INTRODUCTION

Since the early twentieth century, the ascomycetous genus Cryphonectria has been a major concern of several generations of tree pathologists from North America and Europe. This genus includes a number of obligate hemibiotrophs in which the initial biotrophy can develop into necrotrophy (Stauber et al., 2020). The best-known representative of this genus is the highly pathogenic Cryphonectria parasitica, the causal agent of the chestnut blight disease. Since the establishment of the United States Department of Agriculture, it has been the stated policy of the U.S. government to collect plant species from around the world, test...
their economic value, and introduce them to American farmers (Stoner & Hummer, 2007; Williams & Volk, 2020). Thus, starting in 1876, American breeders first introduced Japanese chestnuts (Castanea crenata) and European chestnuts (Castanea sativa) and later Chinese chestnuts (Castanea mollissima), hoping to create a market for these trees (Biermann, 2016). We know nothing about the event that led to the actual introduction, except that sometime between 1876 and 1904 a fungus came to the United States. We are talking about C. parasitica that was first noticed in New York in 1904 on native chestnuts, spreading rapidly throughout the range of chestnut forests and within 40 years, destroyed most of the estimated four billion American chestnut (Castanea dentata) trees (Anonymous, 2006). Later genetic analyses revealed that C. parasitica was mainly introduced to North America from Japan, while only minor gene flow was detected from China (Dutech et al., 2012; Milgroom et al., 1996), confirming earlier reports that almost all Castanea imports came from Japan to the USA prior to the emergence of chestnut blight in North America (Anagnostakis, 1992).

The rapid spread of chestnut blight in the United States led to intensive research and to an early discovery of the original distribution area of the pathogen in China and Japan (Shear & Stevens, 1913, 1916). Nevertheless, Asian chestnut orchards remained popular in Europe and were promoted by governments for decades in the first half of the twentieth century. Already at that time, European chestnut was seriously threatened by the ink disease (Phytophthora spp.), so that in the most affected countries like Italy, France, Portugal and Spain, all hopes were placed on the supposedly resistant Asian chestnuts (Darpoux, 1949; Elorrieta y Artaza, 1949; Pavari, 1949; Taveira Fernandes, 1953; Vannini & Vettraino, 2001). It is unclear where the fungus originally came from. What is known is that the first noticed symptoms of chestnut blight on European territory were discovered in 1938 in the northern Apennine Mountains near the Italian city of Genoa, although this observation was not published until after the Second World War (Biraghi, 1946). Shortly afterwards, in 1940, the owner of a Japanese chestnut plantation in a village in northern Spain also reported symptomatic trees, and the Phytopathological Station of La Coruña (EFA) investigated whether it might be C. parasitica—“the cause of the terrible American canker” (Rodriguez Sardiña, 1943). Thanks to the preservation of the isolates from the Japanese chestnuts at the EFA station (closed in 1964), these isolates were transferred to the Swiss Federal Research Institute WSL, where they are still kept today.

In the shadow of the aggressive C. parasitica, research on other Cryphonectria species occurring in Europe has long been neglected, and little is known about their life strategies. Pathogenicity tests have shown that they can successfully infect tree tissue and live saprotrophically on the dead tree or on fallen branches (Dennert et al., 2020). Despite this saprotrophic behaviour, a genome analysis classified all Cryphonectria species as genetically capable of hemibiotrophy (Stauber et al., 2020). The low-profile infections could, at least to some extent, be the reason why in Europe Cryphonectria naterciae (Bragança et al., 2011) and Cryphonectria carpiniola (Cornejo et al., 2021) have only recently been described as new species. Furthermore, Cryphonectria radialis, which was documented in England and Central Europe as early as the nineteenth century under various names (mostly as species of Endothia), was hardly reported for several decades and was rediscovered as a Cryphonectria species in Switzerland (Hoegger et al., 2002) and Greece (Sotirovski et al., 2004) only at the end of the twentieth century. As a fourth species, Cryphonectria decipiens was recently separated morphologically from other Cryphonectria species mainly based on their ascospore size using herbarium specimens preserved in the U.S. National Fungus Collections (BPI) (Gryzenhout et al., 2009). Although no isolate deposit or DNA data of its holotype BPI 1112743 have been published, C. decipiens was hypothetically linked to DNA data of two strains collected in Portugal and Italy (Myburg et al., 2004). Consequently, other authors have suspected that C. decipiens is conspecific with C. naterciae (Cornejo et al., 2021; Rigling & Prospero, 2018; Stauber et al., 2020). This will be clarified in the present study by molecular analysis of the holotype of C. decipiens.

