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MYCOBIOTA IN THE SEEDS OF NARROW-LEAVED ASH (*FRAXINUS ANGUSTIFOLIA* VAHL)

VRSTE GLJIVA U SJEMENU POLJSKOGA JASENA (*Fraxinus angustifolia* Vahl)

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SUMMARY

Narrow-leaved ash (*Fraxinus angustifolia*), currently the most damaged forest tree species in the Republic of Croatia, is suffering from dieback primarily caused by pathogenic fungus *Hymenoscyphus fraxineus*. Since health status of seeds is very important for future seedling production, objective of this study was to screen narrow-leaved ash seeds for presence of this main pathogen and other potentially parasitic fungi. Seeds were collected from five locations and analysed using three different methods. Results revealed relatively good health status of inspected seeds, with total of 15 different fungal taxa identified in less than 40% of samples and no confirmation of *Hymenoscyphus fraxineus* presence. Most frequently detected fungi were various species of genus *Alternaria* and species *Sphaerulina berberidis*, while other taxa occurred rarely. Although identified fungal species haven't caused visible symptoms on seeds after one to two months of storage, many of them are known seed pathogens or opportunistic ash (*Fraxinus* spp.) pathogens and could have a negative effect on seeds after longer period of storage or storage in unfavourable conditions.

KEY WORDS: fungal isolation, nested PCR, *Alternaria* sp., *Sphaerulina berberidis*

INTRODUCTION
UVOD

Narrow-leaved ash (*Fraxinus angustifolia* Vahl), ecologically and economically very important species in lowland forests, is currently the most damaged forest tree species in the Republic of Croatia with 75% of trees having significantly defoliated crown according to the ICP Forests program data for 2017 (Potočić *et al.* 2018). Existing research revealed that, among other factors, there are several parasitic fungi involved in the decline in roots and stem collars of affected trees (Kranjec 2017), with pathogenic fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya confirmed as the primary causative agent of crown dieback at multiple locations (Diminić 2015, Milotić *et al.* 2016). Presence of this pathogen responsible for large-scale dieback of common (*Fraxinus excelsior* L.) and narrow-leaved ash throughout Europe has been confirmed in roots, stems, branches, shoots, petioles and leaves of both tree species (Kowalski 2006, Gross *et al.* 2014, Chandelier *et al.* 2016), but also in the symptomatic and visually healthy seeds of common ash from Latvia and Sweden (Cleary *et al.* 2013, Hayatgheibi 2013, Marčiulynienė *et al.* 2018).

Yield and health status of narrow-leaved ash seeds are of great importance in the Republic of Croatia, since they are

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necessary for nursery production of seedlings, majority of which are further used for forest stand regeneration or afforestation. For this purpose seeds are collected from adult trees in existing natural stands selected and registered as seed sources, forest stands which are phenotypically above average and specially managed for the purpose of seed collection and thus registered as seed stands and seed orchards established also for the purpose of seed collecting, from the genetically superior individual trees (Anon 2009, 2011, 2013, 2014).

Fungal presence in the seeds of forest tree species in general is considered to be a significant cause of shortened seed longevity during storage (Sutherland *et al.* 2002), reduced seed germination due to embryo or endosperm deterioration and potential cause of diseases that affect other developmental stages of plants, such as increased damping-off, shoot dieback, cankers and dieback of older seedlings (Cram 2009), although number of species just act as endophytes or saprotrophs and do not adversely affect the performance of seeds sown in nurseries (Mittal and Wang 1987).

In Croatian narrow-leaved ash forest stands there was a recorded case of seedlings delivered from a nursery Zalužje, Forestry Office (FO) Vinkovci, which expressed symptoms of *Hymenoscyphus fraxineus* dieback approximately one month after being planted in the field, FO Vinkovci, Management Unit (MU) Vrbanske šume, Subcompartment (SC) 91b, although the pathogen wasn't confirmed on older ash trees sampled in the area nearby SC 132a (MU Vrbanske šume) and SC 49a (MU Kusare) (FO Vinkovci) (Anon 2015). This finding raised a question of infection origin and possibility that pathogen spread from seeds into the plant tissue, eventually causing visible dieback symptoms in grown seedlings.

The objective of this research was to screen narrow-leaved ash seeds for the presence of pathogenic fungus *Hymenoscyphus fraxineus* and simultaneously detect other possible seed-borne pathogens in order to estimate the health status and suitability of seeds collected from registered seed sources and seed stands for further nursery seedling production.

