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Characterisation and pathogenicity of *Cryphonectria parasitica* on sweet chestnut and sessile oak trees in Serbia

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Abstract: The presence of *Cryphonectria parasitica* (Murrill) M.E. Barr was studied in six natural and planted stands of sweet chestnut in Serbia. The fungus was detected on the sweet chestnut in five localities and on the sessile oak in one locality. In total, 77 isolates from the sweet chestnut and five isolates from the sessile oak were obtained. Based on the culture morphology, all the obtained isolates were proven to be free from *Cryphonectria* (Saccardo) Saccardo & D. Saccardo hypovirus. The isolates of *C. parasitica* from the sweet chestnut were compatible with three different vegetative compatibility types, EU-12, EU-2, and EU-1, while the isolates from the sessile oak belonged to EU-12. After inoculation in laboratory conditions, the isolate from the sweet chestnut and sessile oak caused the decline in 88 and 76% of the sweet chestnut plants, respectively. In the case of the sessile oak, both isolates caused the decline in 52% of the plants. In field conditions, both isolates were aggressive to sessile oak trees after previous bark wounds and they were statistically significantly different compared to the control trees. The isolate from the sweet chestnut caused significantly larger cankers compared to both the isolate from the sessile oak and the control.

Keywords: chestnut blight fungus; oak decline; sequencing; vegetative compatibility type diversity; aggressiveness

Cryphonectria parasitica (Murrill) M.E. Barr belongs to the family Cryphonectriaceae, and according to Kirk *et al.* (2008), this family includes a total of 26 species within 12 genera. This fungus is a strong pathogen known worldwide as a cause of chestnut blight. The blight of the American chestnut (*Castanea dentata* (Marsh) Borkhausen), caused by *C. parasitica*, represents one of the greatest botanical catastrophes in the history of humankind. The first trees infected with this fungus were recorded in the New York Zoo

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in 1904 (Griffin 1986). Chestnut blight caused by *C. parasitica* was found in Europe for the first time in 1938 in Italy, from which it has spread to most European countries (Griffin 1986).

Apart from species of the *Castanea* Miller genus, other hosts of *C. parasitica* belong to several families, including Aceraceae, Betulaceae, Fagaceae, Anacardiaceae, Juglandaceae and Magnoliaceae (Nash & Stambaugh 1982; Turchetti *et al.* 1991; Dallavalle & Zambonelli 1999; Radócz & Tarcali 2005). *C. parasitica* is occurring as a saprotroph for most of the species listed above, with the exception of the oak (*Quercus* spp.), for which it is parasitic (Radócz & Tarcali 2005; Adamčíková *et al.* 2010; Tziros *et al.* 2015).

According to Krstić (1950), cankers on the bark of the sweet chestnut in the former Yugoslavia were recorded for the first time in Slovenia in the "Panovac" forest. The presence of *C. parasitica* in Serbia was recorded for the first time in 1975 in the province of Kosovo and Metohija (Marinković & Karadžić 1985). Later field studies and monitoring of sweet chestnut stands have confirmed the decline of sweet chestnut trees all over Serbia (personal observation, unpublished). Generally, *C. parasitica* is present in the most important natural stands of sweet chestnut in Serbia (in the area near Vranje and Prijepolje) as well as in most of the localities where individual sweet chestnut trees occur (Radulović 2013). Additionally, *C. parasitica* has been recorded on sessile oak trees in Serbia (Karadžić & Milenković 2013).

Due to the lack of data about the occurrence and the characteristics of this parasitic fungus on different host trees in Serbia, our study is aimed at (*i*) determining and confirming the presence of this fungus on sweet chestnut and oak trees, (*ii*) determining the vegetative compatibility (VC) types and occurrence of the hypovirus in isolates of this fungus obtained from sweet chestnut and from sessile oak trees in Serbia, and (*iii*) testing the pathogenicity of the selected isolates in the controlled and field conditions.

MATERIAL AND METHODS

Studied localities and sampling. The study was performed in six mixed stands of sweet chestnut and oak trees in Serbia, including three stands mixed with Hungarian oak (*Quercus frainetto* Tenore), two stands mixed with sessile oak (*Quercus petraea* (von Mattuschka) Lieblein), and one stand mixed with both oak species (Figure 1 and Table 1). In two stands, sweet chestnut appears naturally, building autochthonous communities, while this species was artificially introduced in four stands (Table 1). Planted sweet chestnut trees in the natural sessile oak forests near the Vršac Mountains, part of the Carpathian basin in Serbia were sampled and analysed together with sessile oak trees in the same locality (Table 1). Another planted forest established on the natural stand of *Q. frainetto* was sampled in the area near Kruševac in central Serbia (Table 1). Two natural and two planted stands of sweet chestnut trees were sampled in southern Serbia in different localities near Vranje, including Kozji Dol, Milivojce, Muhovac and Sobina, respectively (Table 1), where this species composes one of the rarely found autochthonous communities in Serbia. *Q. petraea* or *Q. frainetto* naturally appear in the mixtures in the latter four stands, and they were also inspected for the presence of the symptoms of the disease.

