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First record of chestnut blight on Quercus petraea (Fagaceae) in Bulgaria

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Abstract. Chestnut blight is one of the most destructive diseases on species of *Castanea* genus (Fagaceae). The causal agent of the disease is the ascomycete fungus *Cryphonectria parasitica* (Valsaceae). In the period 2018-2020, chestnut blight was found on both European (sweet) chestnut (*Castanea sativa*) and sessile oak (*Quercus petraea*) trees in mixed chestnut-oak stands planted in the region of State Forest Enterprise (SFE) Simitli (Pirin Mt.) and SFE Petrich (Belasitsa Mt.). The present study provides new data on the first detection of damage caused by *Cryphonectria parasitica* on *Quercus petraea* plantations in southwestern Bulgaria. The frequency and impact of the disease, the variety of vegetative compatibility types of the pathogen, as well as the molecular identification and mating type of the pathogen from oak samples were characterized.

Key words: *Cryphonectria parasitica, Quercus petraea,* new record, Bulgaria.

Introduction

The ascomycete fungus *Cryphonectria parasitica* (Murrill) M.E.Barr, the causal agent of chestnut blight, was introduced into the North America from Asia in the early 1900s and has destroyed almost all of the American chestnut, *Castanea dentata* (Marsh.) Borah. stands (Anagnostakis, 1987). In Europe, chestnut blight was first observed in northern Italy in 1938, and spread rapidly to most of the European (sweet) chestnut (*Castanea sativa* Mill.) growing areas (Robin & Heiniger, 2001). However, the damage caused by this pathogen in Europe was much less severe than in North America and many European chestnut stands recovered from the disease as a result of the natural occurrence of hypovirulent *C. parasitica* strains (Heiniger & Rigling, 1994).

In Bulgaria, chestnut blight was firstly detected in 1993 in *Castanea sativa* stands in Belasitsa Mt. and the Eastern Rhodopes (Petkov & Rosnev, 2000). In the following years, significant extension of the disease into chestnut natural stands and artificial plantations was observed (Petkov & Rossnev, 2001; Georgieva et al., 2013, Filipova & Georgieva, 2018).

Cryphonectria parasitica affects shoots, branches and stems of the sweet chestnut forming cankers that grow inside the inner bark and cambium. A number of other tree species are also affected by chestnut blight. Some studies have reported that *C. parasitica* has the ability to infect oak trees, but as a pathogen it is not as serious as in chestnut (Batson & Witcher, 1968). The pathogen was found on oak of Virgil (*Quercus virgiliana* (Ten.) Ten.) in the North America.

In Europe, the fungus *C. parasitica* was detected on various *Quercus* species in different countries – Greece (Tziros et al., 2015), Czech Republic (Haltofová et al., 2005), Italy (Biraghi, 1950; Dallavalle & Zambonelli, 1999) and Slovakia

(Adamčíková et al., 2010). In mixed stands of chestnut and other broad-leaved trees in Italy in 1994, *C. parasitica* was recorded on oak species (*Quercus petraea* (Matt.) Liebl., *Q. pubescens* Willd., *Q. robur* L.), hornbeam (*Carpinus betulus* L.), ashes (*Fraxinus* spp.) and maples (*Acer* spp.) (Frigimelica & Faccoli, 1999). Symptoms were detected in mixed plantation of *Q. petraea* and *C. sativa* near Wengen, Switzerland (Bissegger & Heiniger, 1991). Researches in Serbia (Karadžić & Milenković, 2013; Karadžić et al., 2019) have observed damage from *C. parasitica* on *Q. dalechampii* Ten. and *Q. petraea*. Tarcali et al. (2009) have also shown that oaks are susceptible to *C. parasitica*, especially *Quercus rubra* L., *Q. petraea* and *Q. robur*.

Currently, the monitoring of the health status of sweet chestnut stands in Bulgaria has confirmed that the disease is spreading and gaining high impact on natural stands and plantations in different mountain areas: southwest Bulgaria (Belasitsa, Ograzhden, West Pirin, and Rila Mts.), southeast Bulgaria (the Eastern Rhodopes) and West Balkan Range (Filipova, 2021).

