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Cryphonectria carpinicola sp. nov. Associated with hornbeam decline in Europe

Carolina Cornejo [a, *], Andrea Hauser [a], Ludwig Beenken [a], Thomas Cech [b], Daniel Rigling [a]

[a] Swiss Federal Research Institute WSL, Zuercherstrasse 111, 8903, Birmensdorf, Switzerland
[b] Bundesforschungszentrum für Wald, Institut für Waldschutz, Seckendorff-Gudent-Weg 8, 1131, Wien, Austria

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ABSTRACT

Since the early 2000s, reports on declining hornbeam trees (*Carpinus betulus*) are spreading in Europe. Two fungi are involved in the decline phenomenon: One is *Anthostoma decipiens*, but the other etiological agent has not been identified yet. We examined the morphology, phylogenetic position, and pathogenicity of *yellow* fungal isolates obtained from hornbeam trees from Austria, Georgia and Switzerland, and compared data with disease reports from northern Italy documented since the early 2000s. Results demonstrate distinctive morphology and monophyletic status of *Cryphonectria carpinicola* sp. nov. as etiological agent of the European hornbeam decline. Interestingly, the genus *Cryphonectria* splits into two major clades. One includes *Cry. carpinicola* together with *Cry. radicalis*, *Cry. decipiens and Cry. naterciae* from Europe, while the other comprises species known from Asia—suggesting that the genus *Cryphonectria* has developed at two evolutionary centres, one in Europe and Asia Minor, the other in East Asia. Pathogenicity studies confirm that *Car. betulus* is a major host species of *Cry. carpinicola*. This clearly distinguished *Cry. carpinicola* from other *Cryphonectria* species, which mainly occur on *Castanea* and *Quercus*.

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1. Introduction

In last decades, the generic classification of Cryphonectriaceae (Diaporthales) has been reassessed based on molecular data (Gryzenhout et al., 2006a, b; Jiang et al., 2020). Several well-supported clades were recognized within Cryphonectriaceae, which correlate with morphological and eco-geographical features and were proposed to represent distinct generic lineages within the family. Some of these lineages relocated species formerly named as *Cryphonectria* in new erected genera, e.g., *Crysoporthe*, *Rostraureum*, *Elaeocarpus*, *Amphilogia* (Gryzenhout et al., 2006a), or *Microthia*, *Holocryphia* and *Ursicollum* (Gryzenhout et al., 2006b). In other cases, species known from other genera have been re-classified as *Cryphonectria* (e.g., *Cry. citrina*; Jiang et al., 2020), and new species are being discovered continuously, expanding the species list within this genus (e.g., *Cry. quercus* and *Cry. quercicola*, Jiang et al., 2018; *Cry. neoparasitica*, Jiang et al., 2019). The present study follows recent, revised classification and takes a critical look at some species within the genus *Cryhonectria* that occur in Europe.

The genus *Cryphonectria* is best known for its famous member, *Cry. parasitica*, the causal agent of chestnut blight (Rigling and Prospero, 2018). In Europe, three additional *Cryphonectria* species have been reported to occur together with the invasive chestnut blight fungus. One species, *Cry. radicalis*, was first reported for North America but also well-documented for Europe and Japan at the beginning of the 20th century. It has, however, apparently disappeared in North America and seems to be rare in Europe since the introduction of the chestnut blight fungus (Hoegger et al., 2002). The other, *Cry. naterciae*, was recently described based on morphology as well as molecular data and has been confirmed for Portugal on *Castanea sativa* and *Quercus suber* (Bragança et al., 2011), and for Algeria and Italy on declining *Q. suber* (Pinna et al., 2019; Smahi et al., 2018). Both *Cry. radicalis* and *Cry. naterciae* have often been accidently isolated from *Cas. sativa* during sampling campaigns for the chestnut blight fungus (Bragança et al., 2011; Hoegger et al., 2002; Sotirovski et al., 2004). A putative third species is *Cry. decipiens,* which was separated from *Cry. radicalis* based on the ascospore morphology of herbarium samples preserved in the U.S. National Fungus Collections (BPI) (Gryzenhout

Abbreviations: Cryphonectria, Cry.; Castanea, Cas.; Carpinus, Car.; Corylus, Cor..

* Corresponding author. Forest Health and Biotic Interactions, Swiss Federal Research Institute WSL, Zuercherstrasse 111, 8903, Birmensdorf, Switzerland.
E-mail address: carolina.cornejo@wsl.ch (C. Cornejo).

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et al., 2009). While there is no isolate deposition of *Cry. decipiens* linked to the holotype BPI 1112743, it has been assumed that *Cry. decipiens* and *Cry. naterciae* are conspecific (Rigling and Prospero, 2018). The present study focusses on an additional putative *Cryphonectria* species, which has been claimed to be involved in the decline of hornbeam trees in Europe.

The European hornbeam, *C. betulus* L. (Betulaceae), is a widely distributed deciduous tree with a natural range extending from the Pyrenees to southern Sweden and eastwards over the Caucasus to western Iran (Sikkema et al., 2016). It is one of few shade tolerant tree species, playing an important role as a secondary species in mixed stands dominated by oak (Postolache et al., 2017), or as ornamental tree in urban parks, gardens and along roadsides (Imperato et al., 2019; Saracchi et al., 2007). Although the wood of the hornbeam is very hard and strong, trees tend to have an irregular form and are therefore of minor commercial significance (Sikkema et al., 2016). Until recently, no major pest and disease problems were reported to affect European hornbeam. The powdery mildew *Erysiphe arcuata* is known parasitizing the European hornbeam (Braun et al., 2006; Vajna, 2006; Wołczańska, 2007), and *E. kenjiana* found on hornbeam was recently reported as new alien species for Ukraine (Heluta et al., 2009). In addition, Moradi-Amirabad et al. (2018) presented the first detection of the bacteria *Brenneria* spp. and *Rahnella victoriana,* which are associated with hornbeam trees in the western forests of Iran and causes symptoms similar to acute oak decline.

Since the early 2000s, however, declining hornbeam trees have been repeatedly reported in Europe—starting from northern Italy (Dallavalle and Zambonelli, 1999; Ricca et al., 2008; Rocchi et al., 2010; Saracchi et al., 2007, 2008), followed later by several central European countries including Germany (Kehr et al., 2016, 2017; Krauthausen and Fischer, 2018), Austria (Cech, 2019) and Switzerland (Queloz and Dubach, 2019)—as well as from the most eastern distribution limit of *Car. betulus* in Iran (Mirabolfathy et al., 2018). Trees are described to be infected by two fungi, either individually or both at the same time, and die within a few years if heavily attacked. One fungus produces large bark necrosis with red resin-like clumps on trunks and main branches, and could be clearly identified as *Anthostoma decipiens* based on morphological and molecular analyses (Rocchi et al., 2010). The second etiological agent has been reported to produce yellow stromata on the bark, which were assigned to an unknown *Endothiella* or Cryphonectriaceae species (Ricca et al., 2008; Rocchi et al., 2010; Saracchi et al., 2008, 2015). The term *Endothiella* refers to a historical generic name for the asexual form of *Cryphonectria* species and is considered here obsolete according to the *International Code of Nomenclature for Algae, Fungi, and Plants* (Melbourne Code; McNeill et al., 2012). For this reason, hereafter, we refer to the fungus with yellow stromata on the European hornbeam as *Cryphonectria* taxon.