In each locality, 30–50% of the trees were randomly chosen and closely inspected to confirm the presence of the diseases (Table 1). Approximately 10–20% of

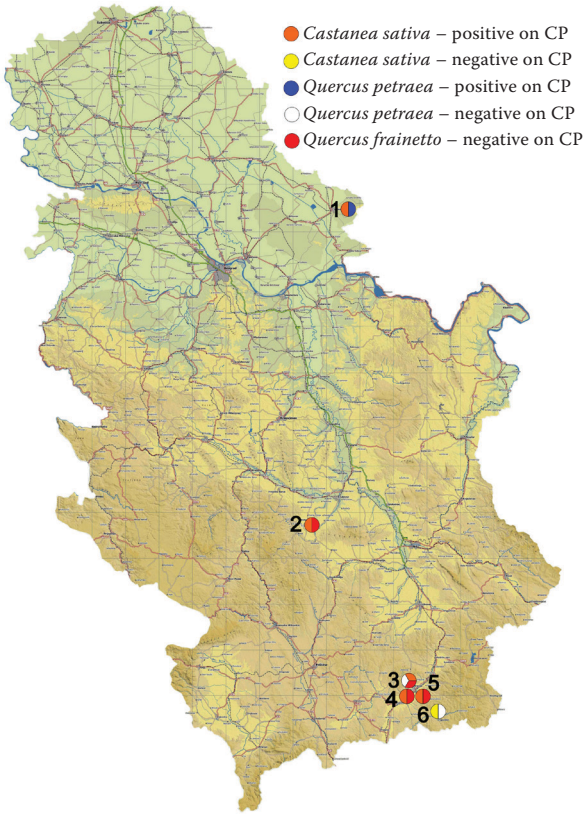

Figure 1. The studied localities and hosts

1 – Vršac Mountains; 2 – Kruševac/Brus; 3 – Vranje/Muhovac; 4 – Vranje/Sobina; 5 – Vranje/Milivojce; 6 – Vranje/Kozji Dol; CP – *Cryphonectria parasitica* (Murrill) M.E. Barr

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Table 1. Characteristics of the studied localities, evaluated trees and the obtained samples

No.	Locality	Year	Coordinates	Stand type	Age of the trees in the stand (year)	No. of observed sweet chestnut/ oak trees	Presence of chestnut blight symptoms on the sweet chestnut/oak trees	No. of trees (cankers) sampled from the *Castanea sativa* Miller/ oak trees	No. of isolates obtained from the sweet chestnut/ oak trees
1	Vršac Mountains	2012–2013	45°07'55''N 21°23'33''E	natural + planted stand	20–40	100/17[2]	100/17	10/5	10/5[2]
2	Kruševac/Brus	2013–2016	43°25'086''N 21°03'251''E	planted stand	50–60	20/10[1]	20/0	5/0	8
3	Vranje/Muhovac	2006–2012	42°34'33''N 21°51'42''E	natural stand	> 120	30/5[1]/7[2]	30/0	10/0	18
4	Vranje/Sobina	2006–2012	42°33'52''N 21°50'58''E	natural stand	> 120	50/7[1]	49/0	20/0	31
5	Vranje/Milivojce	2015	42°31'58.49''N 21°48'47.90''E	planted anti-erosion stand	30–40	30/3[1]	23/0	10/0	10
6	Vranje/Kozji Dol	2016	42°20'35.13''N 22°04'27.87''E	planted stand	50–60	50/20[2]	0/0	0	0

[1]*Quercus frainetto* Tenore; [2]*Quercus petraea* (von Mattuschka) Lieblein

the total number of the trees in each locality was randomly chosen for sampling and isolation (Table 1). Tissue samples were collected from the bark around the upper or lower margins, and one canker per tree was sampled. In total, 55 cankers on the stems and branches of *Castanea sativa* Miller trees and five cankers on *Q. petraea* stems were sampled (Table 1).

Isolation and identification of Cryphonectria parasitica. The collected samples were sterilised in the laboratory by immersion in 96% ethanol for 10 s and passing through an open flame. After sterilisation, one to three pieces from the collected samples were plated on a water-agar media (Torlak, Serbia) and incubated at 25°C in the dark. After 2–3 days of incubation, pieces of the young hypha were transferred from the edges of the growing colonies onto a malt extract agar (MEA) media prepared with 49 g/l of MEA (Merck, Germany) and amended with 100 mg/l methionine and 1 mg/l biotin. Further incubation was performed at 25°C in the dark. The hyphal characteristics, fruiting bodies and spores were observed in the four-week-old cultures under a light microscope.

To determine colony growth patterns, all the obtained isolates were transferred onto a potato dextrose agar (PDA) media prepared with 39 g/l of a PDA (Merck, Germany) and MEA media, and incubation was performed at 20°C in the dark. The main colony characteristics of the younger and older colonies, including pigmentation, colony shape, position of the hyphae and colony colour were evaluated.

The identification of the fungus was carried out based on the morphological features of the fruiting bodies, recorded on the bark of the infected trees, including perithecia with asci and ascospores and pycnidia with conidia, in combination with the characteristics of the pure cultures and comparison with the description in Gryzenhout *et al*. (2009).

Vegetative compatibility of the Cryphonectria parasitica isolates. VC tests were performed according to the methods specified by Bissegger *et al*. (1997). Five different European tester isolates of *C. parasitica* were used in this study: EU-1, EU-2, EU-5, EU-10 and EU-12 (provided by Dr Kiril Sotirovski, Faculty of Forestry, University of Skopje, FYR Macedonia). The incubation of the paired cultures was performed on the MEA media first at 25°C in the dark for seven days and then for an additional seven days in daylight at room temperature (22–25°C). The experiment was repeated twice, and the VC types were scored visually after 14 days according to merging/ barrage response.

