Long-term preserved mycelium establishes the presence of *Cryphonectria carpinicola* in the Balkans and of *Cryphonectria radicalis* in Bulgaria

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Abstract Around the world, isolate collections in mycological institutes have long preserved valuable specimens and information for science. In the present study, we determine *Cryphonectria*-like taxonomic rarities in our 10–24 years old isolate collection from the Balkans to link preserved dry mycelium with DNA data. Using ITS sequences, we confirm for the first time the occurrence of *Cryphonectria carpinicola* on a *Carpinus* tree in the Balkans and extend the range of *Cryphonectria radicalis* found on *Castanea sativa* to Bulgaria. The oldest specimen examined dates from 1998 and molecularly confirms the first reported finding of *Cryphonectria radicalis* for North Macedonia.

Keywords Fagaceae · Betulaceae · Tree pathogen · Species diversity · Barcoding · Culture collection

Members of the ascomycetous genus *Cryphonectria* (Sacc.) Sacc. & D. Sacc. are known to infect important deciduous tree species in the Fagaceae and Betulaceae. *Cryphonectria parasitica* (Murrill) M.E. Barr causes chestnut blight disease and is the best-studied *Cryphonectria* species. This fungus, native to East Asia, invaded North America and Europe in the last century, where it caused pronounced symptoms in *Castanea* Mill. species and severely damaged chestnut stands in eastern U.S.A. and in all major chestnut areas of Europe (Rigling & Prospero, 2018). For this reason, most research has focused on *C. parasitica*, while other *Cryphonectria* species have been overlooked and therefore poorly studied. Examples of neglected species in Europe are *Cryphonectria naterciae* M.H. Bragança, E. Diogo & A.J.L. Phillips (Bragança et al., 2011) and *Cryphonectria carpinicola* D. Rigling, T. Cech, Cornejo & L. Beenken (Cornejo et al., 2021), which have only recently been recognized as species at all. However, little is known about their distribution range in Europe. To date, *C. naterciae* has been reported in Portugal, Italy, and Algeria only, and *Cryphonectria carpinicola* in a few Central European countries (Austria, Italy, Germany, Switzerland) and in the Republic of Georgia on the bark of dead *Carpinus* L. species (for more details, Cornejo et al., 2021).

While investigating the invasion dynamics of *C. parasitica* in the Balkans in the early 2000s, we have conducted several extensive sampling campaigns of symptomatic *Castanea sativa* Mill. in the Balkan Mountains in Bulgaria, North Macedonia, and Kosovo (Fig. 1A). During isolation of fungal strains from bark material, we encountered morphologically atypical mycelia, distinct from *C. parasitica*, suggesting that different *Cryphonectria* species may be present in this region. These taxonomic rarities were preserved as dried mycelium at the plant pathology lab of the Hans Em

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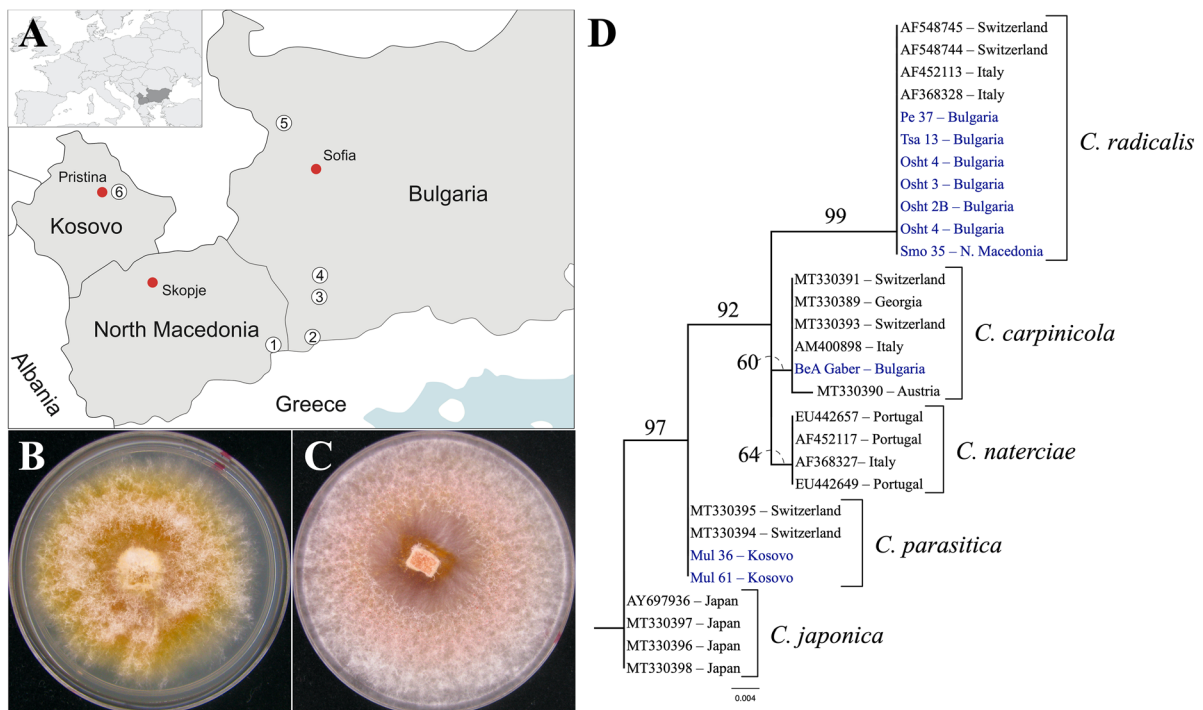
Fig. 1 Collection sites and results of culture screening. A Map section of Europe (framed) and magnification of Bulgaria, Kosovo, and North Macedonia (grey). Shown are the collection sites of *Cryphonectria* specimens in North Macedonia (1: Smolari), Bulgaria (2: Petric; 3: Tsaparevo; 4: Oshtava; 5: Berkowitza), and Kosovo (6: Mulliq). B–C Culture morphology of recultivated specimens grown on potato dextrose agar for 7 days. B *Cryphonectria parasitica*, isolate Mul 36. C *Cryphonectria radicalis*, isolate Osht 2B. D Maximum likelihood tree reconstruction based on the ITS2 region (PhyML v.3.0; 29 polymorphic out of 332 nucleotide sites; K80 substitution model). Blue letters highlight the phylogenetic positions of the ten analysed *Cryphonectria* samples. GenBank accession numbers of related specimens included in the analysis are indicated in the tree. The numbers at nodes are values of 1000 bootstrap replicates