A first species hypothesis for the *Cryphonectria* taxon tested the relationship to *Cry. parasitica*. Dallavalle and Zambonelli (1999) isolated a *Cryphonectria*-like strain from hornbeam trees in the city of Parma and based on mating and vegetative compatibility experiments ruled out that it belongs to *Cry. parasitica*. Another hypothesis related the *Cryphonectria* taxon with *Cry. radicalis* based on the ascospore morphology (Dallavalle et al., 2003). In fact, the fungal collection BPI registers several herbarium samples of *Cry. radicalis* (syn., *Endothia radicalis*) on *Carpinus* species, e.g., on *Car. betulus* for Abkhazia and Slovakia (labelled as Czechoslovakia), or on *Cry. japonica* for Japan, *Car. laxiflora* for Korea, and *Carpinus* sp. for the U.S.A. In contrast to these records, most *Cryphonectria* species are known to occur on members of the family Fagaceae—including mainly *Castanea* and *Quercus* (Gryzenhout et al., 2006b). In fact, only a few *Cryphonectria* species are reported on a wider host range than Fagaceae. Examples include the said *Cry. radicalis* and *Cry. japonica* (syn., *Cry. nitschkei;* Gryzenhout et al., 2009) with six different tree families listed as hosts (Myburgh et al., 2004a). However, many of historical reports should be taken with caution, as the identity of the *Cryphonectria* species remains uncertain due to the lack of molecular identification.

During phytosanitary surveys in Switzerland, several fungal cultures were isolated from the bark of hornbeam trees that showed sporulation as reported for the *Cryphonectria* taxon (Queloz et al., 2019). These isolates shared morphological features with the isolates M9290 from Austria and M5717 from Georgia preserved in our isolate collection at the Swiss Federal Research Institute WSL. First attempts to taxonomically assign the Austrian and Georgian isolates based on the fungal barcode ITS reached high identity scores with undetermined Cryphonectriaceae sp. (KC894698–KC894672) and *Endothiella* sp. (AM400898) (last search on 2020/07/17 on www.ncbi.nlm.nih.gov). Starting from these preliminary data, the present study first investigates the taxonomic position of the *Cryphonectria* taxon on hornbeam under the hypotheses that it belongs (i) to one of the four *Cryphonectria* species present in Europe, or that it represents (ii) a distinctive *Cryphonectria*, yet undescribed species. For this purpose, a molecular phylogeny was generated based on four genetic markers: the large subunit (LSU) and the internal transcribed spacer (ITS) of the ribosomal RNA gene, two different sections of the β-Tubulin gene (TUB) and a partial sequence of the RNA polymerase II gene (RPB2). In addition, we analysed phenotypic traits such as culture morphology and conidia size, which were compared with features of *Cry. naterciae*, *Cry. radicalis* and *Cry. parasitica*, which are present in Europe. Since sexual reproduction is common in *Cryphonectria* species (Milgroom et al., 1993; Wilson et al., 2015), isolates of the *Cryphonectria* taxon were crossed on hornbeam twigs to test their mating behaviour. Finally, the pathogenic characteristics of the isolates from Austria, Georgia and Switzerland was assessed in an inoculation experiment on *Carpinus*, *Corylus*, and *Betula* species as well as *C. sativa*.

2. Material and methods

2.1. Isolates used in this study

Voucher information for all isolates included in this study is listed in Table 1. From the *Cryphonectria* taxon, four isolates were molecularly characterized, one isolate from *Carpinus* sp. in Georgia, and three isolates from *Car. betulus* in Austria and Switzerland. Isolates of *Cry. parasitica* and *Cry. radicalis* from Switzerland, *Cry. japonica* from Japan, and *Cry. naterciae* from Portugal were used to compare morphological features between the different *Cryphonectria* species. The species *Cry. decipiens* was only assessed molecularly at three loci (LSU, ITS and TUB) based on GenBank entries as no isolate of the holotype BPI 1112743 is available.

2.2. DNA extraction, PCR and sequencing

Strains were grown on Potato Dextrose Agar (PDA; 39 g/l; Difco Laboratories, Detroit, U.S.A.) for a period of 7 d at 25 °C in the dark. Thereafter, mycelia were harvested, transferred to 2 ml Eppendorf tubes, and lyophilized overnight. Genomic DNA was extracted from 10 to 20 mg of lyophilized and milled fungal mycelium using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Four genomic regions were amplified by polymerase chain reaction (PCR): (1) LSU (primer used: LR0R/LR5 and LR3/LR7; Vilgalys and Hester, 1990); (2) ITS (ITS1/ITS4; White et al., 1990); (3) TUB (Bt1a/Bt1b and Bt2a/Bt2b; Glass and Donaldson, 1995); and (4) RPB2 (fRPB2-5F/fRPB2-7cR; Liu et al., 1999). All reactions used a 25 µl-mix, containing 12.5 µl

Table 1
Isolates used in the present study.

Species	Specimen ID[a]	Host	Origin	GenBank accession numbers[b]				
				LSU	ITS	TUB		RPB2
						Bt1	Bt2	
Cryphonectria carpinicola	M5717	*Carpinus* sp.	Georgia	**MT311229**	**MT330389**	**MW086463**		**MW086449**
	M9615[c]	*Carpinus betulus*	Switzerland	**MT311233**	**MT330391**	**MW086465**		**MW086451**
	M9290	*Carpinus* sp.	Austria	**MT311230**	**MT330390**	**MW086464**		**MW086450**
	M10525	*Carpinus betulus*	Switzerland	**MT311232**	**MT330393**	**MW086466**		**MW086452**
	GIALLO-A[d]	*Carpinus betulus*	Italy	NA	AM400898	AM920692	NA	NA
	AR2_5[d]	*Carpinus betulus*	Italy	NA	KC894698	NA	NA	NA
	AR9-3_2[d]	*Carpinus betulus*	Italy	NA	KC894699	NA	NA	NA
	AR6-2[d]	*Carpinus betulus*	Italy	NA	KC894700	NA	NA	NA
	AR1_7[d]	*Carpinus betulus*	Italy	NA	KC894701	NA	NA	NA
	AR3_8[d]	*Carpinus betulus*	Italy	NA	KC894702	NA	NA	NA
C. citrina	CBS 109758	*Quercus mongolica*	Russia	EU255074	MN172407	NA		EU219342
C. decipiens	CMW 10484	*Castanea sativa*	Italy	NA	AF368327	AH011606		NA
	CMW 10436	*Quercus suber*	Portugal	JQ862750	AF452117	AF525703	AF525710	NA
C. naterciae	M3659	*Quercus suber*	Portugal	**MT311226**	EU442649	**MW086470**		**MW086455**
	M3660	*Quercus suber*	Portugal	**MT311227**	EU442650	**MW086471**		**MW086456**
	M3664	*Quercus suber*	Portugal	**MT311228**	EU442657	**MW086472**		**MW086457**
	C 0084	*Quercus suber*	Portugal	NA	NA	EU442658		NA
	C 0608	*Quercus suber*	Portugal	NA	NA	EU442659		NA
	C 0614	*Quercus suber*	Portugal	NA	NA	EU442660		NA
	C 0679/CBS 129352	*Castanea sativa*	Portugal	NA	NA	EU442661		NA
	C 0685	*Castanea sativa*	Portugal	NA	NA	EU442662		NA
	C 0691	*Castanea sativa*	Portugal	NA	NA	EU442663		NA
C. japonica	M9605	*Castanea crenata*	Japan	**MT311220**	**MT330397**	**MW086473**		**MW086458**
	M9606	*Castanea crenata*	Japan	**MT311221**	**MT330396**	**MW086474**		**MW086459**
	M9607	*Castanea crenata*	Japan	**MT311222**	**MT330398**	**MW086475**		**MW086460**
	CMW 10527	*Quercus mongolica*	Russia	AF408341	DQ120761	AH015162		NA
	CFCC 52148	*Quercus spinosa*	China	MH514023	MH514033	MH539686	MH539696	NA
	CMW 13742	*Quercus grosseserrata*	Japan	NA	AY697936	AH014588	NA	NA
C. neoparasitica	CFCC 52146	*Castanea mollissima*	China	MH514019	MH514029	MH539682	MH539692	NA
	CFCC 52147	*Castanea mollissima*	China	MH514020	MH514030	MH539683	MH539693	NA
C. macrospora	CMW 10463	*Castanopsis cuspidata*	Japan	NA	AF368331	AH011608		NA
	CMW 10914	*Castanea cuspidata*	Japan	JQ862749	AY697942	AH014594		NA
	CBS 109764	*Quercus mongolica*	Russia	AF408340	EU199182	NA		EU220029
C. parasitica	M2671	*Castanea sativa*	Switzerland	**MT311218**	**MT330394**	**MW086476**		**MW086461**
	M4023	*Castanea sativa*	Switzerland	**MT311219**	**MT330395**	**MW086477**		**MW086462**
	ATCC 38755	*Castanea dentata*		EU199123	AY141856	NA		DQ862017
C. radicalis	M2268	*Castanea sativa*	Switzerland	**MT311223**	NA	**MW086467**	NA	NA
	M2269	*Castanea sativa*	Switzerland	**MT311224**	AF548744	**MW086468**		**MW086453**
	M2270	*Castanea sativa*	Switzerland	**MT311225**	AF548745	**MW086469**		**MW086454**
	CMW 10455	*Quercus suber*	Italy	NA	AF452113	NA		NA
	CMW 7051	*Castanea sativa*	Italy	NA	AF368328	NA		NA
	CMW 13754	*Fagus japonica*	Japan	NA	NA	AH014584		NA
	CMW 10477	*Quercus suber*	Italy	NA	NA	AH011607		NA
C. quercicola	CFCC 52140	*Quercus wutaishansea*	China	NA	MG866026	MG896113	MG896117	NA
	CFCC 52141	*Quercus wutaishansea*	China	NA	MG866027	MG896114	MG896118	NA
C. quercus	CFCC 52138	*Quercus aliena*	China	NA	MG866024	MG896111	MG896115	NA
	CFCC 52139	*Quercus aliena*	China	NA	MG866025	MG896112	MG896116	NA
Amphilogia gyrosa[e]	CMW 10469	*Elaeocarpus*	New Zealand	AY194107	AF452111	AF525707	AF525714	NA
Endothia gyrosa[e]	CMW 2091	*Quercus palustris*	USA	AY194114	AF368325	AH011601	AH011601	NA
Chrysoporthe cubensis[e]	CBS 101281	*Eucalyptus urophylla*	Cameroon	NA	NA	NA		EU219341