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Diversity of the VC types. The diversity of the VC types was estimated using the *S*/*N* ratio, where *S* represents the number of VC types, and *N* the number of isolates. Additionally, for the 59 isolates from the locality in Vranje, the Shannon-Wiener's index of diversity (*H'*) was calculated using Eq. 1 (Shannon & Weaver 1949):

$$H' = -\sum i\left[pi \ln\left(pi \right)\right] \quad (1)$$

where: *pi* – frequency of the VC type *i* obtained by the *X*/*N* quotient; *X* – number of the isolates of that type; *N* – total number of the isolates (ΣX_i)

Determination of the hypovirulent isolates. Occurrence of hypovirulence among the obtained *C. parasitica* isolates in Serbia was determined based on the colony pigmentation method (Robin *et al*. 2000). For this assay, all the obtained isolates were transferred onto the PDA and MEA media and incubated at ~25°C in the dark for 10 days. After 10 days of incubation, they were incubated for an additional 10 days at ~25°C with a photoperiod of 16 hours. The pigmentation of the colonies was determined; if the isolates had an orange colony colour, they were considered virus-free, while isolates with a white colony colour (typical of *Cryphonectria* (Saccardo) Saccardo & D. Saccardo hypovirus infection) were assumed to be infected with the hypovirus.

Molecular identification. DNA was extracted from two representative isolates; one originated from sweet chestnut trees from Vranje (KF276606) and the other from sessile oak trees from the Vršac Mts. (KF276605), and both belonged to the EU-12 VC type. Parts of their mycelium were transferred to a liquid malt extract media prepared with 18 g/l of malt extract (Merck, Germany) and incubated at 22–25°C in the dark. After 5 days of incubation, the fresh mycelium was collected, washed in sterile distilled water and dried on sterile filter paper. The dried mycelium was crushed in liquid nitrogen, and the genomic DNA was extracted following the protocol for the Tissue DNA Mini Kit (Syngen, Poland).

The rDNA sequence containing the internal transcribed spacer (ITS) regions was used for the molecular identification of the *C. parasitica* isolates. The polymerase chain reaction (PCR) was performed using the previously described universal primers ITS1F and ITS4, which amplify the above-mentioned sequence (White *et al*. 1990; Gardes & Bruns 1993). PCR was performed in single-plex conditions in 0.2 ml plastic tubes, using a Bio-Rad iCycler (Bio-Rad, USA) with a reaction mixture consisting of 1 × RedTaq ReadyMix (Sigma Aldrich, USA), 200 µM of dNTPs, 250 nM of each primer and 1 µl of gDNA. The PCR programme was set at 3 min at 94°C followed by 40 cycles of 25 s at 94°C, 25 s at 56°C and 50 s at 72°C. The presence and size of the PCR products was confirmed by analysing 1 µl of product by electrophoresis on a 1% TAE-agarose gel, stained with GelRed™ Nucleic Acid Dye (Biotium, Inc., USA). For sequencing, 25 µl of the PCR product was purified with a CleanUp Kit (A&A Biotechnology, Poland) following the manufacturer's protocol and sequenced with an ABI 3730xl DNA analyzer (Applied Biosystems, USA).

The obtained sequences were aligned using the ClustalW algorithm of the BioEdit programme (Version 7.1.3.0) (Hall 1999) and compared with known sequences in the GenBank nucleotide sequence database (http://ncbi.nlm.nih.gov). Sequence analysis was performed with MEGA software (Version 6.0) (Tamura *et al*. 2013). The nucleotide sequences of the analysed *C. parasitica* isolates were submitted to GenBank (accession numbers: KF276605 and KF276606).

Pathogenicity tests. The two molecularly confirmed *C. parasitica* isolates (KF276606 and KF276605) were used in pathogenicity tests. To prepare the inocula, discs of wood and bark, ca. five to six mm high and nine and five mm in diameter, were taken from living, healthy branches of sweet chestnut and sessile oak using metal cork borers. After autoclave sterilisation, the fragments were plated on the surface of the Petri dishes already overgrown with two *C. parasitica* isolates incubated at 20°C on the MEA media for four weeks. The plated wood fragments were further incubated for an additional four weeks at 20°C in the dark.

To test the pathogenicity in the controlled conditions, two-year-old plants of *C. sativa* and *Q. petraea* were grown in 1-litre individual pots in the laboratory conditions at room temperature. The plants have a local origin, *C. sativa* grown from seeds from the Vranje provenance and *Q. petraea* from the Kruševac provenance. At 5–7 cm above the soil level, the bark was surface sterilised using 70% ethanol, and pieces 5 mm in diameter were removed using a sterilised metal cork borer. Pieces of the wood previously overgrown with the fungal mycelium of the two *C. parasitica* isolates were placed upside-down on the exposed wood tissue and sealed with parafilm. In total, 50 plants of each host were inoculated (25 plants per tested isolate), while the control group consists of 25 plants inoculated with the sterilised fragments.