The aim of the present study was to investigate, confirm and report the first detection of a damage caused by *Cryphonectria parasitica* on sessile oak (*Quercus petraea*) trees in mixed chestnut-oak stands in southwest Bulgaria.

Materials and methods

Studied localities and sampling

The study was conducted in the period 2018-2020. Three sample plots with mixed chestnut-oaks stands were monitored in southwestern Bulgaria: two chestnut plantations planted in natural sessile oak (*Quercus petraea*) forests near Brezhani village (Pirin Mt.) and one natural chestnut stand mixed with sessile oak trees in Belasitsa Mt. (Fig. 1, Table 1).

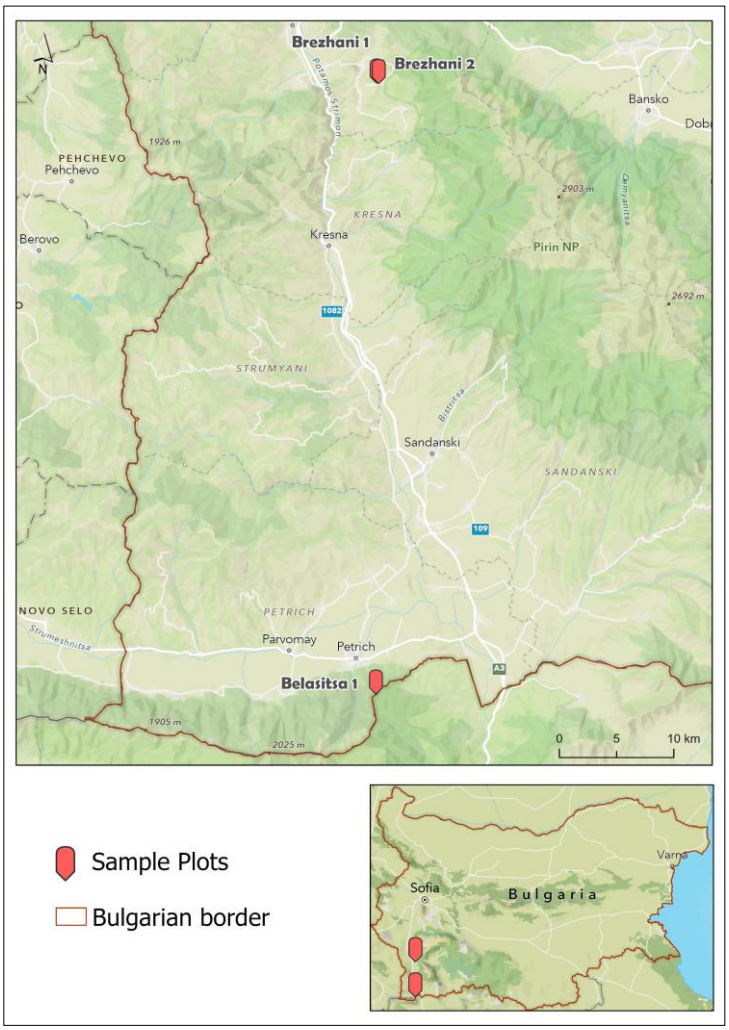

Fig. 1. Map of the sample plots.

Table 1. Characteristics of the sample plots.

N	State Forest Enterprise (Mountain)	Sample plot	Tree species, %	Age of the plantation in years	Geographical coordinates	Altitude, m
1	Simitli (Pirin)	Brezhani 1	*Castanea sativa*, 90 *Quercus petraea*, 10	45	41.850112° N; 23.202389° E	808
2	Simitli (Pirin)	Brezhani 2	*Castanea sativa*, 10 *Quercus petraea*, 90	120	41.849796° N; 23.204615° E	825
3	Petrich (Belasitsa)	Belasitsa 1	*Castanea sativa*, 90 *Quercus petraea*, 10	170	41.370357° N; 23.229687° E	871

Forty sessile oak trees with symptoms of blight on the stems and branches were randomly selected. The degree of defoliation was evaluated according to the methodology of International Cooperative Programme 'Forests' (Eichhorn et al., 2010).