Faculty of Forest Sciences, Landscape Architecture and Environmental Engineering (HEF; North Macedonia)—undetermined but accompanied with valuable information such as the collection year, the collection site and host species. This study aims to clarify the taxonomic identity of dry-preserved mycelium and relate it to the current state of science, its host, and geographical distribution.

During sampling campaigns, pieces of bark (c. 3 × 3 cm) from the margin of cankers or with visible stromata were cut from symptomatic trees. After isolation from the bark samples, the cultures were grown on potato dextrose agar (PDA) overlain with glass fibre filters, which were dried and stored at −20 °C until further use. For the present study, we selected twelve 10–24 years old isolates from Bulgaria, North Macedonia and Kosovo that were documented as *Cryphonectria*-like but could not be clearly identified after isolation and were preserved as dried mycelium on glass fibre filters. The filters were laid out on PDA

(Difco Laboratories, BD) for 7–10 days in the laboratory (21–22 °C) to recultivate mycelium of as many as possible and to observe the cultural characteristics. The DNA of successfully recultivated mycelial cultures was obtained using the kit and instruction by the semi-automated KingFisher 96 Flex (Thermo Fisher Scientific). If the dry samples were no longer alive, the filters were homogenized to powder to disrupt the mycelium using the MP Biomedicals FastPrep-24 (Thermo Fisher Scientific) and subjected to manual single DNA extractions following the instructions of the NucleoSpin Tissue kit (Marchery-Nagel), which is suitable for the small-scale preparation of genomic DNA from any tissue. The barcode internal transcribed spacer (ITS) of the nuclear rRNA gene was sequenced either as the complete ITS-region (primer-pair: ITS1–ITS4) or in two parts (primer-pairs: ITS1–ITS2, and 5.8S–ITS4) using published protocols (Vilgalys & Hester, 1990; White et al., 1990). The resulting forward and reverse sequences were assembled using the software DNA Main Workbench (CLC bio,

Qiagen). The ITS-sequences were verified in GenBank (www.ncbi.nlm.nih.gov, accessed on 27/06/2022) using the nucleotide BLAST search. Assignment to a species was accepted only for matches >99% sequence identity.

Fungal culture collections are important for research and industrial applications, especially because in this way the genetic and ecological potential of microorganisms can be kept alive. But preservation is a difficult task, and many different techniques have been suggested for the long-term preservation of living mycelium such as cryopreservation, lyophilization, mineral oil, water stocks and many others. The preservation of mycelium on glass fibre filter is an inexpensive method that requires very little space and simple storage conditions such as a −20 °C freezer, which is why it is also very popular with mycological research institutes. We obtained the DNA of all twelve analysed specimens (Table 1). Even though, only nine specimens could be recultivated and three were no longer alive, our culture screening shows that cold preservation on glass fibre filters at least conserved the DNA of valuable samples for up to 24 years. In total, our specimen screening confirmed ten samples being a species of the genus Cryphonectria, while two belonged to the ascomycete Epicoccum Link and were, thus, excluded from further analysis. Using the BLAST search in GenBank, the ITS sequence of one specimen was identified as C. carpinicola, two as C. parasitica (Fig. 1B), and seven specimens as C. radicalis M.E. Barr (Fig. 1C). Due to mono-repeats at

the beginning and at the end of the ITS1 region of C. radicalis, only its ITS2 could be sequenced unambiguously. Thus, the phylogenetic analysis was performed using the ITS2 region to verify the position of each specimen in relation to other Cryphonectria specimens obtained from GenBank (Fig. 1D).