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Figure 2. The pathogenicity test of *Cryphonectria parasitica* (Murrill) M.E. Barr in the controlled conditions: plants of *Castanea sativa* Miller three months (**A**), eight months (**B**) after inoculation; plants of *Quercus petraea* (von Mattuschka) Lieblein three months (**C**), eight months (**D**) after inoculation

1 – isolate from *C. sativa*; 2 – isolate from *Q. petraea*; 3 – control

Incubation was at 22–25°C in natural light in the laboratory conditions. The appearance of cankers, fruiting bodies and the decline of the inoculated plants were monitored after 3, 8, 12 and 18 months of inoculation (Figure 2). Reisolations were made from the edges of the necrotic zones and from the fruiting bodies that formed.

To test the pathogenicity in the field conditions, two plots (Vršac Mts.) within the infected stand of the sessile oaks, each containing 60 healthy, young trees, were designated (Figure 3A). At approximately 1.3 m high on the stems, the bark was surface sterilised using 96% ethanol, and pieces 9 mm in diameter were removed using a sterilised cork borer. Fragments of wood and bark overgrown with *C. parasitica* mycelium were placed on the exposed wood tissue of 30 trees in the first plot, while 15 trees were inoculated by placing pieces of the inoculated wood directly on the healthy bark without wounds. The same treatment was performed in the second plot. The control group of the trees numbered 15 in each plot and were wounded with a 9 mm cork borer at 1.3 m but were not inoculated. An isolate of *C. parasitica* originating from *C. sativa* (KF276606) was used in the first plot (Figure 3A), where the tree diameter at breast height (DBH) averaged 7.7 ± 0.16 cm, while an isolate originating from *Q. petraea* (KF276605) was used in the second plot, where the DBH averaged 8 ± 0.25 cm. All the inoculation sites were sealed with plastic folia (Figure 3B). The inoculation was performed during the first half of July 2014. To check the viability of the inocula, the experiment was assessed after three and 11 months, and randomly chosen control reisolations were performed from all the groups, including the control one. The experiment was completed after 27 months and the necroses were measured. Reisolations were made from the edges of the necrotic zones or from the edges of the wounded wood tissues of all the trees subjected to the experiment.

Based on the values of length, and width, measured after the pathogenicity in the field conditions, the area of each necrosis was calculated using the mathematical formula for elliptic surfaces. For each treatment, the mean ($\bar{x}$) and standard error (± SE) of the necrosis length, width and area were calculated. Analysis of the variance was performed using a generalised linear model, with a significance level of $\alpha = 0.05$. Testing of the significance of the differences in the mean necrosis length, width and surface between the different *C. parasitica* isolates and negative control

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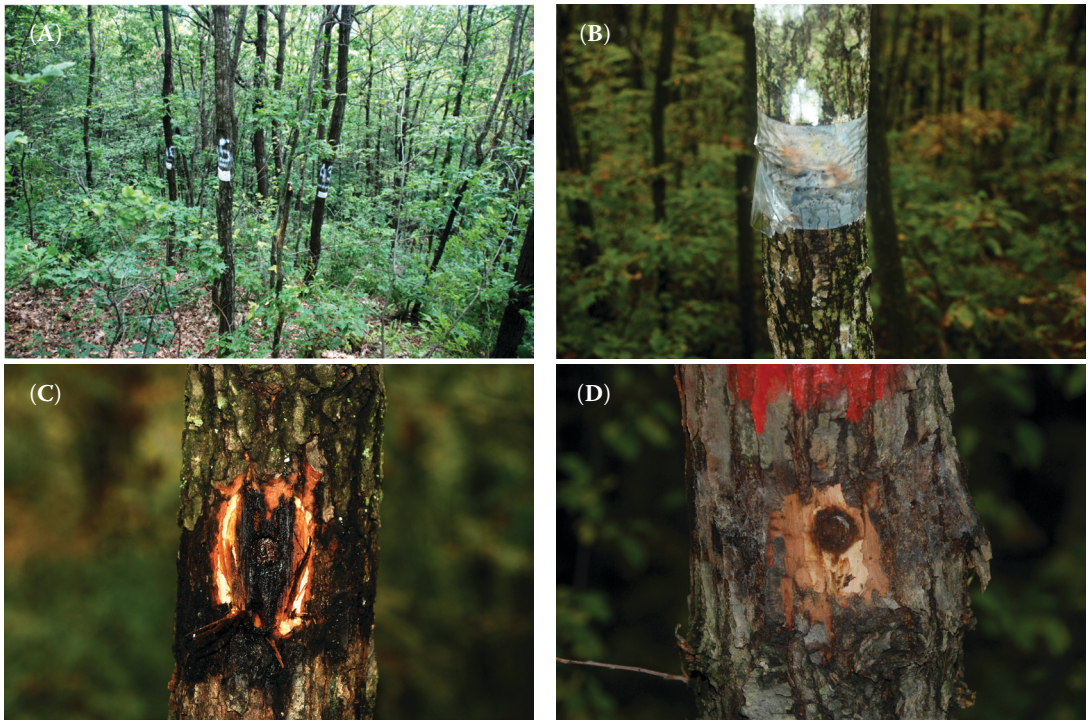

Figure 3. The pathogenicity test of *Cryphonectria parasitica* (Murrill) M.E. Barr in the field conditions: plot with *Quercus petraea* (von Mattuschka) Lieblein trees inoculated with the isolate from the sweet chestnut in field plot 1 (**A**), inoculated *Q. petraea* tree and covered with plastic folia – detail (**B**), a stem necrosis 27 months after inoculation with the isolate from *Q. petraea* (KF 276605) (**C**), a control tree in field plot 2 (**D**)

was performed using Tukey's HSD post hoc test (α = 0.05). Statistical analyses were performed with the RStudio software (Version 1.1.383, 2017).