Samples of bark with necrosis (approximately 3×3 cm) were taken and placed in a separate sterile polyethylene bag on which the locality number and tree number were recorded. The samples were transferred to the Laboratory of Phytopathology at Forest Research Institute, Sofia.

Isolation of mycelium

All collected samples were stored at -20°C until initiation of mycelium isolations according to the methodology of Cortesi et al. (1996).

The samples were surfacely sterilized with 70% ethanol for 10 s, washed with 0.5% sodium hypochlorite and afterwards - with sterile water. Wet chambers were prepared for four samples (three chestnut samples and one oak sample). The chestnut samples were from regions Belasitsa 1 and other two localities from other study while the sesile oak sample was from Brezhani 2 and Belasitsa 1.

One week later, one isolate per tree was randomly chosen and transferred to malt extract agar (MEA) nutrient medium. All isolated colonies of *Cryphonectria parasitica* mycelium were stored at 22°C.

The identification of fungal pathogens was carried out on the basis of their morphological and cultural characteristics.

Vegetative compatibility

All oak isolates were tested for vegetative compatibility with the European strains EU-1, EU-2, EU-5, EU-10, EU-12 and EU-22 that are the most distributed on Balkan Peninsula (Cortesi, 1998).

Molecular identification

Fungal mycelium from Brezhani 2 (oak, 2 samples), Belasitsa 1, and samples from two different localities (chestnut, 1 sample from region) was carefully collected using a scalpel, transferred to 2 ml tubes and frozen at -80°C for further DNA isolation. Material was initially ground in liquid nitrogen and DNA was extracted by DNeasy Plant Mini kit (Qiagen) following the standard protocol suggested by the manufacturer; three eluates were obtained from each DNA preparation. The concentration and purity of eluted DNAs were assessed by NanoDrop 2000 (Thermo Scientific) and the respective eluates were combined. Sample from Brezhani 1 it was not collected, because Brezhani 1 and Brezhani 2 sample plots are close each other.

PCR amplification was performed with universal primers targeting the ITS (internal transcribed spacer) region of ribosomal DNA. Two primer combinations with the standard fungal specific primers ITS1F, ITS1 and ITS4 were tested - ITS1F–ITS4 and ITS1-ITS4. PCR amplification was performed in 20 µl PCR reactions containing 1x HOT FIREPol® Blend Master Mix (Solis BioDyne), 0.3 µM primers, 1.5 mM MgCl2 and 50 ng template DNA under the following amplification conditions: Initial denaturation 12 min. at 95°C, 30-35 cycles - denaturation for 1 min. at 95°C, annealing at 56°C for 1 min. and elongation at 72°C for 1.5 min., followed by final elongation at 72°C for 5 min. PCR amplification was performed in Doppio Gradient Thermal cycler (VWR). PCR products were analyzed by neutral gel-electrophoresis. Four microliters of each PCR reaction were loaded along with appropriate molecular marker on 1% agarose gel stained with GoodView (SBS Genetech) and run in 1xTAE buffer; the rest of material was used for further sequencing.

Gels were visualized and photographed by GBOX-CHEMI-XRQ gel documentation system (Syngene) and densitometrically analysed by ImageQuant TL7 software (GE Healthcare) in order to determine the specificity, approximate length and concentration of the amplified PCR products.