As part of the data digitization of our culture collection, the historical strains M282–M289 of the culture collection of the Swiss Federal Research Institute WSL were verified by barcode sequencing. These strains were compiled by the Italian plant pathologist Mario Orsenigo for a research series on the chestnut blight disease (Orsenigo, 1955a, 1955b) and made available to WSL in 1954, as the spread of chestnut blight had been observed in southern Switzerland since 1948 (Arigoni, 1950). Our laboratory preserved the mycelium of this small collection as lyophilizes in vacuum-sealed glass capsules and also archived the documentation from the 1950s. Nevertheless, these strains remained almost unnoticed for decades and the information in the paper archive was no longer traceable, making it difficult to reproduce the chronology of the strains today.

In this study, we reconstructed the species identity of isolates M282–M289 by DNA barcoding and their history based on the primary literature describing the original source of these isolates. The chronology of M282–M289 lead us on a historical journey from Italy to France, Spain and Portugal in the 1920s to 1940s. While the species classification of the eight isolates based on DNA barcoding was unambiguous, their relationship to the modern species concept and the question of which symptoms they caused in which host remained controversial. On the one hand, the first documented cases of bark canker in Spain in 1940 remained in need of explanation: Was this the first outbreak of chestnut blight in Spain? On the other hand, the chronology of isolate M289 from Portugal also prompted us to examine the legitimacy of the two names C. decipiens and C. naterciae: Are these two taxa conspecific? The present study uses a phylogenetic approach to determine the systematic classification of the isolates M282–M289 and the holotype of C. decipiens BPI 1112743 in relation to other species of the genus Cryphonectria. The preservation of isolates, detailed descriptions and experiments of the pioneers of European tree pathology have left traces of Cryphonectria species causing similar but different tree diseases than C. parasitica.
2 | METHODS

2.1 | Investigated material

Isolates M282–M289 were stored lyophilized in glass capsules and at 4°C since 1967. Pieces of mycelium containing conidia were transferred on PDA (potato dextrose agar, Difco) in the laboratory (21–22°C) for 7–10 days to check their viability.

The herbarium specimen designated as the holotype specimen of *Cryphonectria decipiens* was kindly loaned by the U.S. National Fungal Collection on 19-08-2022: France, Pau 64, Bois Bastard, on *Quercus* sp., 27-Nov-1991, coll. Françoise Candoussau, No: 5615-1, BPI 1112743, determined as *C. radicalis* by G. J. Samuels, Feb 1992.

2.2 | DNA barcoding

The DNA from recultivated mycelial cultures M282–M289 was extracted using the semi-automated KingFisher 96 Flex (Thermo Fisher Scientific) suitable for cultures grown on agar, which contain high amount of DNA. However, to preserve the historical herbarium material and avoid contamination of the expected degraded DNA, sampling and DNA extraction of BPI 1112743 was performed under sterile conditions. A minute piece of 5 x 5 mm containing a conidiophore was removed and subsequently subjected to manual single extraction using the NucleoSpin Tissue Kit (Marchery-Nagel) according to the manufacturer’s instructions, which is suitable for the small-scale preparation of genomic DNA from any tissue, including forensic samples.

The internal transcribed spacers (ITS) of the nuclear rRNA gene cluster were amplified and sequenced as the complete ITS-fragment (primer-pair: ITS1–ITS4) according to published protocols (White et al., 1990). However, DNA gained from BPI 1112743 resulted to be highly degraded. Whereas the ITS1 region (primer-pairs: ITS1–ITS2) could be amplified following published protocols (White et al., 1990), new primer for the ITS2 regions were needed. Two primer pairs were designed using GenBank sequence references of *C. naterciae* and *C. radicalis* and the software DNA Main Workbench v22 (CLC bio, Qiagen) (pair 1: Crad-ITS2-F1/R1: 5’− AAC GGA TCT CTT GGT TCT −3/3’− GCG AGG TGG AAG AAA AAA AA −5’; pair 2: Crad-ITS2-F2/R2: 5’− CTT TCA ACA AGC GAT CTC T −3/3’− TTT ACG GCA AAA GCA ACC −5’) (Figure S1). The resulting forward and reverse amplicon sequences were assembled using the DNA Main Workbench software. All ITS consensus sequences were verified in GenBank (www.ncbi.nlm.nih.gov, accessed on 22-09-2023) using the nucleotide BLASTn search. Species assignment was accepted for matches >99% sequence identity only. The ITS1 sequences of *C. radicalis* specimens contained long stretches of single nucleotide repeats, resulting in a rapid decline in Sanger read quality after the long poly(dA) and poly(dT) stretches. Therefore, only the ITS2 region was used for alignment and phylogenetic analysis under the maximum likelihood criterion as applied in PhyML 3.0 (Guindon et al., 2010) on the ATGC platform (http://www.atgc-montpellier.fr, accessed on 22-09-2023), using the Smart Model Selection and the Akaike Information Criterion. Bootstrap (B) with 1000 pseudoreplicates was performed to estimate the robustness of the individual branches. The resulting tree was displayed in TreeGraph 2 (Stoever & Mueller, 2010). Finally, to further characterize the genotype of *C. parasitica* specimens, we determined the allelic composition of the vic and MAT genes using a published molecular method (Cornejo et al., 2019).