RESULTS

Chestnut blight presence in Serbia

Presence of chestnut blight was confirmed in five out of the six studied localities in Serbia (Table 1). In the stand near the Vršac Mts. both the sweet chestnut and sessile oak were positive for the presence of *C. parasitica* (Table 1). Namely, the symptoms of the decline in this locality were recorded in the natural sessile oak forest at ~0.5 ha, and 17 trees displayed dieback in the crowns and branches, yellowing of the leaves, increased crown transparency, and occurrence of cankers and necroses on the bark. Additionally, the planted sweet chestnut trees are present near the declining sessile oaks in this area, with ca. 100 young sweet chestnut trees showing typical symptoms of chestnut blight, including the appearance of cankers and the fruiting bodies of *C. parasitica*. In the rest of the stands, only the sweet chestnut was infected, while the sessile or Hungarian oak were negative for the presence of *C. parasitica* (Table 1).

Isolation of *Cryphonectria parasitica* from the sweet chestnut and sessile oak in Serbia

In total, five isolates were obtained from sessile oak trees sampled near the Vršac Mts. (Table 1). All isolates obtained from the sessile oak originated from the symptomatic tissue, and no fruiting bodies were recorded on the symptomatic bark. Also, ten isolates from ten randomly chosen trees were obtained from the declining sweet chestnut trees in this area (Table 1). Eight isolates were obtained from five sampled cankers in the declining planted forest near Kruševac, while 59 isolates were obtained from the cankers of the declining sweet chestnut trees near Vranje (Table 1).

Vegetative compatibility types

Among the isolates obtained from the area of the Vršac Mts. and from the area of Kruševac, only one VC type was recorded for both hosts after the compat-

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ibility tests: all the isolates from both the sessile oak and sweet chestnut belonged to the EU-12 VC type.

Among the isolates from Vranje, three different VC types were recorded: EU-1, EU-2 and EU-12. The low value of the Shannon-Wiener's index (H' = 0.647836) reflects the small diversity of the VC types in this area. Of 59 total isolates, 43 were compatible with EU-12 (~72.9%), 15 with EU-2 (~25.4%), and a single isolate with EU-1 (~1.7%). The overall S/N ratio for the studied localities was 0.04.

Occurrence of hypovirulence

All the isolates had a typical orange colony colour after 20 days of incubation; hence, they were considered *Cryphonectria* hypovirus-free.

Molecular identification

The sequence analysis of the ITS region covered 582 bp. After BLAST analyses of the obtained sequences, which were compared to the closest sequences in GenBank (KM199767, KF220299 and KF220298), the identities of the *C. parasitica* sequenced isolates were 100% with E-values of 0.0. The sequences of two studied isolates are deposited in Genbank (accession numbers KF276605 and KF276606).

Pathogenicity test

Pathogenicity in the controlled conditions. Eighteen months after inoculation, over 70% of the inoculated plants showed a decline, and the experiment was concluded. Numerous fruiting bodies were recorded at the stem bases of the killed sweet chestnut trees. Reisolations were successful from all the inoculated chestnuts (100%), and both isolates were infective to the tested plants (Table 2), causing the decline of 88 and 76% of the chestnuts.

However, in the case of the sessile oak, each isolate caused the decline of 52% of the plants (Table 2). No fruiting bodies were recorded on the oaks. Reisolations were successful from all the inoculated sessile oak plants (100%).

The control plants of both species remained healthy, showed no signs of infection, the inoculation sites were covered with callus tissue. The reisolations from the controls were negative.

Pathogenicity in the field conditions. Both isolates were pathogenic to sessile oak trees under the condition of the previous bark wounds (Figure 3C), while no infections of the tissues occurred in the control groups (Figure 3D). Based on control reisolations, *C. parasitica* was active in the necrotic tissues and could easily be isolated from the necrotic margins. After 27 months of inoculation, numerous symptomatic trees within the inoculated groups were recorded (Table 3), and

Table 2. The pathogenicity of *Cryphonectria parasitica* (Murrill) M.E. Barr on the sweet chestnut and sessile oak in the controlled conditions

Treatment	No. of inoculated plants		No. of declining plants (%)		No. of plants without infection	
	sweet chestnut	sessile oak	sweet chestnut	sessile oak	sweet chestnut	sessile oak
KF276606	25	25	22 (88)	13 (52)	3	12
KF276605	25	25	19 (76)	13 (52)	6	12
Control	25	25	0	0	25	25

KF276606 – isolate from the sweet chestnut; KF276605 – isolate from the sessile oak

Table 3. The mean values, standard errors, and Tukey's post hoc tests for the bark necroses caused by *Cryphonectria parasitica* (Murrill) M.E. Barr and the results of the underbark inoculation pathogenicity test on *Quercus petraea* (von Mattuschka) Lieblein in the field conditions after 27 months