[a] **ATCC** = American Type Culture Collection, Manassas, Virginia, U.S.A.; **CBS** = Centraalbureau voor Schimmelcultures, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; **CFCC** = China Forestry Culture Collection Center, Research Institute of Forest Ecology, Environment and Protection, Beijing, China; **CMW** = Culture Collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa; **C** = Collection of the Instituto Nacional de Recursos Biologicos (INRB), I.P., Oeiras, Portugal; **M** = culture collection of the Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Birmensdorf, Switzerland.

[b] Sequences obtained in the present study are highlighted in bold type. All other sequences were acquired from GenBank (https://www.ncbi.nlm.nih.gov/). **LSU** = 28S large subunit of the nrDNA gene; **ITS** = internal transcribed spacer of the nrDNA gene, including the 5.8S gene; **TUB** = β-tubulin gene, Bt1 and Bt2 according to Glass and Donaldson (1995); **RPB2** = RNA polymerase II gene.

[c] Holotype specimen MB837752.

[d] Strain IDs of specimens used in Rocchi et al. (2010).

[e] Sequences of *Amphilogia gyrosa*, *Endothia gyrosa*, and *Chrysoporthe cubensis* were used as outgroup in phylogenetic analyses.

JumpStart REDTaq ReadyMix (Sigma Aldrich: Merck KGaA, Darmstadt, Germany), 1 μl of DNA template, 8.5 μl of molecular-grade water (Merck) and 1 μl each primer (10 μM). Thermal cycling parameters for all reactions were: 2 min 94 °C initial denaturation, 35 cycles of 30 sec 94 °C, 30 sec 55 °C, 1 min 72 °C, and 10 min at 72 °C final elongation. PCR products were purified by an exonuclease I and alkaline phosphatase treatment following the manufacturer's instructions (GE Healthcare, Chicago, Illinois, U.S.A.).

The forward and reverse DNA strands were Sanger sequenced using the same primer as for the PCR reactions, except for locus RBP2 that exceeded the available sequencing length. Therefore, an internal forward primer Cryph_RPB2seq_F (5′- TCTACGGCTGGTGT CTCTCA-3′) was newly designed and combined with the

Cryphonectria specific variant of the degenerated primer fRPB27cR (Cryph_RPB2seq_R, 5′-TCCTCGTCATCTTTCTTTCT-3′), for nested sequencing reactions. Sequencing reactions were then conducted in 10 μL mixtures, using the Big Dye Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Waltham, Massachusetts, U.S.A.). Cycle sequencing products were purified using the BigDye XTerminator Solution (Applied Biosystems). Sequences were detected on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Forward and reverse sequences were assembled using the program CLC Main Workbench v.7 (Qiagen). If sequences contained long runs of a single nucleotide repeats, the Sanger read quality declined rapidly after the so-called homopolymer. This was the case within some ITS and TUB sequences, which contained long poly (dA) and poly (dT) stretches. In such cases, reference sequences were used for assembling the forward and reverse reads. The homopolymer itself was regarded as missing data and denoted with poly (dN) for each of four nucleotides according to guidelines of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Homopolymer regions and other ambiguous alignments were excluded from analyses by processing all datasets with Gblocks 0.91b (Castresana, 2000; Talavera and Castresana, 2007) on the Phylogeny.fr platform (Dereeper et al., 2008).

2.3. Phylogenetic reconstructions

Phylogenetic trees were reconstructed by Bayesian and maximum likelihood (ML) analyses, using BEAST 1.8.4 (Drummond and Rambaut, 2007) on desk computer and PhyML 3.0 (Guindon and Gascuel, 2003; Guindon et al., 2010) on the ATGC platform (www.atgc-montpellier.fr). To select the model that best fitted our data, the Smart Model Selection SMS (Lefort et al., 2017) and the Akaike Information Criterion (AIC) (Akaike, 1973) were used on the ATGC platform. BEAST analysis was run with 10 million generations and sampled every 1000th generation, following a discarded burn-in of 2500 generations. Convergence and the consequent proportion of burn-in were assessed using Tracer v1.7 (available from http://beast.community/). To obtain the Bayesian posterior probabilities (PP), a maximum clade credibility tree was generated by analysing the BEAST tree file in TreeAnnotator v.1.8.4 (available in the BEAST package). Bootstrap confidence values (B) were calculated in PhyML for 100 pseudoreplicates (Felsenstein, 1985). Phylograms were displayed in TreeGraph 2 (Stoever and Mueller, 2010).

The neighbor joining (NJ) algorithm (Saitou and Nei, 1987) was applied exclusively to assess the genetic diversity of all available sequences linked to the hornbeam decline in Europe. Early Italian studies on hornbeam decline submitted several ITS sequences (KC894698–KC894672) and one TUB sequence (AM920698) to GenBank named as Cryphonectriaceae sp. or *Endothiella* sp. (cf. Table 1). The analyses were performed with SplitsTree v.4.11.3 (Huson and Bryant, 2006) on each data matrix separately. Support values for branch lengths were computed from 1000 bootstrap replicates.