2.3 | Literature research

This study uses a qualitative research approach based on the evaluation of the primary sources in which the strains were first described. The search for primary sources began with the transcription of information contained in the paper documents archived at WSL since the 1950s. The transcription followed the rules of the Ad fontes learning platform of the University of Zurich for all those who work with historical materials (UZH, 2001, accessed on 30-07-2023). After bibliographic research, the references of the primary sources could be retrieved thanks to the scientific library service of the ETH-domain (https://www.lib4ri.ch/). Additional information on the strains compiled by Mario Orsenigo was kindly provided by the curator of the international culture collections of the Westerdijk Fungal Biodiversity Institute (hereinafter, CBS). In order to be able to follow the historical naming, the names valid today are as follows (old name: current name): *Endothia fluens*: *Cryphonectria radicalis*. *Endothia parasitica*: *Cryphonectria parasitica*. *Endothia radicalis*: *Cryphonectria radicalis*. *Endothiella Sacc.*: anamorph form of *Endothia*.

3 | RESULTS

3.1 | Barcode sequencing

All lyophilizates M282–M289 stored at 4°C in the WSL collection could be revived on PDA, and the growing mycelium of each sample was used for DNA sequencing of the ITS region (GenBank accessions for M282–M289: OR840573–OR840580). While the isolates M283 (Stauber et al., 2020) and M285 (Hoegger et al., 2002) were used in earlier studies, the others were first time revived for this study. All primers designed to amplify short fragments from degraded DNA of the holotype of *C. decipiens* (BPI 1112743) were able to amplify parts of the ITS1 and ITS2 region (GenBank accession for BPI 1112743: OR840581). GenBank accessions AF368328.2 and MH857310.1, both corresponding to *Cryphonectria radicalis* CBS 240.54 were used to identify ambiguos sites in the amplicons of BPI 1112743 (for technical details see Figure S1). Sequences producing significant alignments to the ITS2 among closely related *Cryphonectria* species, as a result of the BLASTn search, were downloaded directly from GenBank and used for phylogenetic analysis. The maximum likelihood tree included M284 in a clade of *C. parasitica* specimens with a bootstrap
support of B: 94.9%, M289 in a clade of C. naterciae sequences (B: 89.4%) and all other WSL-cultures examined in a clade of C. radicalis sequences (B: 95.3%). BLASTn search revealed that the ITS2 region of the holotype of C. decipiens (BPI 1112743) was 100% identical to other C. radicalis sequences (Figure S1). Phylogenetically, it forms a monophyletic group together with specimens of C. radicalis with 95.3% bootstrap support (Figure 1). This result is also confirmed by analysis of the genetic distance based on the ITS2 alignment (Figure S2). Cryphonectria decipiens must therefore be synonymized with C. radicalis.

3.1.1 | Taxonomie

Cryphonectria radicalis (Schwein.: Fr.) M.E. Barr, Mycol. Mem. 7: 144 (1978).
Basionym: Sphaeria radicalis Schwein.: Fr., Elenchus Fung. 2, 73. 1828.

Synonyms:

3.2 | Literature research

Primary sources were evaluated to compile information on collector, year of collection, location, host, symptoms, and other aspects of interest such as results of experiments conducted or causes for tree sampling. Information on M282–M289 preserved in the WSL

FIGURE 1 Phylogeny of Cryphonectria species, including isolates M282–M289, and habitus of herbarium specimen of BPI 1112743.
(a) Maximum likelihood tree reconstruction based on the ITS2 region of 40 Cryphonectria taxa (PhyML v.3.0; 16 polymorphic out of 294 nucleotide sites; GTR substitution model; starting from five random Neighbor-Joining trees). Bold red letters mark the phylogenetic positions of the eight strains M282–M289, while bold black letters indicate the position of BPI 1112743 and blue letters the sequences of Myburg et al. (2004) for C. radicalis sensu lato. The GenBank accession numbers and the country of the specimens included in the analysis are indicated in the tree. The numbers on the branches are the values of 1000 bootstrap repeats. (b) Herbarium sheet of BPI 1112743 labelled by hand by the collector Françoise Candousau and others. (c) Herbarium specimen BPI 1112743, Cryphonectria radicalis.
archives and from other primary sources is summarized in the Table S1. The tortuous paths taken by the isolates are outlined in Figure 2 and their collection sites are plotted in Figure 3, while the reappraisal of the historical significance of M282–M289 is described below.