Treatment	Trees with symptoms in the crown	Trees with necroses	*Q. petraea* necroses (mean ± SE + Tukey's test)		
			length (mm)	width (mm)	area (mm^2)
Control	0	0	1.14 ± 0.03^a	1.14 ± 0.03^a	1.03 ± 0.052^a
KF276606	24	30	6.68 ± 0.29^c	4.0 ± 0.18^c	20.92 ± 1.193^c
KF276605	29	30	5.36 ± 0.28^b	3.41 ± 0.19^b	15.23 ± 1.373^b

KF276606 – isolate from the sweet chestnut; KF276605 – isolate from the sessile oak; the different letters behind the values indicate significant differences ($\alpha = 0.05$)

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Table 4. The estimates, *t*-values, *P*-values and residual deviances from the generalised linear model for the tested parameters in the underbark inoculation trial with *Quercus petraea* (von Mattuschka) Lieblein

Necroses		Treatment			Residual deviance
		control	KF276606	KF276605	
Length	estimate	0.13	1.77	1.55	5.63
	t-value	3.13	29.87	26.16	
	P-value	**< 0.010**	**0.000**	**0.000**	
Width	estimate	0.13	1.26	1.1	4.64
	t-value	3.007	20.88	18.25	
	P-value	**< 0.010**	**0.000**	**0.000**	
Area	estimate	0.03	3.01	2.69	14.47
	t-value	0.489	31.25	27.95	
	P-value	**0.63**	**0.000**	**0.000**	

KF276606 – isolate from the sweet chestnut; KF276605 – isolate from the sessile oak; the significant effects are noted in bold; the degree of freedom for the residual deviance = 87

the experiment was concluded. The dieback of one or more branches was the most frequent symptom of both isolates, but two trees inoculated with the isolate from the sweet chestnut showed dieback at the top of the crowns. Additionally, all 60 inoculated trees showed cankers and necroses (Figure 3C) that were measured 27 months after inoculation. Reisolations were successful from the cankers of all the inoculated trees (100%), while reisolations from the control group were negative. The statistical analyses showed that different *C. parasitica* isolates and the negative control significantly influenced the tested parameters, except of the control on the surface areas of the necroses (Table 4). The isolate from the sweet chestnut caused statistically significantly larger cankers compared to both the isolate from the sessile oak and the control (Table 3). The results of Tukey's test are shown in Table 3.

DISCUSSION

The presence of *C. parasitica* on the sessile oak and the sweet chestnut in Serbia was confirmed by the isolation, the morphology and the molecular identification, and the EU-12 VC type was the most frequent in the studied localities. After the record of the chestnut blight fungus in 1975 for the first time in Serbia (Marinković & Karadžić 1985), this pathogen sped to the other natural stands and planted forests. In later studies, *C. parasitica* has been recorded on sessile oak trees in Serbia (Karadžić & Milenković 2013). Also, Radulović (2013) reported the presence of *C. parasitica* among the other mycosis in natural stands and localities where individual sweet chestnut trees occur. However, no detailed studies were performed to characterise this pathogen on sweet chestnut trees in Serbia. In addition to one previously studied locality where *C. parasitica* has been recorded on the sessile oak (Karadžić & Milenković 2013), five additional localities were studied for the presence of *C. parasitica* in Serbia (Table 1). In this study, *C. parasitica* was very common in the natural and planted sweet chestnut stands near Vranje (southern Serbia). In this area, the sweet chestnut appears in the *Querco-Castanetum metochiense* (Glišić 1975) community, together with the sessile oak (*Q. petraea*), the black ash (*Fraxinus ornus* Linnaeus), the oriental hornbeam (*Carpinus orientalis* Miller) and other xerothermic species, but the only infected species in these sites was the chestnut, the oak trees were disease free. However, near the Vršac Mts., sessile oak trees occur in several natural stands, while the sweet chestnut has been introduced artificially in some areas. The majority of the sweet chestnut stands in Serbia, including both natural and planted forests, are affected by chestnut blight, and almost all the trees in these forests are affected with the disease (personal observations). Nevertheless, one very rare sweet chestnut stand that remained healthy and free of the chestnut blight fungus, the planted forest in Kozji Dol (Table 1), was also evaluated during the current study.

In the overview studies by Robin and Heiniger (2001), 40 different VC types were recorded in Europe, and EU-2 was dominant in western and north-western Europe, while EU-12 was dominant in southern and eastern Europe. The results obtained in these studies corresponded to our data, in which EU-12 was the most dominant VC type in Serbia in both recorded hosts. Also, the diversity of the VC type was low in Serbia, and only three different VC types were recorded. Moreover, the *S*/*N* ratio for all the studied localities was 0.04. A similar situation of a low VC type diversity was recorded in the neighbouring countries in south-eastern Europe, including FYR Macedonia (Sotirovski *et al.* 2004), Albania (Myteberi *et al.* 2013), Bulgaria (Risteski *et al.* 2013), and Romania (Adamčíková *et al.* 2015). However, a higher VC type diversity was recorded in the neighbouring Bosnia and Herzegovina (Trestic *et al.* 2001) and Croatia (Krstin *et al.* 2008) in the west and Hungary in the north (Radócz 2001). In Slovenia, although not a

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neighbouring country, a high VC type diversity was also recorded (Krstin *et al*. 2011), with the longest chestnut blight history from the former Yugoslav countries. Moreover, the trend of an increasing VC type diversity was recorded in Croatia, where Mlinarec *et al*. (2018) recorded significantly a higher level of VC type diversity among populations of *C. parasitica* using a multi-locus PCR assay, compared to the results previously obtained by Krstin *et al*. (2008). Also, in the future, more detailed samplings and VC type determinations, and the inclusion of advanced molecular techniques are required to determine the real VC type diversity in Serbia.