MATERIALS AND METHODS
MATERIJALI I METODE

Fraxinus angustifolia seeds were collected in period from August to November 2017 from visually healthy trees in four natural forest stands registered as narrow-leaved ash seed sources and one registered narrow-leaved ash seed stand (Table 1). Seeds were examined for fungal presence after one to two months of storage at room temperature, using both classical method of mycelia isolation on artificial media and a nested PCR method to analyse DNA directly from seeds. Seeds were additionally screened for presence of pathogenic fungus *Hymenoscyphus fraxineus* using species specific primers (Johansson *et al.* 2010).

Isolation of fungi from seeds – *Izolacija gljiva iz sjemena*

Twelve seeds from each of five locations were used for fungal isolation on malt extract agar medium (MEA, Oxoid, Basingstoke, UK) supplemented with streptomycin sulphate (200 mg l^{-1}, Sigma-Aldrich, St. Louis, USA). Seeds were surface sterilized in a solution of sodium hypochlorite (approx. 4% active chlorine) for one minute and then rinsed three times in sterile distilled water. Seeds cut in half were plated on medium in Petri dishes (9 cm diameter) and incubated in dark at 20 °C for four weeks (Bulovec 2018). Petri dishes were checked weekly for fungal growth and emerging mycelia were subcultured to MEA medium. Pure cultures were grouped into morphotypes and at least one isolate of each morphotype group was used for molecular identification. Extraction of DNA was performed according to Allemann *et al.* (1999) with modifications (Kranjec *et al.* 2017) and PCR amplification was conducted with primers ITS 1 and ITS 4 (White *et al.* 1990) in 25 µl reactions containing 200 µM deoxyribonucleoside triphosphates, 0.4 µM of each primer, 0.5 U of Taq DNA polymerase with reaction buffer (Sigma-Aldrich, St. Louis, USA), 1.5 mM $MgCl_2$ and 1 µl of 100-fold diluted DNA template. Cycling conditions were as follows: an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s, extension at 72 °C for 90 s and a final

Table 1. Locations and dates of narrow-leaved ash seed collection
Tablica 1. Lokacije i datumi sakupljanja sjemena poljskoga jasena

Location		Dates of seed collection (year 2017)
Forestry Office	Seed source/stand	
Gunja	natural stand HR-FAN-SI-121/011	22 August – 6 September
Lipovljani	seed stand HR-FAN-SS-123/160	21 August – 15 September
Novoselec	natural stand HR-FAN-SI-123/366	25 August – 3 November
Vukovar	natural stand HR-FAN-SI-111/030	23 August – 30 August
Županja	natural stand HR-FAN-SI-121/305	23 August – 6 September

Table 2. Percentage of narrow-leaved ash seeds with detected fungal presence
Tablica 2. Udio sjemena poljskoga jasena u kojemu je utvrđena prisutnost gljiva

Location *Lokacija*	Percentage of seeds with fungal presence detected using nested PCR approach *Udio sjemena u kojem su potvrđene gljive korištenjem nested PCR metode*	Percentage of seeds with fungal presence detected by isolation on MEA medium *Udio sjemena u kojem su potvrđene gljive izolacijom na MEA hranjivu podlogu*
Gunja	35%	50%
Lipovljani	55%	50%
Novoselec	20%	25%
Vukovar	30%	58%
Županja	25%	25%

extension step at 72 °C for 5 min. The resulting PCR products were sequenced using primer ITS 4 at the DNA sequencing facility of Macrogen Europe (Amsterdam, Netherlands). After processing raw data using the BioEdit Sequence Alignment Editor v.7.2.5 software (Hall 1999), sequences were identified by comparison with reference sequences in NCBI GenBank using BLAST tool (Altschul *et al.* 1990). Sequences with 98 – 100% similarity were identified to the species level and with 94 – 97% of similarity to the genus level (Bakys *et al.* 2011).

