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SHORT COMMUNICATION

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First report of *Cryphonectria carpinicola* in Hungary and Slovakia (Central Europe)

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Abstract

Hornbeam (*Carpinus betulus*) is an economically significant tree species, serving as a valuable resource for timber and ornamental wood products. It plays a crucial ecological role within forest communities and exhibits wide distribution across Central Europe. During October 2022 and March 2023, we encountered instances of declining hornbeam trees in Hungary and Slovakia. In both cases, characteristic symptoms associated with *Cryphonectria* canker were observed. Subsequent morphological and molecular-genetic analyses of fungal samples and isolates confirmed their identification as the recently described species *Cryphonectria carpinicola*. This study represents the first documented report of this pathogen in Hungary and Slovakia, contributing to our understanding of its presence and impact in Central Europe.

KEYWORDS
Carpinus betulinus, *Cryphonectriaceae*, *Diaporthales*, hornbeam decline, ITS, molecular phylogeny

1 | INTRODUCTION

Members of the ascomycete fungal genus *Cryphonectria* (Sacc.) Sacc. & D. Sacc. are significant forest pathogens infecting trees in the Fagaceae and Betulaceae families. The most infamous and destructive species of the genus is *C. parasitica* (Murrill) M.E. Barr, which is the causal agent of chestnut blight and has inflicted significant harm on the chestnut stands in Europe and North America (Rigling & Prospero, 2018). Recently, a new *Cryphonectria* species has been described by Cornejo et al. (2021), which plays a role in the hornbeam (*Carpinus betulus* L.) decline in Europe, in addition to the pathogenic ascomycete, *Anthostoma decipiens* (DC.) Nitschke (Rocchi et al., 2010). The holotype of the recently described *C. carpinicola* D. Rigling, T. Cech, Cornejo & L. Beenken, was originally collected in Switzerland; however, subsequent investigations have revealed the pathogen's presence in Austria and Georgia (country), and its occurrence in Italy has been confirmed through ITS sequence analysis (Cornejo et al., 2021). Additionally, recent DNA barcoding of old samples from 2007 confirmed that *C. carpinicola* has been present in Bulgaria for at least 15 years (Cornejo, Risteski, et al., 2023). The pathogen (and its sexual stage) has also been discovered in Japan. The Japanese isolates did not diverge from the European samples based on the ITS sequence, which raises questions about the place of origin of *C. carpinicola*. Research works on *C. carpinicola* underscore the need for ongoing monitoring of the pathogen's spread and urge the development of genetic tools to clarify the origin and history of the pathogen (Cornejo, Otani, et al., 2023).

In late October of 2022, signs of hornbeam decline were detected in the Pilis Mountains of Hungary. The afflicted trees exhibited symptoms of desiccation, accompanied by the emergence of conidiomata from two distinct fungal species on their trunks. Through analysis, one of the species was identified as *Anthostoma decipiens* (DC.) Nitschke (=*Cytospora decipiens* Sacc.), which had been previously documented in Hungary (Tóth, 1967, 1994). The

other species displayed marked macromorphological similarities to the recently described *C. carpinicola*. Later, in March 2023, during a survey conducted in the vicinity of Zvolen, Slovakia, we also observed dead hornbeam trees with fungal structures on their trunks showing identical morphological characteristics. Accordingly, the objective of this investigation was to determine the identity of these fungal specimens from Hungary and Slovakia through the use of micromorphological and molecular phylogenetic methods.

2 | MATERIALS AND METHODS

The fungal specimens utilized for the investigations were collected on two separate occasions and locations. The Hungarian location was a severely infected hornbeam stand with numerous heavily infected dead trees (both young and old). The sampled tree was a dead, old individual growing in the understory (specimen examined: Hungary, Pilis Mts, Esztergom, Lencse Valley, in Pannonic wood with *Quercus petraea* and *Carpinus betulus*, 47°44′03.7″ N, 18°46′35.8″ E, 170 m.a.s.l., leg. Csaba Németh, 27 October 2022, on *Carpinus betulus*, BP 112582, pers. herb. C. Németh 11154). In the Slovakian locality only a few young, infected individuals were observed in the territory. The sampled tree was a dead, young individual growing in the understory (specimen examined: Slovakia, Kremnica Mts, Turovské predhorie, Budča, Mt Bukovina, in beech-dominated forest, 48°34′07.7″ N, 19°01′14.9″ E, 540 m.a.s.l., leg. Csaba Németh, 23 March 2023, on *Carpinus betulus*, BP 112583, pers. herb. C. Németh 11416B).