Molecular identification of the mating type of *Cryphonectria parasitica* isolates was determined by PCR with the primer pairs Cp-MAT1–1F-R (For ACATCAGTCGTGGAAATGAAAA - Rev ACATCACAAGTCGGCTCCCA) and Cp-MAT1–2F-R (For AGGAATGTGATCTGTGGTGTTCT – Rev AATTTACTTTCACCGCGATTATG) targeting MAT1-1 and MAT1-2 locus, respectively (Cornejo et al., 2019). Initial optimization was performed regarding annealing temperature and MgCl2 concentration; final reactions were performed in 1x HOT FIREPol® Blend Master Mix (Solis BioDyne), 0.3 µM primers, 2 mM MgCl2 and 50 ng DNA template; amplification conditions were: initial denaturation 12 min. at 95°C , 30 cycles - denaturation at 95°C for 1 min., annealing at 58°C for 40 sec. and elongation at 72°C for 1 min., followed by final elongation at 72°C for 5 min. Resulting PCR fragments were resolved in 1.6% agarose gel and analyzed as described above.

Primer synthesis and Sanger sequencing of PCR fragments were performed by Microsynth (Switzerland). Sequence data were edited and analyzed by MEGA 11 software (Tamura et al., 2021); sequence identification and similarity search were performed by BLASTn against NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Assessment of the health status of Quercus petraea trees

The health status of *Quercus petraea* trees in both sample plots Brezhani 1 and Brezhani 2 was significantly deteriorated. There were 80% severely damaged trees with stem necrosis and 20% dead trees. The average crown defoliation score was 88.3% in Brezhani 1 and 86.6% in Brezhani 2.

Necrotic wounds resembling those of chestnut trees and dry branches in the upper part of the crowns were observed. Open wounds with peeling of the bark were detected on the stems (Fig. 2).

In sample plot Belasitsa 1, the number of slightly and moderately damaged *Q. petraea* trees amounted to 87.5%. The percent of dead trees was 2.5. Most of oak trees were with 30% defoliation (average 33.3%).

Fig. 2. Necrotic wound on *Quercus petraea* trees

Isolation of Cryphonectria parasitica from Quercus petraea trees

A total of 27 mycelium isolates of the fungal pathogen *Cryphonectria parasitica* were isolated from *Q. petraea* bark samples. Of these, 23 mycelium isolates were detected from the two sample plots near Brezhani village (85.2%) and 4 mycelium isolates from Belasitsa (14.8%).

Cryphonectria parasitica was identified by the characteristic features of its reproductive organs: a yellow to dark orange stroma, 0.5–3–4 mm in diameter and 1.5–2.5 mm in height, emerging from the cortex with only the upper part protruding, and yellow-orange mycelium formed under the bark.

The asexual structures (pycnidia) observed were 100–300 µm in diameter (single or clustered), red-orange in color, formed as irregular locules, which, when moistened, extruded homogeneous yellowish shoots containing sticky conidia. After a 4-day stay in a humid chamber, the pycnidia developed a yellow dense mycelium, which indicated the presence of the highly virulent strain of the fungus *C. parasitica*. Conidia were observed in all samples with orange mycelia at day 14 post-inoculation.

Vegetative compatibility test

The vegetative compatibility tests were made to sessile oak (*Quercus petraea*) samples from Brezhani 1 and 2 and Belasitsa. The results showed that only strain EU-12 was distributed in the three localities (Fig. 3).

Fig. 3. Results of vegetative compatibility tests.

Molecular identification of Cryphonectria parasitica isolates based on ribosomal ITS sequences

Sequencing of the nuclear ribosomal ITS region was performed in order to confirm the affiliation of four isolates to *C. parasitica*, obtained from oak bark. The ITS1–ITS4 primer combination failed to amplify any fragment (data not shown), whereas the ITS1F–ITS4 primer pair amplified a specific PCR fragment of ~700 bp in length in all four isolates which was further subjected to sequencing (Fig. 4A).