Analysis of DNA from seeds – *Analiza DNA iz sjemena*

Twenty seeds from each of five locations were analyzed for fungal presence using a nested PCR method. After surface disinfection of samaras by immersing them in 35% H_2O_2 for three minutes, seeds were aseptically removed, cut into small pieces (1 – 2 mm long), placed in separate 2 ml centrifuge tubes and freeze-dried for 24 h (Cleary *et al.* 2013). Samples were homogenized in TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for two minutes. DNA was extracted following the protocol according to Minas et al. (2011). First PCR was conducted using the primers ITS1-F (Gardes and Bruns 1993) and ITS 4 (White *et al.* 1990) under the same cycling conditions and with same reagents concentrations as in the described PCR protocol used for DNA analysis of isolated mycelia. The PCR products were size separated by gel electrophoresis on 2% agarose gels stained with GelStar Nucleic Acid Gel Stain (Lonza, Rockland, USA) and visualised under UV light. All bands were aseptically excised from the gel, purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and re-amplified in a second PCR using the primers ITS 1 and ITS 4 (White *et al.* 1990) under the same cycling conditions and with same reagents concentrations as in the first one. The resulting PCR products were sequenced using primer ITS 4 at the DNA sequencing facility of Macrogen Europe (Amsterdam, Netherlands) and identified using NCBI GenBank database as already described in this paper.

Detection of *Hymenoscyphus fraxineus* in seeds – *Utvrđivanje prisutnosti gljive Hymenoscyphus fraxineus u sjemenu*

DNA extracted from seeds, as previously described, was additionally checked for the presence of *Hymenoscyphus fraxineus* in a PCR reaction with species specific primers: forward (5′AGCTGGGGAAACCTGACTG) and reverse (5′ACACCGCAAGGACCCTATC) (Johansson *et al.* 2010), and with same reagents concentrations as in previous analysis. The thermal cycling was carried out as follows: an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 60 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min (Hayatgheibi 2013). DNA of confirmed *Hymenoscyphus fraxineus* isolate obtained from earlier research (isolated from *Fraxinus angustifolia* stem collar, Kranjec 2017) was used as a positive control in each PCR reaction. PCR products were run on 1% agarose gels stained with GelStar Nucleic Acid Gel Stain (Lonza, Rockland, USA) and visualised under UV light.

RESULTS
REZULTATI

Analysis of *Fraxinus angustifolia* seeds by mycelia isolation on MEA medium and nested PCR revealed fungal presence in 20 – 58% of screened seeds, depending on the method used and location they originated from (Table 2). Isolation of mycelia on MEA medium resulted in growth of 26 fungal isolates belonging to 15 different taxa, 10 of which were identified to the species level (Table 3). The nested PCR analysis resulted in identification of 19 different fungal taxa, 10 of which were identified to the species level (Table 4).

Most frequently detected taxa were *Sphaerulina berberidis* and *Alternaria* sp. with *Alternaria alternata* and *A. tenuissima* identified to the species level. Among the most frequently detected were also seven sequences obtained in nested PCR which corresponded to Fungal endophyte isolate 4480 according to NCBI GenBank and might be a species of genus *Sphaerulina,* which is next closest match in the given database. Species of *Alternaria* occurred in the seeds from all five locations included in this research and *Sphaerulina berberidis* occurred in seeds from four of those locations (not confirmed only in seeds from stand HR-FAN-SI-111/030 in Vukovar).

Neither of sequences obtained by first two described methods belonged to *Hymenoscyphus fraxineus*. Presence of this pathogenic fungus in seeds was not confirmed by using

Table 3. Identified fungal taxa in narrow-leaved ash seeds by mycelia isolation on MEA medium
Tablica 3. Taksoni gljiva identificirani u sjemenu poljskoga jasena izolacijom micelija na MEA hranjive podloge

Fungal taxa identified according to NCBI GenBank *Identificirani takson gljive prema NCBI GenBank bazi podataka*	Accession number in NCBI GenBank *Identifikacijski broj sekvence u NCBI GenBank bazi*	Percentage of *Fraxinus angustifolia* seeds where fungus is present *Udio sjemena poljskoga jasena na kojem je gljiva prisutna*
Alternaria sp.	MH137756	13,3%
Alternaria tenuissima (Kunze) Wiltshire	MH137745 MH137746	3,3%
Cercospora beticola Sacc.	MH137755	1,6%
Cladosporium cladosporioides (Fresen.) G.A. de Vries	MH137753	1,6%
Cladosporium herbarum (Pers.) Link	MH137759	1,6%
Cladosporium sp.	MH137748	1,6%
Colletotrichum sp.	MH137751	1,6%
Phomopsis velata (Sacc.) Traverso	MH137754	1,6%
Phomopsis cucurbitae McKeen 1957	MH137752	1,6%
Botryosphaeria stevensii Shoemaker	MH137758	1,6%
Fusarium oxysporum Schltdl.	MH137749	1,6%
Lophiostoma sp.	MH137750	1,6%
Penicillium sp.	MH137760	1,6%
Sphaerulina berberidis (Niessl) Quaedvl., Verkley & Crous	MH137747	6,6%
Venturia fraxini Aderh.	MH137761	1,6%

species specific primers for PCR amplification either, as there were no visible PCR products on agarose gels besides positive controls included in each reaction.