Cryphonectria radicalis has been reported for Central Europe over the last two centuries. During the twentieth century, C. radicalis was reported in Europe, North America and Japan (Hoegger et al., 2002) only in the early years after the discovery of C. parasitica, but was rediscovered in Switzerland (Hoegger et al., 2002), Greece and North Macedonia (Sotirovski et al., 2004) early in the twenty-first century. Mostly, C. radicalis has been isolated from dead chestnut wood showing sporulation on the bark. In the present case, C. radicalis has been accidently isolated from C. sativa during sampling campaigns for the chestnut blight fungus in the Balkans. It seems that C. radicalis can co-exist at low prevalence together with the dominant C. parasitica as previously found in other regions in Europe (Hoegger et al., 2002). The voucher specimen Smo 35, collected in 1998 in North Macedonia, was identified at that time as C. radicalis morphologically and by its characteristic pattern following restriction fragment length polymorphisms (RFLP) (Sotirovski et al., 2004). Here, we confirm its identity as C. radicalis based on the ITS barcode. In addition, we report for the first time the occurrence of

Table 1 Host, location and collection year of the isolates used in this study, and the species determined based on ITS sequences

Host	Country	Region	Voucher	Year	Species	ITS length (nu) / GB identity	GB accession	Collection identifier[a]
Carpinus sp.	Bulgaria	Berkovitsa	BeA Gaber	2007	Cryphonectria carpinicola	551 / 100%	OP007129	Not grown
Castanea sativa		Oshtava	Osht 2A	2007	C. radicalis	333 / 100%	OP007132	M10583
		Oshtava	Osht 2B	2007	C. radicalis	332 / 100%	OP007133	M10584
		Oshtava	Osht 3	2007	C. radicalis	332 / 100%	OP007134	M10585
		Oshtava	Osht 4	2007	C. radicalis	278 / 100%	OP007135	Not grown
		Tsaparevo	Tsa 13	2007	C. radicalis	332 / 99.40%	OP007136	M10586
		Petric	Pe 37	2005	C. radicalis	332 / 100%	OP007137	M10587
	North Macedonia	Raven	Rav 3	2016	Epicoccum sp.	n/a	n/a	n/a
		Smolari	Smo 17C1	2015	Epicoccum sp.	n/a	n/a	n/a
		Smolari	Smo 35	1998	C. radicalis	276 / 100%	OP007138	Not grown
	Kosovo	Mulliq	Mul 36	2014	C. parasitica	595 / 99.66%	OP007130	n/a
		Mulliq	Mul 61	2014	C. parasitica	516 / 100%	OP007131	n/a

[a] **Accession number** of the Phytopathology culture collection of the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Birmensdorf, Switzerland

Abbreviations: nu, nucleotides. GB, GenBank. n/a, not assessed

C. radicalis in Bulgaria, isolated from *C. sativa* in 2005 and 2007.

Cryphonectria carpinicola has only been detected in the last two decades on hornbeam (*Carpinus*) in urban areas of Italy and Central Europe (for a review, Cornejo et al., 2021). The first reports came from northern Italy in the early 2000s as an unidentified *Endothiella* Sacc. fungus. To date, *C. carpinicola* has been found in Europe only on *Carpinus betulus* L. and on an undetermined *Carpinus* species in the Caucasus region. In pathogenicity tests, lesions of *C. carpinicola* on hornbeam were small and did not cause host mortality (Cornejo et al., 2021). In Italy and Switzerland, *C. carpinicola* was reported to be pathogenic on drought-stressed hornbeam in urban environments, often together with *Anthostoma decipiens* (DC.) Nitschke. Based on our culture screening, we report for the first time the occurrence of *C. carpinicola* in 2007 in the Balkans (Bulgaria) on a *Carpinus* tree that was sampled because of conspicuous *Cryphonectria*-like orange-yellow stromata breaking through the bark. At that time, its culture morphology and RFLP pattern could not be assigned to any known species. Advances in DNA sequencing have helped to recognize additional *Cryphonectria* species and hence to identify the voucher specimen *BeA Gaber* as *C. carpinicola,* even though its mycelium could not be recultivated. This is not surprising, since isolation, growth, and conidiation in *C. carpinicola* are generally rather slow and difficult compared to other *Cryphonectria* species.

Determining whether *C. carpinicola* is a fungus native to Europe is difficult because it has been found only recently in a few countries, although the host tree *C. betulus* is widely distributed in Europe. In our study, we report the occurrence of *C. carpinicola* in Bulgaria. Although this is a single finding, it is important information that closes the previously reported distribution gap between Central Europe and the Caucasus (Republic of Georgia). Even though additional observations of live hornbeam trees, exhibiting orange-yellow stromata, were made in Bulgaria in the 2007-campaign, they were not sampled at that time. Further surveys are needed to determine the distribution of *C. carpinicola* in the Balkans and its association to dieback symptoms on hornbeams in this region. Screening of poorly studied taxonomic rarities preserved in mycological institutes around the world can also make an important contribution to filling geographical and temporal knowledge gaps.

Declarations

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