2.4. Morphology, growth and mating behaviour

Mycelial plugs (0.5 cm diameter) of the *Cryphonectria* taxon M9290, M5717, and M9615 were excised with a sterile cork borer from the edge of actively growing PDA cultures and placed in the centre of a 9 cm PDA plate. One set of subcultures was incubated in a climate chamber at 25 °C with a cycle of 10 h at dark and 14 h at light and the morphology was recorded for a period of three weeks. The second set was incubated at 10, 15, 20, 25, and 30 °C in the dark to assess the effect of temperature on fungal growth. Ten replicated plates were prepared for each fungal strain and temperature. The radial growth (mm) of the colonies was assessed after 2 and 4 days and the mean and standard deviation calculated. Isolates of *Cry. japonica, Cry. naterciae, Cry. parasitica* and *Cry. radicalis* were included in this experiment for comparison of culture morphology, but growth–temperature correlation of these species was not assessed, since this has been already studied elsewhere (cf. Bragança et al., 2011; Gryzenhout et al., 2009; Hoegger et al., 2002).

To test the development of sexual fruiting bodies, three isolates (M9290, M5717, and M9615) were crossed on *C. betulus* stem segments, either with themselves or with each other. Small stems of *Car. betulus* with a diameter of approx. 2 cm were cut into 5 cm long segments, split lengthwise and then autoclaved for 15 min at 121 °C. The autoclaved segments were individually placed in 9 cm diameter petri dishes and PDA medium was poured around them. The isolates to be crossed were inoculated onto the agar medium at both ends of the segments. The plates were incubated at 25 °C under a 16 h photoperiod for 3 weeks. Conidia produced by the isolates were then suspended in sterile water and distributed over the stem segments to induce mating. The mating plates were sealed with parafilm and incubated at 20 °C under a 12 h photoperiod. The plates were periodically examined for the presence of perithecia under a dissecting microscope for one year. To prevent desiccation, sterile water was added to the plates if necessary.

The mating plates were also used to harvest conidia of the *Cryphonectria* taxon for size measurements. After incubation for one year, conidia were taken under sterile conditions from the blister-like conidiomata produced on the hornbeam stems and dissolved in a water drop on a glass-slide. A Zeiss Axio Scope A1 microscope was used to measure 50 conidia of each isolate at 1000 times magnification with the software ZEN 2.3 (Carl Zeiss Microscopy GMBH, Germany). The mean diameter of the conidia was determined, and the standard deviation was calculated.

Morphology of field collections and cultures were investigated using a Zeiss Discovery.V8 SteREO microscope and hand sections of stromata were studied at 1000 times magnification using Zeiss Axio Scope A1 microscope. The ZEN 2.3 digital equipment was use for photography.

2.5. Pathogenicity studies

To assess the pathogenicity of the *Cryphonectria* taxon, three isolates (M9290, M5717, and M9615) were inoculated into *C. betulus* and two additional tree species belonging to the family Betulacea, *Corylus avellane* and *Betula pendula.* Because *Castanea* spp. are major hosts for many *Cryphonectria* species, we also included *C. sativa* in this inoculation experiment. Two-year-old seedlings of Swiss provenances were used, except for *Cas. sativa*, which was of a German provenance. The stem of each seedling was wound-inoculated in a greenhouse chamber as described by Dennert et al. (2019). For each isolate, five seedlings of each tree species were used. As negative controls, five seedlings of each species (three for *B. pendula*) were inoculated with an agar plug. Two months after inoculations, the length and width of the lesions were measured and the lesion size calculated using the formula of an ellipse area. Sporulation of the isolates was assessed by recording presence or absence of fungal stromata on each lesion. In the end of the experiment, all lesions were sampled to recover the inoculated fungus as described by Dennert et al. (2020). The identity of the re-isolated cultures was assessed visually by their typical orange culture morphology when growing on PDA plates. Linear model with Scheffe post hoc test (calculated using DataDesk 6.3, Data-Description Inc, Ithaca, NY) were used to test for significant differences ($P \leq 0.05$) in mean lesion size between isolates and tree species.

3. Results

3.1. Phylogenetic analyses

In total, ten ITS, 15 RPB2, 16 LSU, and 16 TUB sequences were obtained and submitted to GenBank (Table 1). The ITS and TUB sequences contained highly variable and repetitive regions that poses analytical problems related to the substitution model, which does not account for fast-evolving, repetitive mutations. Therefore, homopolymers and ambiguously aligned regions were processed with the software Gblocks. Information on data matrices, such as the number of excluded and polymorphic sites, is listed in Table 2.

The RPB2 dataset was mainly composed of one exon coding for a 354 amino acid sequence and containing around 18% polymorphic single nucleotides (SNP) as well as one indel (insertion/deletion)—a codon that was present in the outgroup species *Chrysoporthe cubensis* but not in *Cryphonectria* spp. TUB sequences included four introns and five exons, which resulted in protein sequences of 160–163 amino acids. After the exclusion of homopolymers and ambiguously aligned regions, the TUB dataset was composed of *c.* 24% and the ITS matrix of *c.* 11% informative SNPs. Although the LSU sequences were highly conserved, the *c.* 2.5% informative SNPs were mainly concerned to the studied lineages *Cryphonectria* taxon, *Cry. decipiens*, *Cry. naterciae* and *Cry. radicalis* as well as the outgroup species. In single-locus analyses (Supplemental Fig. S1), RPB2 topology resulted in well-supported monophyletic clades for the six analysed species. On contrary, the reduced ITS and TUB datasets failed to discriminate between already described species—such as *Cry. naterciae* and *Cry. decipiens* (ITS and TUB) or *Cry. quercus* and *Cry. quercicola* (TUB). The LSU tree resulted in a flat topology that did not resolve most species, except for specimens of the *Cryphonectria* taxon, *Cry. decipiens* and *Cry. radicalis*, but not among *Cry. decipiens* and *Cry. naterciae*. In the present study, a species was considered strongly supported if a lineage exhibited monophyly in a majority of sampled loci (*genealogical concordance*), which was not contradicted by phylogenetic patterns in other loci (*genealogical non-discordance*) (Dettman et al., 2003, 2006; Taylor et al., 2000). Since, no well-supported (≥70%) conflicting branching was detected among single locus trees, multilocus analyses were performed based on a concatenated dataset.

The concatenated dataset included only specimens that were represented by three or four sequences in order to improve the detection of monophyly. For this reason, species like *Cry. quercus* and *Cry. quercicola*, which were described on two loci (ITS and TUB) only, were not included in this dataset. The resulting data matrix comprised 2804 sites and was composed of 23 sequences of nine *Cryphonectria* species and two outgroup species, *Endothia gyrosa* and *Amphilogia gyrosa*. Of the 2804 sites, 329 were polymorphic. Both the Bayesian and PhyML analyses resulted in almost identical topologies. Therefore, the Bayesian tree was selected for representation in Fig. 1. This phylogeny confirms the monophyly of the genus *Cryphonectria* (PP = 1.0; B = 98%), which splits into two highly supported lineages (Fig. 1, A, and B). Within lineage A, the isolates from declining hornbeam trees are separated from *Cry. decipiens*, *Cry. naterciae* and *Cry. radicalis* in a strongly supported monophyletic clade (PP = 1.0; B = 98%). Within lineage B, specimens of *Cry. japonica*, *Cry. macrospora*, *Cry. neoparasitica*, and *Cry. parasitica* each also represented a well-supported monophyletic clade.

