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Cryphonectria parasitica, the chestnut blight fungus, causes cankers on *Quercus frainetto* in Greece

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Abstract In 2011, cankers were observed on Hungarian oak trees (*Quercus frainetto*) in three localities in Greece. The pathogen was identified as *Cryphonectria parasitica* on the basis of morphology and sequence analysis of the internal transcribed region (ITS), part of the elongation factor 1a (EF-1a) and part of the beta-tubulin region (βT). A pathogenicity test was performed and Koch's postulates were confirmed by re-isolation of the fungus *C. parasitica* from artificially inoculated detached twigs. This is the first report of *C. parasitica* as the cause of cankers on Hungarian oak trees in Greece.

Keywords *Quercus frainetto* · *Cryphonectria parasitica* · Sequence analysis · Pathogenicity

The Hungarian oak (*Quercus frainetto*) is a deciduous tree of the beech family (Fagaceae) native to southeastern Europe and Turkey. It is considered as one of the most important forest trees in Greece as it is the dominant oak species, covers large semi-mountainous areas in the entire mainland country and produces highly valued timber (Konstantinidis et al. 2002; Chatziphilippidis and Spyroglou 2006).

Chestnut blight, a bark disease caused by the fungus *Cryphonectria parasitica*, has caused serious damage in coppice forests and chestnut orchards in Europe (Anagnostakis 1987; Locci 2003). In Greece it is spread all over the country where European chestnut (*Castanea sativa*) is present (Perlerou et al. 2002; Perlerou and Diamandis 2006). Nonetheless, data on economic losses attributed to chestnut blight are not available for Greece.

In 2011, cankers were observed on approximately 20 % of Hungarians oak trees in mixed chestnut-oak forests during an evaluation survey aiming to determine the establishment and dissemination of introduced hypovirulence of *Cryphonectria parasitica* on chestnut blight on European chestnut. Cankers were found in the following three localities: Karditsa, Ioannina and Halkidiki. Sixty two oak trees were surveyed in total in the three tested areas (21, 30 and 11 in Karditsa, Ioannina and Halkidiki respectively). The symptoms on infected oaks were similar to those on chestnut but less severe and less conspicuous. Diseased trees exhibited sunken cankers on the trunk, with swelled margins and subsequent cracking of the outer bark (Fig. 1). However, symptoms did not include crown dieback and dead or dying trees.

For pathogen isolation and identification small pieces of infected bark tissue from the growing edge of the cankers were surfaced disinfested in 1 % sodium hypochlorite for 1 min and plated on water agar (WA). Petri dishes with WA medium were then incubated for 3 days at 24 °C and the isolates derived were subcultured on potato dextrose agar (PDA) medium and gave rise to orange colonies. Conidia were straight or slightly curved, hyaline, 2–3 μm long and 0.5–1 μm wide. Six isolates were deposited at the Forest Research Institute Culture Collection (57006, Vassilika, Thessaloniki, Greece) as FRI1536 - FRI1541. In addition, two representative isolates, one from Ioannina and one from Karditsa, were deposited at the Benaki Phytopathological Institute Culture Collection (Athens, Greece) as BPIC 2703 and BPIC 2704 respectively (Table 1). The morphological descriptions and measurements of the

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Fig. 1 Sunken canker on a *Quercus frainetto* tree at the locality of Karditsa (Central Greece) caused by *Cryphonectria parasitica*

fungus were similar to Cryphonectria parasitica (Roane et al. 1986). After close examination of the bark, in the areas surveyed, no perithecia were detected. This finding is in accordance with the previous finding that the teleomorph (perithecial stage) of *C. parasitica* is not known from Greece (Perlerou and Diamandis 2006). The vegetative compatibility (vc) type of all isolates was conducted twice by pairing *C. parasitica* isolates with European vc type tester strains (Cortesi et al. 1998) following the technique described by Bissegger et al. (1997). Only a single vc type was identified from the three localities, the EU-12 type. EU-12 was found to be the dominant vc type in the surrounding chestnuts

Table 1 Isolates used in this study

Isolate	Location of infected trees	GenBank Accession Number		
		ITS	β-tubulin	EF-1a[a]
FRI1536[b]	Karditsa	KM199764	KP659394	KP659400
FRI1537	Karditsa	KM199765	KP659395	KP659401
FRI1538[c]	Ioannina	KM199766	KP659396	KP659402
FRI1539	Ioannina	KM199767	KP659397	KP659403
FRI1540	Ioannina	KM199768	KP659398	KP659404
FRI1541	Halkidiki	KM199769	KP659399	KP659405