High-quality sequences of 632 bp obtained from each PCR fragment were aligned and the results showed that regarding the amplified ITS fragment all isolates were completely identical. BLAST analysis confirmed their close relationship to the ITS sequences of *C. parasitica* deposited in the NCBI GenBank (Figure 5). BLAST search was performed with the ITS sequences of the isolate obtained from oak and the results were filtered to match records with percent identity of 99-100% and query coverage of 95-100%. Evolutionary relationships tree was constructed based on 25 sequences (4 from the current study and 21 retrieved from NCBI) producing significant alignments, applying the UPGMA method (Sneath & Sokal, 1973). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. All ambiguous positions were removed for each sequence pair (pairwise dele-

tion option). There were a total of 597 positions in the final dataset. Evolutionary analyses were conducted in MEGA 11 (Tamura et al., 2021). All sequences were deposited to The Barcode of Life Data System (BOLD).

Mating type identification of Cryphonectria parasitica isolates

Four fungal samples were analyzed for mating type (three chestnut samples and one sessile oak sample). It has been shown that the primer set Cp-MAT1–1F-R amplified a 145 bp fragment from the MAT1-1 locus and the Cp-MAT1–2F-R primer pair amplified a PCR product of 400 bp from the MAT1-2 locus. In our study, three of the isolates (two from chestnut and one from sessile oak) gave a single band of ~ 150 bp in length with the MAT1-1 specific primers (Fig. 4B, lanes 2, 4 and 5) and no PCR product with the MAT1-2 specific primers., thus establishing the affiliation of these fungal isolates to mating type 1. On the other hand, the fourth isolate from Western Stara Planina (Filipova, 2021) showed a single PCR product with the expected length of ~ 400 bp only with the MAT1-2 specific primers revealing it as belonging to the mating type 2 (Fig. 4B, lane 8).

The phylogenetic tree based on ITS barcode showing the sequence identity between the isolates identified in this study and the *Cryphonectria parasitica* strains published in the NCBI database is shown in Figure 5.

