pathology* 40: 419-429.

Szabo, I. 2009. First report of *Chalara fraxinea* affecting common ash in Hungary. *Plant Pathology* 58: 797.

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* 41: 14-20.

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