[a] Elongation factor 1a

[b] Isolate deposited as BPIC 2704 and [c] as BPIC 2703

(Perlerou and Diamandis 2006). Mycelial DNA was extracted using the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. The ITS1-5.8S-ITS2 region of six single spore cultures was amplified with primers ITS1 and ITS4 (White et al. 1990) while two more loci were used to further characterize the isolates. Partial DNA amplification of the beta-tubulin region (βT) and elongation factor 1a (EF-1a) was performed with primers Bt2B (Glass and Donaldson 1995) and EF1-728F and EF1-968R (Carbone and Kohn 1999) respectively. The amplicons were directly sequenced and deposited in GenBank under the Accession Numbers presented in Table 1. A BLAST search of the GenBank database revealed close identity (99–100 %) with the sequences of various *C. parasitica* isolates (e.g. KF220299 for ITS, AF273470 for b-tubulin and KC851936 for EF-1a). Therefore, the pathogen was identified as *C. parasitica* on the basis of the morphological characteristics and sequence analysis of three genomic regions.

The above *C. parasitica* isolates were examined for occurrence of hypovirulence assessing colony pigmentation. They were considered to be hypovirus infected if they had the white culture morphology typical of infection with CHV1-type hypoviruses, and to be hypovirus free if they had the orange culture morphology (Day et al. 1977; Choi and Nuss 1992; Robin et al. 2000). For this purpose colony pigmentation was evaluated after 10 days of growth in the dark at 25 °C followed by incubation for 10 days at 25 °C with a photoperiod of 16 h. All isolates examined gave rise to orange colored colonies and thus none of the isolates was found to be infected by the *Cryphonectria* hypovirus (CHV1) which causes hypovirulence.

Mating type of these *C. parasitica* isolates was assayed following the PCR method described by McGuire et al. (2004) using primers m1-GS1n and M1-GS3-rev for *MAT-1* and primers M2-GS3 and gsl-d-l for *MAT-2*. *MAT-1* specific primers amplified the expected 1.649 kb PCR product while *MAT-2* specific primers revealed no profile. Thus, all six isolates assayed had the same mating type, *MAT-1*. This finding is similar to previous findings for mating type of *C. parasitica* isolates from diseased European chestnut (*Castanea sativa*) trees in Greece (Xenopoulos 1982; Sotirovski et al. 2004). According to these only one mating type, *MAT-1*, occurs in the country.

For pathogenicity tests, three isolates obtained from diseased trees were used for artificial inoculation of detached twigs of *Q. frainetto* following the method described by Hunter et al. (2013) for inoculation of *Castanea sativa* with *C. parasitica*. PDA plugs, 5 mm in diameter, with actively grown mycelium were transferred on

Fig. 2 Necrotic lesions on, artificially inoculated used for pathogenicity tests, *Q. frainetto* twigs **a** control and **b** twig inoculated with *C. parasitica*

wounds made by a cork borer on previously sterilized twig surfaces. Each treatment consisted of ten twigs and the pathogenicity test was repeated three times. Twigs inoculated in the same way using PDA disks were kept as controls. Following inoculation, twigs were incubated in a growth chamber at 20 °C (60–70 % relative humidity; with a photoperiod of 15 h) and 5 weeks after inoculation lesions developed on all inoculated twigs, while control twigs remained symptomless (Fig. 2). Lesion development was evaluated by measuring lesion length. The lesions caused by the three *C. parasitica* isolates were significantly different from the control but not from each other. Additionally, the isolates derived from artificially inoculated twigs were amplified and the amplicons were sequenced. A BLAST search of the GenBank database revealed 99 % homology with various *C. parasitica* isolates. Koch's postulates were thus fulfilled with the above described procedure.

There are reports of *C. parasitica* causing cankers on *Q. frainetto* in Italy (Gobbi et al. 2002) and on other broad leaf trees like various *Quercus* species worldwide (Farr and Rossman 2014). Nevertheless, to our knowledge, this is the first report of *Cryphonectria parasitica* on Hungarian oak in Greece. Although *C. parasitica* did not kill Hungarian oaks in Greece, infected oaks could serve as reservoirs of virulent *C. parasitica* inoculum. This fact is very important particularly in forest stands where chestnut co-exists with oak trees and in areas where biological control, using hypovirulent strains, has been implemented.

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