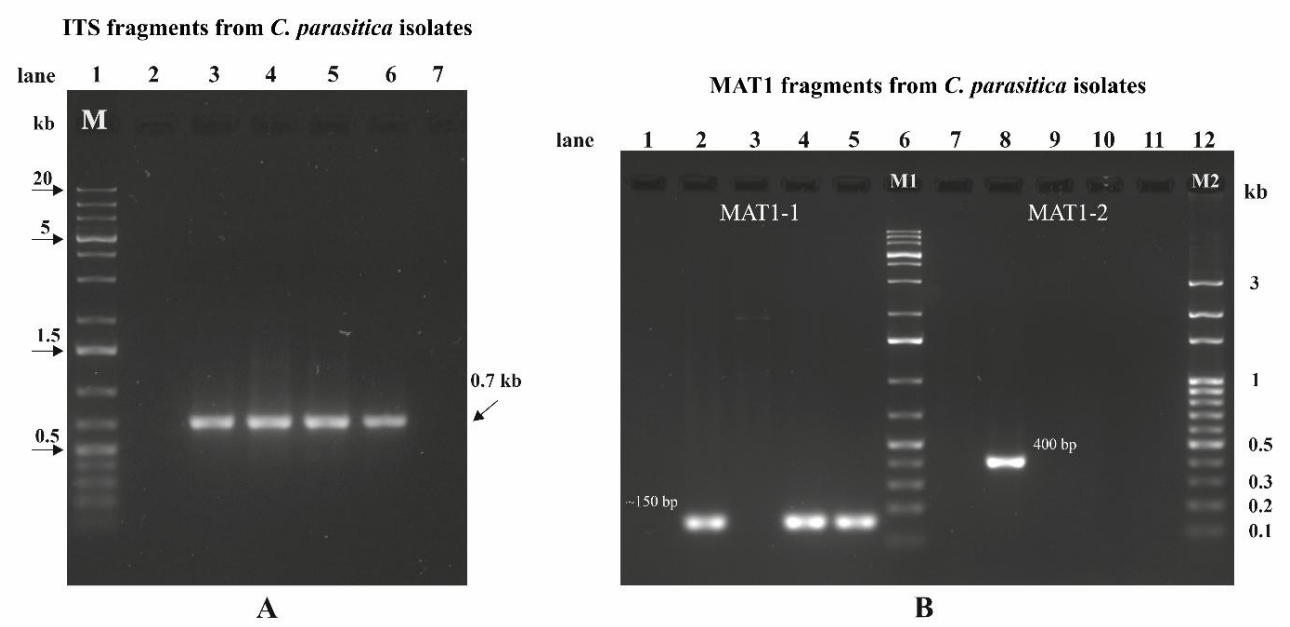

Fig. 4. Molecular identification of *Cryphonectria parasitica* isolates. **(A)** Lane 1 – molecular marker Generuler 1kb+ (Thermo Scientific); lanes 2 and 7 – non-template controls (mQ); lanes 3, 4 and 6 – chestnut isolates; lane 5 – sessile oak isolate. **(B)** MAT1 fragments in *C. parasitica* isolates. Lanes 1-5, MAT1-1 specific primers; lane 6 M1 - DNA маркер GeneRuler 1kb+; lanes 7-11 - MAT1-2 specific primers; lane 12 M2 - 100 bp DNA Ladder (Solis Biodyne); lanes 2, 3, 5, 7, 8, 10 - chestnut isolates; lanes 4 and 9 - sessile oak isolate; lanes 1 and 11 – non-template controls (mQ).