During field surveys, pieces of infected bark fragments with visible fungal conidiomata were cut from symptomatic trees. The collected samples were deposited at the Fungarium of the Hungarian University of Agricultural Sciences and Life Sciences, in the herbarium of the Hungarian Natural History Museum (BP) as well as

FIGURE 1 Morphological features of *Cryphonectria carpinicola*. (a) declined hornbeam trees in the Pilis Mts, Hungary. (b–c) asexual conidiomata with orange tendrils that contain conidial mass (C. Németh 11154). (d) multilocular stroma breaking through the bark of *Carpinus betulus* (C. Németh 11416B). (e) culture morphology of specimens isolated from the Pilis Mts, Hungary (C. Németh 11154). (f) bacilloid conidia of *C. carpinicola* (C. Németh 11154; scale bar: 10 μm). Photos: Cs. Németh (a–d), D. Papp (e), B. Palla (f).

the private herbarium of the last author. Micromorphological data were obtained from dried specimens using a Zeiss Axio Imager.A2 light microscope equipped with AxioVision release 4.8.2 software. Measurements were made with a 100× oil immersion objective (1000× magnification) from slide preparations.

Subsequently, a small piece of mycelium (approx. 4 mm long) from the inner bark of an infected hornbeam twig was aseptically detached from the host tissue under laminal flow. The mycelium piece was then surface sterilized by merging it with 96% alcohol for a few seconds. After the sterilization, it was cultured on a Potato Dextrose Agar (PDA) plate at 20°C in complete darkness for 2 months. To extract DNA from the clean fungal culture, a 1 cm^2 large piece of mycelia was excised from the agar. DNA extraction was performed using the E.Z.N.A.® Plant DNA Mini Kit (Omega Bio-tek), following the manufacturer's protocol. The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) were amplified. The PCR reactions were carried out in a thermal cycler with the following settings: denaturation for 4 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 56°C and extension for 2 min at 72°C with a final extension at 72°C for 10 min. The PCR products were purified using an ExoSAP-IT purification kit (Amersham Biosciences). The purified samples were sent to the Biological Research Centre, Szeged for sequencing, where amplicons were sequenced using a Sanger Sequencing 3500 Dx Series Genetic Analyser (Applied Biosystems™, Thermo-Fisher, Waltham, MA, USA). The resulting chromatograms were carefully inspected, assembled, and edited using CodonCode Aligner 7.0.1 (CodonCode Corporation, Centerville, MA, USA). The obtained ITS sequences from the two isolates have been deposited