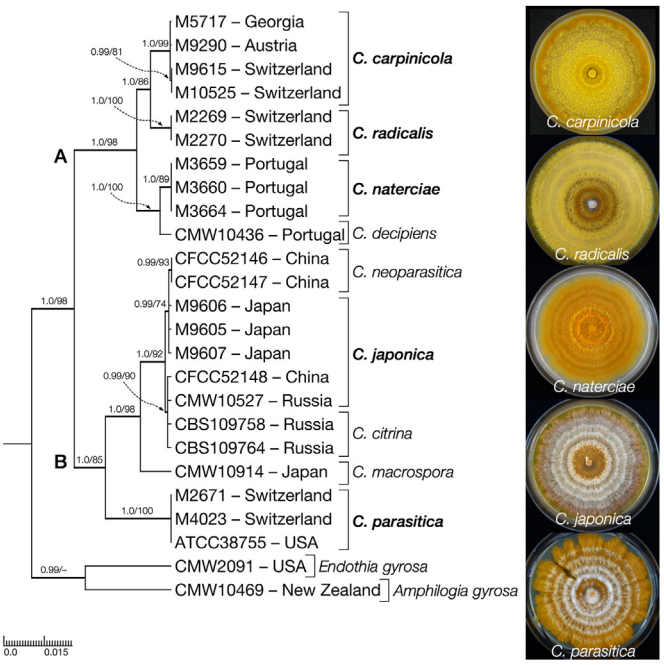


Fig. 1. Phylogeny and culture morphology of specimens isolated from declining hornbeam trees, named as *Cryphonectria carpinicola*. Left: Phylogram resulting from Bayesian analysis of combined ITS, LSU, RPB2 and TUB sequences. Bifurcations with posterior probabilities <0.9 were collapsed. Numbers above branches represent posterior probabilities and bootstrap values of maximum likelihood analysis (bootstraps values > 70%). Species names written in bold highlight cultures shown at right. Right: Photographs of some representative species of the genus *Cryphonectria* grown on PDA plates under lab conditions. At top the habit of *Cry. carpinicola*.

The ITS dataset for NJ analysis contained 19 sequences of *Cryphonectria* taxon, *Cry. decipiens, Cry. naterciae* and *Cry. radicalis*. Thirty-four ambiguous positions were excluded from the dataset and, of the 503 analysed characters, 34 were polymorphic. On contrary, 78% of all positions were excluded from the TUB dataset due to ambiguously aligned sites and highly repetitive homopolymers. Finally, the dataset contained 20 sequences and reached a length of 339 positions including nine polymorphic sites. Similar to the single-locus topology, the ITS-tree failed to separate *Cry. decipiens* from *Cry. naterciae* at species level, but TUB data contained some genetic variability within the *Cryphonectria* taxon and abundant polymorphism between *Cry. decipiens* and *Cry. naterciae*.

Table 2
Summary of molecular data matrices used in phylogenetic analyses including the substitution model applied.

Alignment per locus	LSU	ITS	TUB	RPB2	Combined
Number of sequences	27	35	32	19	25
Number of characters	581	549	833	1098	–
Excluded ambiguous sites[a]	–	127	142	–	–
Final alignment	569	423	691	1098	2804[b]
Polymorphic sites	22	46	154	153	329
Substitution model	GTR + I	GTR + I	HKY + G	GTR + I	GTR + G + I

[a] Ambiguous aligned sites were automatically excluded using Gblocks.
[b] Data matrices were combined after Gblocks analyses.

Isolates from declining hornbeam trees from Italy were clearly positioned together with specimens from Austria, Georgia and Switzerland in both NJ-trees (Fig. 2).

3.2. Morphology, growth and mating behaviour

The culture habit of five *Cryphonectria* species examined in the present study are shown in Fig. 1 (right side). To assess growth characteristics and culture morphology, all isolates were grown on PDA plates incubated at 25 °C under a 14 h photoperiod. Under these conditions, the *Cry. parasitica* and *Cry. radicalis* isolates grew the fastest and reached the margin of the 90 mm agar plates after one-week incubation. While all other isolates reached the margin after two weeks, the Georgian isolate M5717 grew very slowly and reached the margin only after three weeks. The pigmentation of all species started after two to three days from the centre of the plates and extended to the edge of the cultures after two weeks. The Georgian isolate M5717 developed pigmentation in the third week. The *Cryphonectria* taxon showed orange pigmentation on a beige background, whereas the saturation of the orange colour was higher around the central area and faded out towards the margins of the culture. The Georgian isolate M5717 was beige to brown pigmented and had only a small central orange area. The mycelium of *Cry. parasitica* was orange-brown, similar to *Cry. naterciae*, whereas *Cry. radicalis* showed luteous to orange pigmentation with a dark brown central area. After the first week, *Cry. japonica* developed a transient slightly violet pigmentation, which disappeared and turned into brown pigmentation. The mycelium was, together with *Cry. parasitica*, fluffy with clearly visible white growth rings with a flat central area. In contrast, *Cry. radicalis* and *Cry. naterciae*, as well as the *Cryphonectria* taxon had rather less visible growth rings. The margins of the cultures were smooth except for the crenate margins of *Cry. parasitica* and the Georgian isolate M5717. The bright orange-beige coloured conidiomata of *Cry. japonica* were grouped as round droplets along the growth rings, whereas in the other cultures conidiomata were less visible.

The effect of temperature on mean colony diameter of the *Cryophonectria* taxon after two and four days is shown in Fig. 3. Initially, colonies expanded fastest at 25–30 °C, but this early behaviour decreased rapidly and, after four days, all isolates grew optimally at 20–25 °C. The Georgian isolate M5717 exhibited a slower growth than the other two isolates and did not grow above temperatures of 25 °C. In contrast, the Swiss isolate M9615 grew up to 30 °C and the Austrian isolate M9290 even at 35 °C.

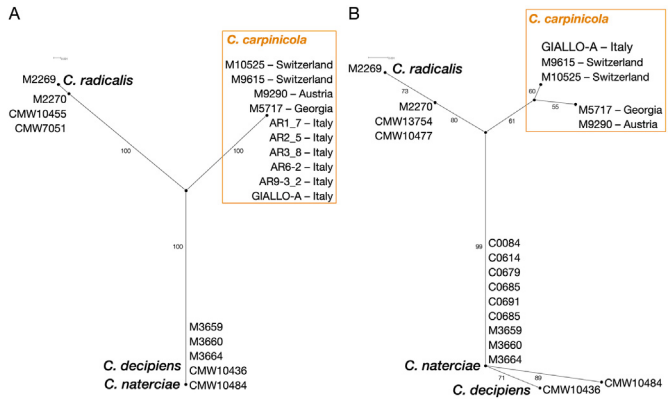


Fig. 2. Unrooted phylograms resulting from NJ analyses of (A) ITS, and (B) TUB sequences. Numbers beside branches represent bootstrap values. The species nova *Cryphonectria carpinicola* is highlighted in orange rectangles. Specimen vouchers are listed beside nodes. Italian specimens shared identical ITS (A) or highly similar TUB (B) sequences with isolates from Austria, Georgia and Switzerland analysed in the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

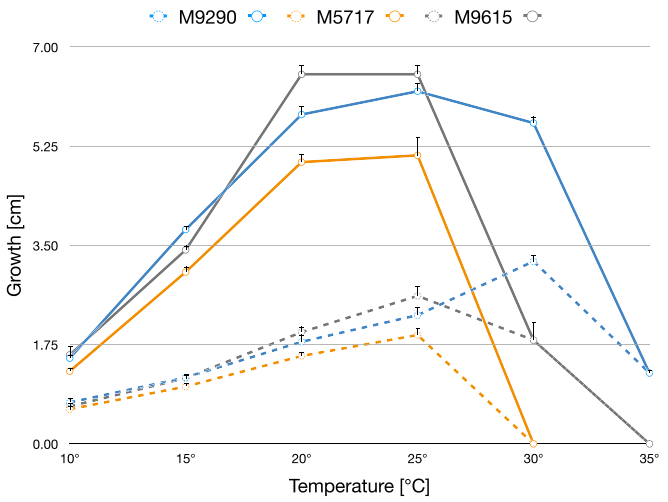


Fig. 3. Relationship between culture growth (cm) and temperature (°C) after 2 days (dashed line) and 4 days (dragged line) on PDA medium. Each data point represents the mean value of ten repetitions and vertical bars the corresponding standard deviation.