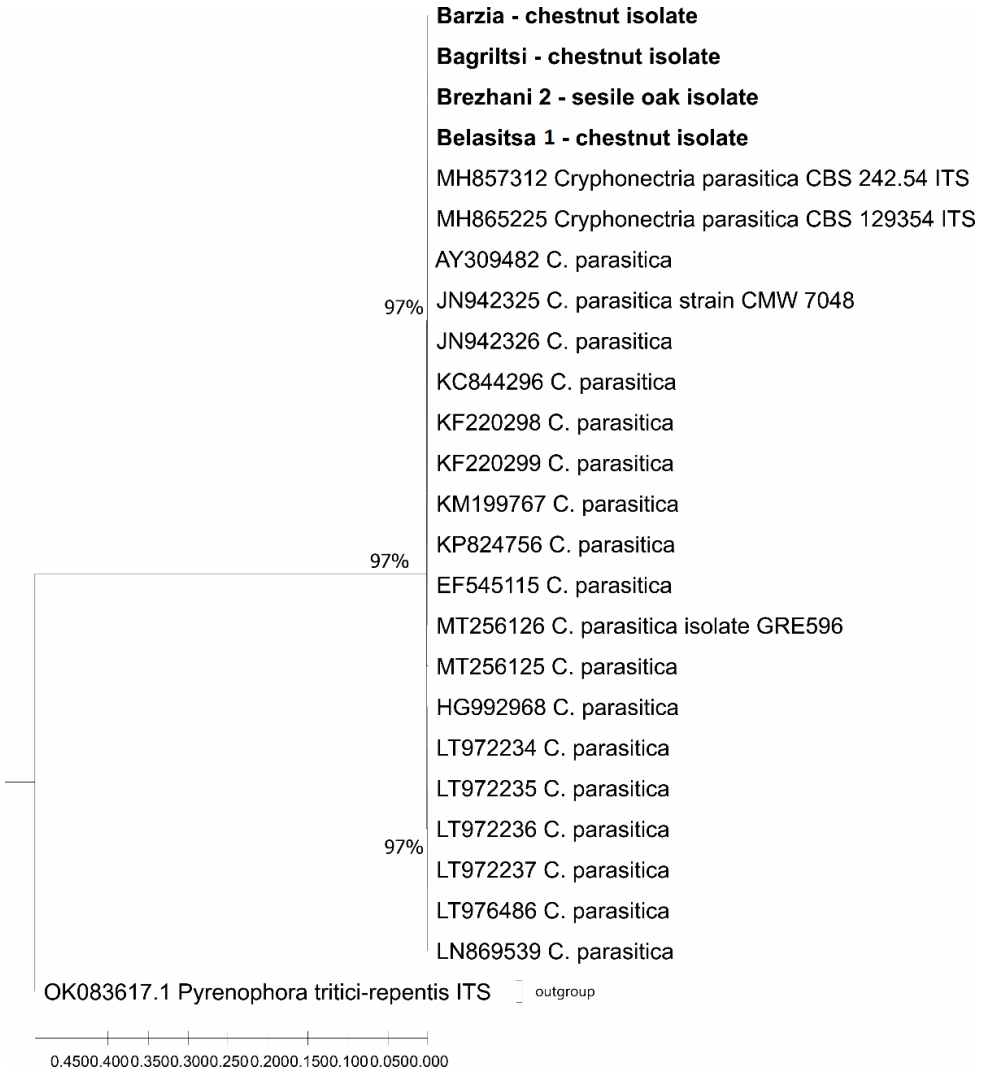

Fig. 5. Phylogenetic tree revealing the evolutionary relationships based on ITS locus. Taxonomic assignment of studied isolates (three from chestnut and one from sessile oak) was performed against accessions in NCBI database (shown with their accession number and organism name). *Pyrenophora tritici* was selected as an outgroup.

Discussion

The European chestnut (*Castanea sativa*) stands in Bulgaria have been managed as the native forests or plantations afforested in the beginning of the 20th century. In the last decades, deterioration of chestnut's health condition was observed due to the influence of different natural and non-native pests and pathogens causing chestnut blight, ink disease, branch and twig deterioration, damages on nuts and leaves, etc. (Petkov & Rossnev, 2000; 2001; Georgieva et al., 2013). Non-native pathogens and pests have caused irreversible damages to chestnut stands in the result of a continuing pathological process for several years. Among them, the most dangerous is *Cryphonectria parasitica* causing chestnut blight disease. With these new data on damage caused by the pathogen *C. parasitica* on sessile oak (*Quercus petraea*) trees, the range of hosts has been expanded. This information is of great importance for future studies of the pathogen distribution in the country.

This study reports new data for the first detection of damage caused by *C. parasitica* on *Quercus petraea* plantations in southwestern Bulgaria. Incidence and impact of the disease, vegetative compatibility type diversity of the pathogen, as well as molecular identification and mating type of the pathogen from oak samples were characterized.

The results of present study showed that only EU-12 vc type of *C. parasitica* was distributed on *Quercus petraea* trees. The virulent EU-12 strain has been reported as dominant in Eastern European countries, as well as in countries in the western and north-western parts of the continent (Robin & Heiniger, 2001). In the neighboring countries of Bulgaria, the dominance of the same virulent strain was found: EU-12 - in the Republic of North Macedonia it occupies 96% (Sotirovski et al., 2004), and in Greece - 88% (Perlerou & Diamandis, 2006). Studies in Serbia found damage from *C. parasitica* on *Quercus dalechampii* and *Q. petraea*, where only EU-12 vc type was isolated (Karadžić & Milenković, 2013; Karadžić et al., 2019). In Greece, *C. parasitica* was reported on *Q. frainetto* in mixed chestnut-oak forests. The pathogen has been found not to kill trees, but infected trees can serve as a reservoir for virulent inoculum of the fungus, with the *C. parasitica* strain detected being EU-12 (Tziros et al., 2015).

Mating type was determined by PCR analysis applying the short-fragments based *C. parasitica* specific assay developed by Cornejo et al. (2019). By means of PCR amplification, the type of sexual reproduction MAT-1 of *C. parasitica* was identified in isolates from sessile oak trees in Pirin Mt. In chestnut stands in the Belasitsa, Pirin and Eastern Rhodope Mountains only MAT-1 type was isolated, but in Western Stara Planina – MAT-2 (Filipova, 2021).

Conclusions

Current knowledge of the incidence and severity of the chestnut blight disease in new host species is important for implementation of timely and effective management strategies applying both biological control with hypovirulent strains of *C. parasitica* and silvicultural methods. The intended outcome of such control programs is the recovery of valuable chestnut-oak ecosystems in Bulgaria.

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