The large number of VC types is the largest problem in the biological control of the disease. Sexual reproduction includes genetic recombination, which most often creates many loci, while asexual reproduction simplifies the application of the hypovirulence as a biocontrol (Sotirovski *et al*. 2004; Perlerou & Diamandis 2006). In this respect, research into the VC type diversity in Serbia is very important, since no biocontrol application trials have been applied in Serbia against this pathogenic fungus so far.

The EFSA Panel on Plant Health (PLH) (2014) published a scientific opinion on the pest categorisation of *C. parasitica*. This pathogen is listed in Annex IIAII of Directive 2000/29/EC. According to this opinion, the potential consequences of the damage caused by *C. parasitica* mainly concern the yield losses of the fruit and wood of the chestnut, the reduced biodiversity and habitat loss for the associated organisms, but the main threat in the future is against the oak stands.

During the artificial inoculation of oaks and sweet chestnuts in Slovakia, Adamčíková *et al*. (2010) recorded smaller cankers and slower development of the necrosis on the inoculated oaks, and these results also showed that the virulence of the isolates from the oak was the same as that of the isolates from the sweet chestnut. Moreover, the isolate QR2, obtained from the oak, caused the largest cankers on both the tested hosts in the above-mentioned experiment. However, these results disagree with the results obtained in our pathogenicity test, where the isolate from the sweet chestnut was more aggressive, causing statistically significantly larger necroses (Table 3). This result can be explained by a difference in the virulence among the isolates; for this trial, we used an isolate originating from the sweet chestnut near Vranje, not from the infected chestnuts near the Vršac Mts.

Although more trees were symptomatic in the case of the isolate originating from the sessile oak, these symptoms constituted the decline of one or a few branches in most cases, and no signs of dieback at the top of the crowns were recorded.

Based on the results of the pathogenicity test in the laboratory conditions, the sessile oak was more resistant to *C. parasitica* because, compared to sweet chestnut plants, fewer sessile oak plants declined within the same period. The isolate from the sweet chestnut was more aggressive towards sweet chestnut plants.

The source of the oak tree infections in Serbia is probably the previously infected sweet chestnut trees. However, the occurrence of *C. parasitica* was confirmed in the infected sweet chestnut trees near the Vršac Mts. by the recorded symptoms and the isolations of this fungus (Table 1). Moreover, our vegetative tests revealed that all the isolates belonged to the same VC type, EU-12 that was obtained from the sessile oak. Similar results were obtained in previous studies in Slovakia (Adamčíková *et al*. 2010) and Greece (Tziros *et al*. 2015).

Based on the results of the field observations and pathogenicity tests in the Vršac Mts. locality, *C. parasitica* spreads relatively slowly in sessile oaks after infection, and a longer period is required before the trees decline. However, this progression is different than that of the infection in sweet chestnut trees, where *C. parasitica* infects readily and spreads quickly from the infection sites (Griffin 1986).

Additionally, Tziros *et al*. (2015) reported the presence of *C. parasitica* on the Hungarian oak (*Q. frainetto*) in Greece, where it caused cankers but not mortality in this oak species. Since the Hungarian oak shares a climatogenic community with the Turkey oak (*Quercus cerris* Linnaeus) over large areas of Serbia, and in some areas, these trees mix with sessile oaks, there is a high risk of disease spreading in these stands, which should be carefully monitored in the future.

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References

Adamčíková K., Kobza M., Juhásova G. (2010): Characteristics of the *Cryphonectria parasitica* isolated from *Quercus* in Slovakia. Forest Pathology, 40: 443–449.

Adamčíková K., Ondrušková E., Kádasi-Horáková M., Botu M., Kobza M., Achim G. (2015): Distribution and popula-

https://doi.org/10.17221/38/2018-PPS

tion structure of the chestnut blight fungus in Romania. Plant Protection Science, 51: 141–149.

Bissegger M., Rigling D., Heiniger U. (1997): Population structure and disease development of *Cryphonectria parasitica* in European chestnut forests in the presence of natural hypo-virulence. Phytopathology, 87: 50–59.

Dallavalle E., Zambonelli A. (1999): Epidemiological role of *Cryphonectria parasitica* isolated from hosts other than chestnut. European Journal of Forest Pathology, 29: 97–102.

EFSA Panel on Plant Health (PLH) (2014): Scientific opinion on the pest categorisation of *Cryphonectria parasitica* (Murrill) Barr. EFSA Journal 2014, 12: 3859. doi: 10.2903/j.efsa.2014.3859

Gardes M., Bruns T.D. (1993): ITS primers with enhanced specificity for Basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology, 2: 113–118.

Glišić M.V. (1975): Sweet chestnut (*Castanea sativa*) in Serbia and its biological and ecological variability. Glasnik Instituta za *šumarstvo* i drvnu industriju, 36 (Special Issue): 1–211. (in Serbian with French and German summary)

Griffin G.J. (1986): Chestnut blight and its control. Horticultural Reviews, 8: 291–336.

Gryzenhout M., Wingfield B.D., Wingfield M.J. (2009): Taxonomy, Phylogeny, and Ecology of Bark-inhabiting and Tree-pathogenic Fungi in the *Cryphonectriaceae*. St. Paul, APS Press.