DISCUSSION
RASPRAVA

With total of 15 different fungal taxa present in less than 40% of samples (in 58 out of 160 in total), narrow-leaved ash seeds revealed relatively good health status in comparison with common ash (*Fraxinus excelsior*) seeds analysed in similar European studies, where larger number of taxa were identified in smaller number of samples and with averagely higher individual presence frequency (Cleary *et al.* 2013, Hayatgheibi 2013).

Species of the most frequently detected genus in this research, *Alternaria* sp., haven't caused visible symptoms on seeds although the identified *Alternaria alternata* and *Alternaria tenuissima* are reported as seed pathogens on *Betula* spp. and *Robinia pseudoacacia* L. (Lilja 1979, Sunita 1998) and causative agents of *Malus* spp. and *Punica granatum* L. fruit rot during storage (Zambounis *et al.* 2015). *Alternaria alternata* has also been found in symptomatic bark, wood and buds of declining *Fraxinus excelsior* (Pukacki and Przybył 2005, Davydenko *et al.* 2013, Kowalski *et al.* 2016), indicating that it can act as an opportunistic pathogen in already declining ash tissue, possibly in the narrow-leaved ash seeds as well if they are under the influence of negative biotic and abiotic factors while on a tree or stored in unfavourable conditions after the harvest. Other frequently detected species, *Sphaerulina berberidis*, has so far been reported only as leaf endophyte of several tree species (Eo *et al.* 2014) and most probably has the same role in the narrow-leaved ash seeds since it hasn't induced any visible symptoms in the analysed samples.

The remainder of identified species in narrow-leaved ash seeds were present in only one to three samples, but included some of the well known tree pathogens such as *Phomopsis velata* (synonym *Diaporthe eres*) and *Botryosphaeria stevensii* (synonym *Diplodia mutila*), which were also found in *Fraxinus excelsior* seeds in Latvia and Sweden (Cleary *et al.* 2013). Former is known for causing stem canker and dieback of several tree species (Quaroni *et al.* 1980, Anagnostakis 2007, Thomidis and Michailides 2009), fruit deterioration (Ristić *et al.* 2016) and being present in necrotic tissue and collar rots of *Fraxinus excelsior* (Kowalski *et al.* 2016, Langer 2017). Latter is known as a parasite involved in bark necrosis, canker formation and dieback of *Fraxinus excelsior* and *Fraxinus ornus* L., *Quercus* spp. and other tree species (Ragazzi *et al.* 1999, Przybył 2002, Sidoti and Granata 2004, Sims *et al.* 2016). Some of identified species are reported to be seed or fruit pathogens on other plant species, like *Fusarium oxysporum* on *Robinia pseudoacacia* seeds (Sunita 1999), *Cladosporium cladosporioides* on tobacco seeds (*Nicotiana tabacum* L.) (Wang *et al.* 2014) and stored hazelnuts (*Corylus avellana* L.) (Moghaddam and Taherzadeh 2007), and *Cladosporium herbarum* on stored figs (*Ficus carica* L.) (Montealegre *et al.* 2000) and *Prunus* spp. fruits (Tonini and Capriotti 1996). *Venturia fraxini*, known primarily as endophyte (Schlegel *et al.* 2016), but

Table 4. Identified fungal taxa in narrow-leaved ash seeds by nested polymerase chain reaction (PCR)
Tablica 4. Taksoni gljiva identificirani u sjemenu poljskoga jasena u ugniježđenoj lančanoj reakciji polimerazom (PCR)