Conidia dimensions are listed in Table 3. The mean conidia width of the *Cryphonectria* taxon and *Cry. naterciae* was similar but the mean length was shorter compared with the conidia length of *Cry. naterciae* and *Cry. parasitica*, but longer than *Cry. radicalis*.

No sexual fruiting bodies (perithecia) were produced on the mating plates, even after an extended incubation time of more than one year. In one cross (M9290 × M9290), perithecial necks typically of *Cryphonectria* spp. were observed (Fig. 4), however, no mature perithecia were present associated with the necks.

3.3. Pathogenicity studies

The lesions produced by the *Cryphonectria* taxon after wound inoculations varied depending on the isolate and the host species (Table 4, Fig. 5). The general linear model revealed significant differences between isolates ($P = 0.0015$) and host species ($P = 0.0008$). The largest lesions were produced by the isolates M9290 on *Car. betulus* and to smaller extend on *Cas. sativa*. The other two isolates did not produce significantly larger lesions than the control on both of these host species. There was no lesion larger

Table 3
Conidia size measure of *Cryphonectria carpinicola* and comparison with related species.

Taxon	Isolate–ID	n	Mean + SD (µm)	Range (µm)[a]
C. carpinicola		150	3.5 ± 0.3 × 1.3 ± 0.1	3.0–4.7 × 0.9–1.8
	M5717	50	3.5 ± 0.2 × 1.4 ± 0.1	3.1–3.6 × 1.2–1.5
	M9615	50	3.4 ± 0.3 × 1.2 ± 0.1	3.0–4.6 × 0.9–1.5
	M9290	50	3.7 ± 0.4 × 1.4 ± 0.1	3.1–4.7 × 1.2–1.8
C. decipiens[b]				3.0–5.0 × 1.5–2.0
C. naterciae[c]		150	3.7 ± 0.4 × 1.3 ± 0.1	2.9–4.9 × 1.0–1.6
C. radicalis[c]		150	3.4 ± 0.3 × 1.4 ± 0.1	2.7–4.1 × 1.2–1.8
C. parasitica[c]		150	3.6 ± 0.4 × 1.4 ± 0.1	2.9–4.5 × 1.1–1.7

[a] Range is given as minimum and maximum dimension measured.
[b] Gryzenhout et al. (2009).
[c] Bragança et al. (2011).

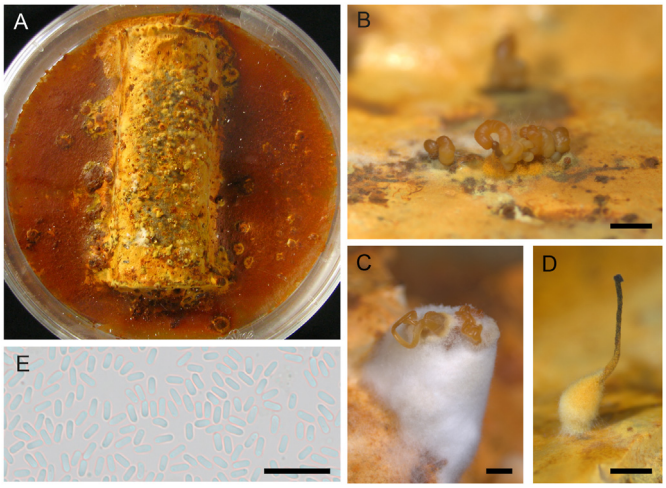


Fig. 4. *Cryphonectria carpinicola* in culture. (A) Halved stem of *Carpinus betulus* overgrown with mycelium. (B–C) Conidiomata with conidial tendrils. (D) Long neck on a fake perithecia. (E) Conidia.

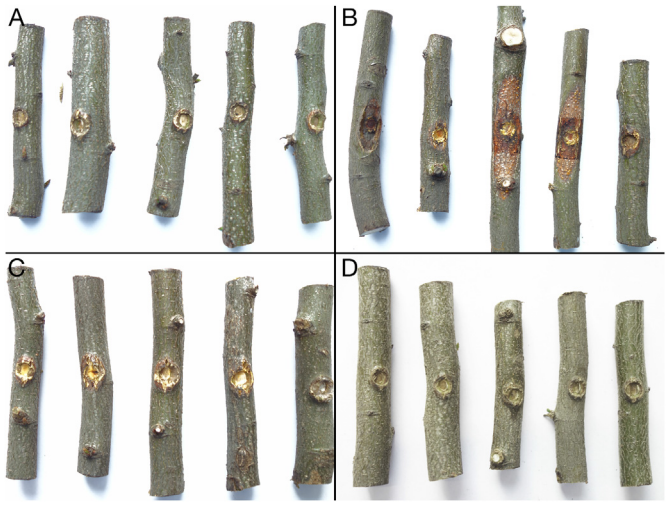


Fig. 5. Pathogenicity test on *Carpinus betulus* using three isolates of *Cryphonectria carpinicola* (A) M5717, (B) M9290, and (C) M9615. For each isolate, five two-year-old seedlings were inoculated. (D) As negative control, five seedlings of *Car. betulus* were inoculated with an agar plug.

Table 4
Pathogenic characteristics of *Cryphonectria carpinicola* isolates on different tree species, assessed two months after inoculation.

Host, Isolate	N	Lesion size (cm^2)	Sporulation[a]	Re-Isolations[b]
Carpinus betulus				
M5717	5	0.57 ± 0.21 a[c]	0	0
M9290	5	3.85 ± 3.05 b	2	5
M9615	5	1.00 ± 0.30 ab	0	4
Control	5	0.60 ± 0.12 a	0	0
Corylus avellana				
M5717	5	0.48 ± 0.16 a	0	0
M9290	5	0.40 ± 0.05 a	0	2
M9615	5	0.43 ± 0.07 a	0	1
Control	5	0.43 ± 0.07 a	0	0
Betula pendula				
M5717	5	0.51 ± 0.15 a	0	3
M9290	5	0.50 ± 0.07 a	0	3
M9615	5	0.52 ± 0.11 a	0	4
Control	3	0.46 ± 0.10 a	0	0
Castanea sativa				
M5717	5	1.05 ± 0.23 a	0	1
M9290	5	1.94 ± 0.63 b	2	5
M9615	5	1.00 ± 0.05 a	0	3
Control	5	0.93 ± 0.27 a	0	0

[a] Number of lesions with fungal stromata development.
[b] Number of lesions from which *C. carpincola* was successfully re-isolated.
[c] Means followed by different letters were significant different ($P < 0.05$).

than the control on *Cor. avellana* and *B. pendula* for all isolates. In many cases, the inoculated wounds became completely overgrown by the callusing reaction of trees (Supplemental Fig. S2). Overall, lesions were significant larger on *Car. betulus* compared to *Car. avellane* ($P = 0.009$) and *B. pendula* ($P = 0.015$) but not compared to *Cas. sativa* ($P = 0.84$). Sporulation was observed only for isolate M9290 on two lesions on *Car. betulus* and two lesions on *Cas. sativa* (Table 4). This isolate also showed the highest re-isolation rate (15 out of 20 lesions), followed by M9615 (12 out of 20 lesions). The isolate M5717 was only recovered from three lesions on *B. pendula* and one lesion on *Cas. sativa* (totally 4 out of 20 lesions). Re-isolations were successful from many lesions, which were not larger than the control. During the entire duration of the experiment, no mortality of the inoculated plants was observed.