Hall T.A. (1999): BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95–98.

Karadžić D., Milenković I. (2013): *Cryphonectria parasitica* (Murill.) Barr occurrence of parasitic fungus on sessile oak in Serbia. Šumarstvo, 1–2: 1–8. (in Serbian with English abstract and summary)

Kirk P.M., Cannon P.F., Minter D.W., Stalpers J.A. (2008): Dictionary of the Fungi. 10th Ed. Wallingford, CABI Publishing.

Krstić M. (1950): *Endothia parasitica* in our country. Zaštita bilja, 2: 113–116. (in Serbian)

Krstin L., Novak-Agbaba S., Rigling D., Ćurković-Perica M. (2011): Diversity of vegetative compatibility types and mating types of *Cryphonectria parasitica* in Slovenia and occurrence of associated *Cryphonectria hypovirus 1*. Plant Pathology, 60: 752–761.

Krstin L., Novak-Agbaba S., Rigling D., Krajacic M., Curcovic-Perica M. (2008): Chestnut blight fungus in Croatia: Diversity of vegetative compatibility types, mating types and genetic variability of associated *Cryphonectria hypovirus 1*. Plant Pathology, 57: 1086–1096.

Marinković P., Karadžić D. (1985): The most important diseases in nurseries, plantations and forests of Kosovo. Šumarstvo, 2–3: 29–38. (in Serbian with English summary)

Mlinarec J., Ježić M., Ćosić J., Ćurković-Perica M. (2018): Multilocus PCR assay reveals high diversity of vegetative compatibility types in populations of *Cryphonectria parasitica* in Croatia. Plant Pathology, 67: 741–749.

Myteberi I.F., Lushaj A.B., Keča N., Lushaj A.B., Lushaj B.M. (2013): Diversity of *Cryphonectria parasitica*, hypovirulence, and possibilities for biocontrol of chestnut canker in Albania. International Journal of Microbiology Research and Reviews, 1: 11–21.

Nash B.L., Stambaugh W.J. (1982): Disease incidence, symptomology, and vegetative compatibility type distribution of *Endothia parasitica* on oak and chestnut hosts in North Carolina. In: Clay Smith H., MacDonald W.L. (eds): Proceedings of the USDA Forest Service American Chestnuts Cooperators Meeting, Jan 5–7, 1982, Morgantown, USA: 74–82.

Perlerou C., Diamandis S. (2006): Identification and geographic distribution of vegetative compatibility types of *Cryphonectria parasitica* and occurrence of hypovirulence in Greece. Forest Pathology, 36: 413–421.

Radócz L. (2001): Study of subpopulations of the chestnut blight (*Cryphonectria parasitica*) fungus in the Carpathian basin. Forest Snow and Landscape Research, 76: 368–372.

Radócz L., Tarcali G. (2005): Identification of natural infection of *Quercus* spp. by chestnut blight fungus (*Cryphonectria parasitica*). Acta Horticulturae, 693: 617–619.

Radulović Z. (2013): Most often mycosis and pseudomycosis on chestnut, with special review to the species of *Cryphonectria parasitica* (Murrill) Barr. [Ph.D. Thesis.] Belgrade, University of Belgrade. (in Serbian with English summary)

Risteski M., Milev M., Rigling D., Milgroom M.G., Bryner S.F., Sotirovski K. (2013): Distribution of chestnut blight and diversity of *Cryphonectria parasitica* in chestnut forests in Bulgaria. Forest Pathology, 43: 437–443.

Robin C., Heiniger U. (2001): Chestnut blight in Europe: Diversity of *Cryphonectria parasitica*, hypovirulence and biocontrol. Forest, Snow and Landscape Research, 76: 361–367.

Robin C., Anziani C., Cortesi P. (2000): Relationship between biological control, incidence of hypovirulence, and diversity of vegetative compatibility types of *Cryphonectria parasitica* in France. Phytopathology, 90: 730–737.

Shannon C.E., Weaver W. (1949): The Mathematical Theory of Communication. Urbana, University of Illinois Press.

Sotirovski K., Papazova-Anakieva I., Grunwald N., Milgroom M. (2004): Low diversity of vegetative compatibility types and mating type of *Cryphonectria parasitica* in the southern Balkans. Plant Pathology, 53: 325–333.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. (2013): MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30: 2725–2729.

https://doi.org/10.17221/38/2018-PPS

Trestic T., Uscuplic M., Colinas C., Rolland G., Giraud A., Robin C. (2001): Vegetative compatibility type diversity of *Cryphonectria parasitica* populations in Bosnia-Herzegovina, Spain and France. Forest Snow and Landscape Research, 76: 391–396.

Turchetti T., Maresi G., Santagada A. (1991): *Cryphonectria parasitica* blight on different hosts in the Cilento region. Monti E Boschi, 42: 54–58. (in Italian)

Tziros G.T., Nakopoulou Z.G., Diamandis S. (2015): *Cryphonectria parasitica*, the chestnut blight fungus, causes cankers on *Quercus frainetto* in Greece. Australasian Plant Disease Notes, 10: 19. doi: 10.1007/s13314-015-0171-4

White T.J., Bruns T., Lee S., 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. (eds): PCR Protocols: A Guide to Methods and Applications. San Diego, Academic Press: 315–322.

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