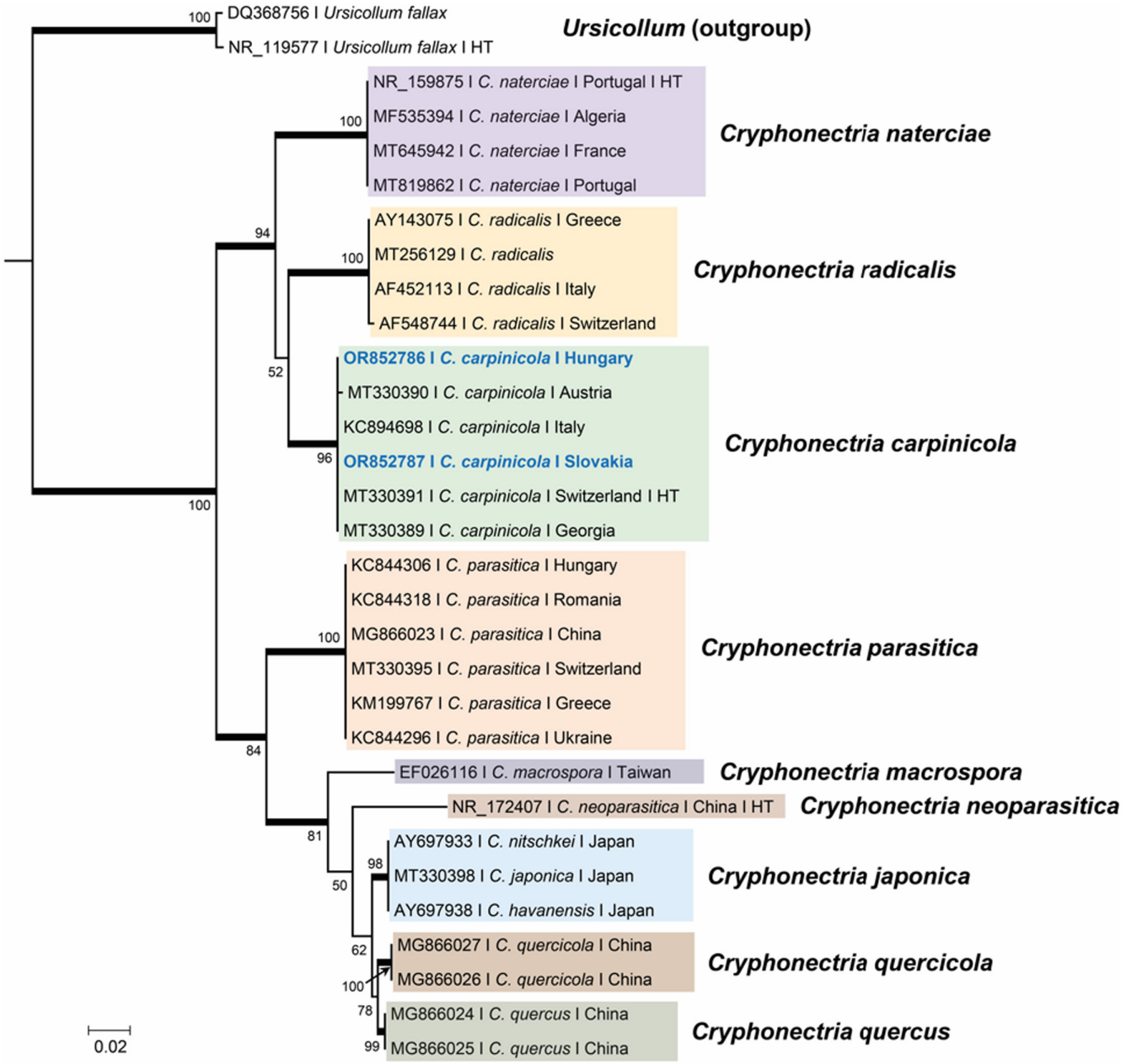

FIGURE 2 Phylogeny of *Cryphonectria carpinicola* and related species inferred from RAxML analyses of nrDNA ITS sequences. Topology is from the best-scoring maximum likelihood (ML) tree. The samples collected in Hungary and Slovakia are marked in blue. HT, holotype.

in the GenBank database under the accession numbers OR852786 and OR852787. For the phylogenetic investigation, we retrieved a supplementary dataset consisting of 27 representative *Cryphonectria* sequences from the GenBank database. As outgroups, we selected ITS sequences from two specimens of *Ursicollum fallax* Gryzenh. & M.J. Wingf. The alignment of the ITS data was performed using the online version of MAFFT v. 7, followed by manual correction, trimming, and concatenation using AliView. Subsequently, the dataset was subjected to maximum likelihood (ML) analyses using RaxmlGUI version 7.3.0. The ML analysis yielded the best-scoring tree, which was further refined and edited using MEGA 7 and Adobe Illustrator CS4, respectively. The finalized tree is depicted in Figure 1.

3 | RESULTS AND DISCUSSION

In the examined collections of *Cryphonectria carpinicola*, we only found conidiomata, and we were unable to observe the sexual ascomata. In the case of the Hungarian sample collected in autumn (C. Németh 11154), we observed well-developed orange tendrils that contain conidial mass (Figure 1a–c). In the Slovakian spring collection (C. Németh 11416B), however, these structures were absent, with only the distinctive multilocular stroma being present, which penetrates the bark (Figure 1d). The macromorphological characteristics of the isolated strains (Figure 1e) matched those described by Cornejo et al. (2021).

During the microscopic examination of conidiomata, characteristic micromorphological features of the *Cryphonectria* genus were observed. The conidiophores were straight, cylindrical and septate, and did not exceed a length of 50 µm and a width of 2 µm. The bacilloid conidia were hyaline, aseptate, and measured 3.08–4.59 × 1.12–1.76 µm ($N = 60/2$) in size (Figure 1f). This conidial size completely overlaps with the measurements reported in previous studies of *C. carpinicola* samples (Cornejo et al., 2021; Cornejo, Otani, et al., 2023; Cornejo, Risteski, et al., 2023).

The shape and size of the conidia can be used as a clear diagnostic feature to distinguish it from other species occurring on hornbeam with a similar appearance; as an example, *Anthostoma decipiens* (*Diatrypaceae*, *Xylariales*) has larger-sized and differently shaped, lunate conidia (Rocchi et al., 2010). Furthermore, this species develops thicker, darker, slightly orange-reddish-coloured conidiomata, reminiscent of resin flow.

For the molecular phylogenetic studies, the ITS sequence dataset gave total alignments of 548 bp including gaps. The studied isolates from Hungary (GenBank no. OR852786) and Slovakia (GenBank no. OR852787) clustered together with other representative isolates of *C. carpinicola* (Figure 2), including the holotype of the species (GenBank no. MT330391) in a strongly supported clade (MLBS = 96%). Therefore, the macro- and micromorphological examinations, along with the phylogenetic data, establish the first documented presence of *C. carpinicola* in Hungary and Slovakia. These recent findings from both countries contribute valuable information to our current understanding of the distribution of *C. carpinicola* in Central Europe. Furthermore, based on our current knowledge, the data from Slovakia represents the northernmost known occurrence of the species in Europe.

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CONFLICT OF INTEREST STATEMENT

The author declares that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/efp.12845.

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