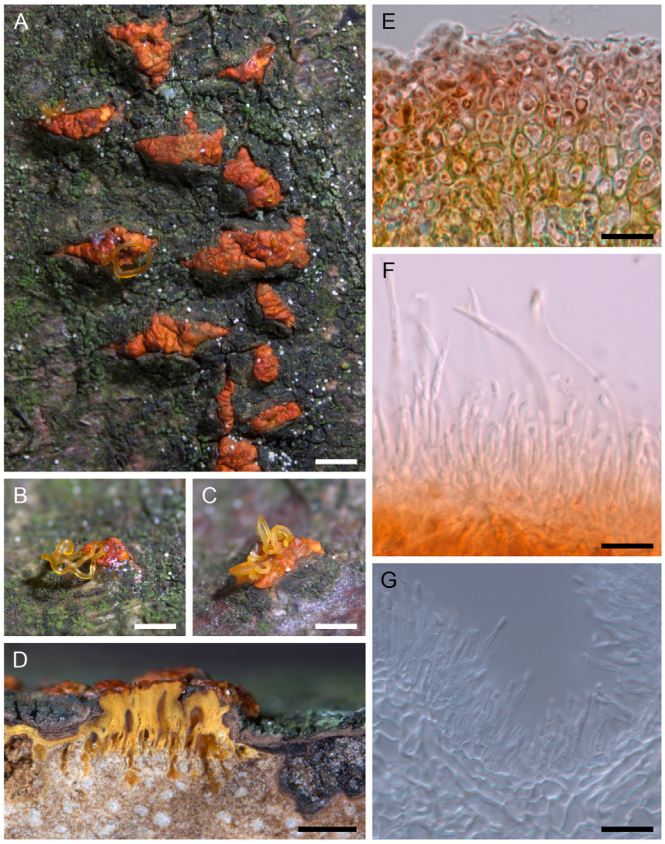


Fig. 6. *Cryphonectria carpinicola* from field collections. (A–C) Conidiomata breaking through the bark of *Carpinus betulus*, conidial mass emerging in orange tendrils. (D) Section through a multilocular stroma. (E–G) Microscopic view of section of stroma. (E) Prosenchymatose outermost layer. (F) Conidiogenous cells. (G) Part of pseudoparenchymatous layer surrounding conidial locules. Scale bars: A–D = 1 mm; E–G = 10 μm.

3.4. Taxonomy

Cryphonectria carpinicola *D. Rigling, T. Cech, Cornejo & L. Beenken,* ***sp. nov.***

MycoBank MB837752 (Fig. 6)

Similar to *Cryphonectria radicalis*, but occurs on species of the family Betulaceae.

Etym.: carpinicola means growing on *Carpinus*, the host genus.

Sexual state: ascomata not observed. *Asexual state*: uni- to multilocular, stromatic conidiomata immersed in the bark, pustular, erupting through the bark, surface shiny smooth, bright orange, discolouring wine-red to purple with 2.5% KOH, 1–5 (10) × 0.5–1.5 mm (sometimes several confluent to larger patches), up to 1 mm high; outermost layer pseudoparenchymatous up to 60 μm thick, with globose to angulate cells 2–6 μm in diameter, cell walls up to 1.5(2) μm thick, orange coloured; inner part pulvinate, eustromatic throughout, only basal, close to the substrate pseudostromatic, prosenchymatose, hyphae (1) 1.5–2.5(3.5) μm wide, with colourless or yellow content, orange incrustations on the hyphal walls dissolving and discolouring pink to purple in 2.5% KOH; many refracting, colourless crystal grains (roundish, up to 40 μm in diameter) between the stromal hyphae. Up to 8 locules per conidioma, locules ovoid bottle to irregularly shaped and convoluted, 250–850 μm wide, 150–900 μm high, non-ostiolate, locules surrounded by a colourless pseudoparenchymatous layer of angulate cells 2–5 × 4–10 μm, cell walls up to 1 μm thick. *Conidiophores* straight, cylindrical, 10–50 μm long, 1.5–1.8 μm wide, septate, rarely branched. *Conidiogenous cells* slightly tapered towards apex, phialidic. *Conidia* minute, bacilloid, 3.0–4.7 × 0.9–1.8 μm, hyaline, cylindrical, aseptate, conidial mass emerging in orange tendrils of 70–250 μm diameter.

Host and distribution: occurring on *Carpinus betulus* L. in Europe (Austria, Italy, Switzerland) and *Carpinus* sp. in Georgia.

Type: **SWITZERLAND**, Basel, Cemetery Wolfgottesacker, 47.54020 N, 7.60829 E, 278 m altitude, on dead trunk of *Carpinus betulus*, 16 Jan. 2018, leg. S. Ramin (M9615). **Holotype** ZT Myc 61307, culture ex-holotype CBS 147194. GenBank accession numbers: MT311233 (LSU), MT330391 (ITS), MW086451 (RPB2) and MW086465 (TUB).

Additional specimens examined: **SWITZERLAND**, canton of Basel Landschaft, Birsfelden, forest «Oberi Hard», 47.53900 N, 7.65550 E, 275 m altitude, on dead trunk of *Carpinus betulus*, 13 Jun. 2019, V. Queloz (M10525), ZT Myc 61308, culture CBS 147195.

—canton of Jura, Delémont, Le Béridier, forest la Vigne, 47.37836 N, 7.35399 E, 550 m altitude, on dead trunk of *Carpinus betulus*, 03 May 2020. V. Queloz (WSS 13609)

AUSTRIA, Biedermannsdorf, 48.090261 N, 16.348137 E, 190 m altitude, on dead trunk of *Carpinus betulus* in a hedge, 24 Nov. 2009, Thomas Cech (M9290), CBS 147196. **GEORGIA**, Tkibuli, N 42.372981, E 43.016217, 1050 m altitude, on a dead stem of *Carpinus* sp. 31 March 2010. D. Rigling (M5717), CBS 147197.

Notes: The anamorph of *Cryphonectria carpinicola* shows only slight differences in morphology and anatomy to the closely related species *Cry. radicalis* and *Cry. naterciae* (Fig. 1) (Bragança et al., 2011). Small differences can be found in the dimensions of the conidia that show a large overlap (Table 3). The teleomorph of this new species, which could show more differentiating features, has not yet been found and mating experiments resulted in fake perithecia that did not produce any asci and ascospores. Therefore, the differentiation of the new species is mainly based on molecular sequence data and to some extend to its host specificity. While the other *Cryphonectria* species mainly occur on tree genera of the family Fagaceae, *Cry. carpinicola* was only found on *Carpinus* spp. of the family Betulaceae.