Closest sequence match in NCBI GenBank *Sekvenca s najvećom podudarnošću u NCBI GenBank bazi podataka*	Fungal taxa identified *Identificirani takson gljive*	Accession number in NCBI GenBank *Identifikacijski broj sekvence u NCBI GenBank bazi*	Percentage of *Fraxinus angustifolia* seeds where fungus is present *Udio sjemena poljskoga jasena na kojem je gljiva prisutna*
Alternaria alternata	*Alternaria alternata* (Fr.) Keissl.	MH137762 MH137763 MH137764 MH137765	4%
Alternaria brassicicola/A. alternata	*Alternaria* sp. FA_N8V7	MH137766 MH137767	2%
Alternaria sp. isolate B6-25	*Alternaria* sp. FA_L9	MH137768	1%
Alternaria alternata/A. porri/A. gaisen/ A. tenuissima/A. brassicae/A. mali/ A. ochroleuca	*Alternaria* sp. FA_Z11	MH137769	1%
Alternaria sp./*Phoma* sp./*Talaromyces* sp.	*Ascomycota* sp. FA_L18	MH137770	1%
Aspergillus ruber	*Aspergillus ruber* (Jos. König, Spieck. & W. Bremer) Thom & Church	MH137771	1%
Cladosporium sp. *A144*	*Cladosporium* sp. FA_V9	MH137772	1%
Cladosporium cladosporioides	*Cladosporium cladosporioides* (Fresen.) G.A. de Vries	MH137773	1%
Cladosporium herbarum	*Cladosporium herbarum* (Pers.) Link	MH137774	1%
Cryptococcus tephrensis	*Vishniacozyma tephrensis* Vishniac ex Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout	MH137775	1%
Diaporthe eres	*Phomopsis velata* (Sacc.) Traverso	MH137776	1%
Didymella heteroderae	*Didymella heteroderae* (Sen Y. Chen, D.W. Dicks. & Kimbr.) Qian Chen & L. Cai	MH137777	1%
Diplodia mutila	*Botryosphaeria stevensii* Shoemaker	MH137778 MH137779	2%
Mycosphaerella coacervata	*Mycosphaerella coacervata* Syd.	MH137780	1%
Fungal endophyte isolate 4480	Fungal endophyte FA_2017	MH137781 MH137782 MH137783 MH137784 MH137785 MH137786 MH137787	7%
Uncultured Ascomycota isolate FL7.5	*Ascomycota* sp. FA_Z19	MH137788	1%
Phoma sp. ZP-40	*Phoma* sp. FA_G14	MH137789	1%
Phomopsis sp. RJ-2015 isolate 310Jb14	*Phomopsis* sp. FA_N6	MH137790	1%
Sphaerulina berberidis	*Sphaerulina berberidis* (Niessl) Quaedvl., Verkley & Crous	MH137791 MH137792 MH137793 MH137794	4%

also confirmed in leaf blotches and other necrotic tissue on *Fraxinus* spp. (Anselmi 2001, Bakys *et al.* 2009), is first time reported in ash seeds. For the rest of the identified species there is no documented evidence of their presence in trees. Instead they are known for being pathogens of agricultural plants (*Cercospora beticola*, *Phomopsis cucurbitae*) (Bertetti *et al.* 2012, Vaghefi *et al.* 2017), pathogens of stored *Pisum sativum* L. seeds (*Aspergillus ruber*) (Harman *et al.* 1972), saprotrophs in forest soil (*Vishniacozyma tephrensis*) (Mašínová *et al.* 2017), parasites of nematodes (*Didymella heteroderae*) (Chen *et al.* 1996) and leaf spot causing agents on *Coprosma robusta* Raoul (*Mycosphaerella coacervata*) (Hood 1985). Since most of the described species were present at low frequencies their effect on general health status of seeds cannot by very significant, but ability of those known as potential parasites to induce symptoms and decline of seeds and later seedlings under the unfavourable conditions remains possible.

Hymenoscyphus fraxineus was not found in the seeds of *Fraxinus angustifolia* analysed in this research performed

by applied methodology, thus not supporting the hypothesis that fungus has spread from infected seeds to seedlings planted in the field from the local nursery. Still, these findings do not exclude the possibility that the fungus could be present and thus spread on the surface of samaras, since this aspect of transmission was not investigated. The fact that this pathogen has been confirmed in both symptomatic and visually healthy seeds from trees of various levels of susceptibility to the fungus in similar research conducted on *Fraxinus excelsior* (Cleary *et al.* 2013, Hayatgheibi 2013, Marčiulynienė *et al.* 2018) and not in the *Fraxinus angustifolia* seeds analysed in this research, could be due to high summer temperatures (July and August 2017 maximum > 35 °C) (DHMZ 2017b, a) characteristic for the narrow-leaved ash distribution area in the Republic of Croatia, which seems to be a limiting factor for the spread of pathogen (Hauptman *et al.* 2013, Grosdidier *et al.* 2018) or due to seed collection method, where only seeds from visually healthy narrow-leaved ash trees from registered seed stands and natural stands registered as seed sources are collected for further purpose of nursery seedling production. In addition, recent surveys conducted by Marčiulynienė *et al.* (2018) found no evidence of fungus being able to spread from infected seeds to grown plants, which still doesn't exclude this possibility in the opinion of authors.