4. Discussion

4.1. Phylogenetic analyses

During last decades, many cases of dieback of *C. betulus* trees were reported in northern Italy and central Europe. Even though, there was major effort to characterize both etiological agents associated with dieback, only *A. decipiens* could be identified at species level (Rocchi et al., 2010). The present work has studied the second fungus causing hornbeam dieback and confirms the species nov. *Cryrphonectria carpinicola* as etiological agent. A comprehensive phylogenetic analysis, including all *Cryphonectria* species known to date (Jiang et al., 2020), show that this fungus belongs to the genus *Cryphonectria* as it is clearly integrated within the ingroup and forms monophyletic clades in three of four sampled loci (Supplemental Fig. S1). Additionally, NJ analysis of the ITS sequences from Italian isolates named in GenBank as Cryphonectriaceae sp. and *Endothiella* sp. were identical to all our isolates of *Cry. carpinicola* as well as the TUB sequence AM920692 from the Lombardy (Italy). The phylogeny of *Cryphonectria* splits into two major clades (Fig. 1). One includes *Cry. carpinicola* together with *Cry. radicalis*, *Cry. decipiens* and *Cry. naterciae* from Europe, while the other comprises species spread in eastern Asia, such as *Cry. citrina*, *Cry. japonica*, *Cry. macrospora* or *Cry. parasitica*.

Morphologically, *Cry. carpinicola* shared many characteristics with *Cry. radicalis*, demonstrating the close relationship between both species. On PDA, the mycelium was flat in both species, but *Cry. radicalis* developed purple colour when grown in the dark (Hoegger et al., 2002). However, we also observed some variation in culture morphology among the *Cry. carpinicola* isolates. For example, the Georgian isolate grew very slowly and only up to 25 °C (Fig. 3). However, molecular data clearly confirmed its taxonomic position together with all other isolates of *Cry. carpinicola* (Figs. 1 and 2). Additionally, the conidia of all three isolates had similar shape and size. Culture morphology can be influenced by many factors and it is well-known that it can change during sub-culturing in the laboratory. Virus infection is also known to affect culture morphology in *Cryphonectria* spp. (Hillman and Suzuki, 2004).

4.2. Host range and distribution of Cry. carpinicola

So far, *Cry. carpinicola* was found in Europe only on *Car. betulus* and in the Caucasus region on an unidentified *Carpinus* species, probably either *Car. betulus* or *Car. orientalis*. *Carpinus* spp. (family Betulaceae) noticeably is the main host of *Cry. carpinicola*. This clearly distinguishes this species from other *Cryphonectria* species, which mainly occur on *Castanea* and *Quercus* in the family Fagaceae. In regions, where *Cry. carpinicola* was found in Europe, extensive sampling of *Cas. sativa* has been done to study the

chestnut blight fungus, but *Cry. carpinicola* has never been reported on European chestnut in these studies. However, our inoculation tests and a field study by Saracchi et al. (2010) demonstrate that *Cry. carpinicola* possesses the potential to invade bark tissue of *Cas. sativa*, although it acts rather as weak pathogen because no girdling cankers were observed. Likewise, *Cry. naterciae* and *Cry. japonica* were primarily reported to colonise chestnut wood saprotrophically (Dennert et al., 2020).

Cryphonectria japonica has also been reported as weak parasite on *Carpinus tschonoskii* in Japan (as the syn. *Cry. nitschkei*; Myburgh et al., 2004a). Although, *Car. tschonoskii* is only a minor host amongst the main host plants of the Fagaceae, the ability to colonise both Fagaceae and Betulaceae trees seems to be an ancestral character state in the genus *Cryphonectria* because, e.g., *Cry. carpinicola* and *Cry. japonica* belong to different lineages within this genus and the most recent common ancestor is placed at the basal genus node (Fig. 1). For this reason, we assume that, depending on the prevailing environmental conditions, *Cryphonectria* species have the potential to behave as pathogen, as weak parasite or as saprophyte on both Fagaceae and Betulaceae in the sense of the *endophytic continuum* (Schulz and Boyle, 2005). This concept hypothesizes that there are no neutral interactions, but rather that endophyte–host interactions involve a balance of antagonisms with at least a degree of virulence on the part of the fungus enabling infection. The ability to maintain a wide host range facilitate surviving under dynamic environmental conditions over a long-term timescale. Indeed, host jumps are common for plant pathogenic fungi (Burgess and Wingfield, 2016; Sieber, 2007; Slippers et al., 2005) and previous studies have shown that different species in the Cryphonectriaceae undergo regularly host jumps (Chen et al., 2016; Gryzenhout et al., 2009; Heath et al., 2006; Vermeulen et al., 2011). Hence, for *Cry. carpinicola*, we assume that it can colonise different host families at least as weak parasite or saprotroph, but it was first discovered as conspicuous pathogen on hornbeam trees.

Additionally, *Cry. radicalis* has been documented on *Carpinus* trees in old herbarium specimens. An explanation for these records is that in the past several closely related species were jointly interpreted as *Cry. radicalis* due to scarce morphological features useable for species discrimination. In fact, Myburg et al. (2004a, b) reported phylogenetically distinctive lineages of specimens labelled as *Cry. radicalis* that resulted in the separation of the new species *Cry. decipiens* from *Cry. radicalis sensu stricto* (Gryzenhout et al., 2009). It is thus possible that *Cry. carpinicola* was reported on *Car. betulus* under the name of the morphologically very similar *Cry. radicalis* and not recognized as a distinctive species.

4.3. *Pathogenic potential of* Cry. carpinicola

Due to heavy dieback in the Lombardy and Piedmont at early 2000s, the disease affecting *Car. betulus* trees was called *hornbeam decline* in Italy (Ricca et al., 2008; Rocchi et al., 2010; Saracchi et al., 2007). In Torino, e.g., mortality increased by 11% from 2004 to 2007, and 54% of the 300 surveyed hornbeam trees were in 2007 symptomatic (Ricca et al., 2008). Our pathogenicity tests confirmed that *Car. betulus* is a main host species of *Cry. carpinicola*. Two isolates (one each from Austria and Switzerland) produced clearly visible lesions, when inoculated into the stems of *Car. betulus* (Table 4, Fig. 5) and both could be re-isolated at high frequency two months after inoculation. This result is consistent with a previous inoculation study using a *Cry. carpinicola* (named *Endothiella* sp.) isolate from Italy, which produced significant larger lesions on *Car. betulus* than on other potential host trees (Saracchi et al., 2015). The isolate from Austria proved to be particularly virulent by producing sporulating lesions on both *Car. betulus* and *Cas. sativa*. None of the isolates caused lesions on hazelnut and birch, but still could be re-isolated to some extend in the end of the experiment, most notably from inoculated birch seedlings.

To assess the pathogenicity potential of *Cry. carpinicola*, we used a wound inoculation method, which has been widely applied to determine virulence and host specificity of the chestnut blight fungus, *Cry. parasitica* (e.g. Dennert et al., 2019; 2020; Peever et al., 2000). Upon inoculations of susceptible chestnut seedlings, *Cry. parasitica* isolates typically produced large lesions within a few weeks, which lead to high seedling mortality (Dennert et al., 2019). In comparison, lesions produced by *Cry. carpinicola* on hornbeam were much smaller and did not cause host mortality, suggesting that *Cry. carpinicola* is rather a secondary than a primary pathogen on its main host tree. The isolates used in this study were all obtained from dead hornbeams trees, which in the cases of the Swiss isolates suffered from drought periods. In Italy, *Cry. carpinicola* has been mainly reported on stressed hornbeam trees in urban environment often together with *A. decipiens* (Saracchi et al., 2010). Which combination of environmental factors incites the pathogenic potential of *Cry. carpinicola*, however, remains to be determined.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2020.11.012.

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