CONCLUSION
ZAKLJUČAK

Analysed narrow-leaved ash seeds collected from visually healthy trees from registered seed sources and seed stand revealed relatively low level fungal presence in comparison to other similar studies, indicating good health status and usability for further nursery seedling production regarding this particular aspect. Identified fungal species haven't caused visible symptoms on seeds after one to two months of storage, not excluding their possible negative effect on seeds after longer period of storage or storage in unfavourable conditions, since some of them are known as seed pathogens and some are reported as opportunistic parasites in necrotic tissues of *Fraxinus* spp. Presence of pathogenic fungus *Hymenoscyphus fraxineus* in seeds was not confirmed, so it can be concluded that potential dieback of seedlings caused by this pathogen in nurseries or in the field is a consequence of infections from affected narrow-leaved ash stands in the vicinity rather than spread of fungus from infected seeds.

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SAŽETAK

Poljski jasen (*Fraxinus angustifolia* Vahl) je u Republici Hrvatskoj trenutno najoštećenija šumska vrsta drveća, sa 75 % stabala značajno osute krošnje prema podacima međunarodnog programa ICP Forests iz 2017. godine. Dosadašnja su istraživanja potvrdila patogenu gljivu *Hymenoscyphus fraxineus* kao primarnog uzročnika odumiranja krošanja poljskoga jasena na više lokacija te utvrdila njenu prisutnost u listovima, izbojcima, granama, bazi debla te korijenu stabala. Cilj ovog istraživanja bio je ispitati prisutnost navedenog patogena u sjemenu poljskoga jasena, a također i identificirati ostale vrste potencijalno parazitskih gljiva, kako bi se s navedenog aspekta moglo procijeniti zdravstveno stanje i uporabljivost sjemena u rasadničkoj proizvodnji poljskoga jasena za obnovu sastojina i pošumljavanje. Sjeme je prikupljeno na pet lokacija u sastojinama kategoriziranim kao sjemenski izvor ili sjemenska sastojina na području šumarija Novoselec, Lipovljani, Gunja, Županja i Vukovar. Za analizu sjemena skladištenog jedan do dva mjeseca korištene su tri različite metode, uključujući klasičnu metodu izolacije gljiva iz tkiva na hranjive podloge te molekularne metode izolacije ukupne stanične DNK iz sjemena i umnažanja ciljanih sekvenci u lančanoj reakciji polimerazom korištenjem univerzalnih početnica (ITS 1, ITS 1 – F, ITS 4) i početnica specifičnih za gljivu *Hymenoscyphus fraxineus*.

Analizom je utvrđeno ukupno 15 različitih taksona gljiva u manje od 40 % ispitivanog sjemena, ukazujući na njegovo relativno dobro zdravstveno stanje. Najčešće su identificirani pripadnici roda *Alternaria,* od kojih su *A. alternata* i *A. tenuissima* identificirane do razine vrste, te vrsta *Sphaerulina berberidis*. Ostali identificirani taksoni zabilježeni su na svega jednoj do tri sjemenke. Iako utvrđeni taksoni gljiva nisu uzrokovali vidljive simptome ili propadanje sjemena nakon jednog do dva mjeseca skladištenja, velik broj njih se u literaturi navode kao patogeni sjemena i plodova različitih vrsta drveća, a dio i kao oportunistički paraziti prisutni u nekrotičnom tkivu jasena (*Fraxinus* spp.), zbog čega se ne može u potpunosti isključiti njihov negativan utjecaj na sjeme tijekom duljih perioda skladištenja ili izlaganja nepovoljnim uvjetima. Vrsta *Hymenoscyphus fraxineus* niti jednom korištenom metodom nije utvrđena u analiziranom sjemenu, te nije dokazana mogućnost njena širenja na uzgojene sadnice ovim putem. Time nije isključena mogućnost njene prisutnosti na površini plodova, tj. perutki, koje su u ovom istraživanju površinski sterilizirane kako bi se smanjio utjecaj uobičajeno prisutnih epifitnih gljiva na rezultate.

KLJUČNE RIJEČI: izolacija gljiva, ugniježđeni PCR, *Alternaria* sp., *Sphaerulina berberidis*