3.2.1 | Cryphonectria radicalis strains M285 and M286 from Italy

Orsenigo (1955a) refers to Antonio Biraghi as the supplier of these isolates, which were given to him under the name Endothia fluens 84 and Endothia fluens 86 (Figure 2a). The same names are also listed online at CBS for the two strains 239.54 and 240.54, which were deposited at this institution by Mario Orsenigo in 1954. Biraghi informed Orsenigo that both strains were isolated in 1952 from a Ca. sativa tree in the province of Catanzaro (Figure 3) that had died of ink disease and that the tree was absolutely not infected with C. parasitica (Orsenigo, 1955a). According to this description, M285 and M286 are most likely genetic clones of the same infection on a single chestnut tree. Additionally, CBS also lists strain 238.54 deposited by A. Biraghi, which was isolated in 1952 from the bark of a Ca. sativa tree "killed by Phytophthora" (ink disease) in the province of Catanzaro, Italy. In accordance with this description, CBS 238.54 is assumed here to be a culture replicate of the same isolation from Catanzaro, together with CBS 239.54 and CBS 240.54 (Table 1).

3.2.2 | Cryphonectria radicalis M282, M283, M287 and M288 from Galicia, Spain

For C. radicalis (Figure 2b) of the provinces Galicia, two places are mentioned in our paper documentation of the 1950s: La Coruña (M282), and Córrego (M283, M287 and M288) (Figure 3). Of particular historical interest are the isolates from Córrego de Valdeorras, as this was the first time in Spain that canker-like symptoms were documented on Japanese chestnuts imported as seedlings from France. Rodríguez Sardiña (1943) identified the causal agent for the Ca. crenata cankers as Endothiella gyrosea Sacc. form Tamba: Endothiella used for the asexual form of the genus Endothia (Cryphonectria) and form Tamba for the variety Tamba of Ca. crenata. However, the author emphasized that this form was distinct from Endothia parasitica because no symptoms were observed in European chestnuts that grew in close proximity to the symptomatic Japanese chestnuts. Eighty years after Rodriguez Sardiña’s studies, these strains have been identified by barcode sequencing as C. radicalis, which is known to cause symptoms on Ca. crenata, but infects the European chestnut asymptotically and is visible saprophytically only on dead branches (Dennert et al., 2020; Hoegger et al., 2002).

3.2.3 | Cryphonectria naterciae M289 from Portugal

Another historical rarity in the WSL-collection is C. naterciae strain M289 that is documented in our hard copy of the 1950s under the label of Endothiella gyrosea Sacc. and the note "de Oliveira" (de: Spanish of) (Figure 1c). The present literature search revealed, on the one hand, that a record named "Endothiella gyrosea Sacc. (?)” was included in the list of new fungi for Portugal as “… In trunci corticatis Quercus suber L., pr. Azambuja (Mata das Virtudes), leg. Branquinho de Oliveira, februario, 1929” (Cámara, 1930). This isolate was studied morphologically and experimentally with inoculations by the collector d’Oliveira (1931), which concluded that the Q. suber isolate did not fit to any Endothiella species described by Shear et al. (1917), “… although the pycnidial forms of Endothiella gyrosea (Schw.) Fries and Endothia fluens (Sow.) S. and S. were very close”. On the other hand, CBS lists strain No. 165.32 from Portugal, isolated from Q. suber and deposited in this collection by d’Oliveira in April 1932 under the name Endothiella gyrosea.

Returning to the cankers in Ca. crenata plantations in Galicia: As these cankers could have been the first outbreak of chestnut blight in Spain, Rodríguez Sardiña assembled several strains of Endothia spp. from the United States and Europe delivered from CBS because the Second World War "... made direct communication to scientists in the U.S. difficult". In this context, Rodríguez Sardiña (1943) listed “… a strain Endothiella gyrosea Sacc., isolated by the Portuguese Braquinho d’Oliveira” received from CBS in 1940. Subsequently, Rodríguez Sardiña deposited all the strains involved in canker study in the culture collection of the EFA station, which were sent by the EFA to Mario Orsenigo in 1953 (Orsenigo, 1955a), and by Orsenigo to WSL (M289) and CBS (250.54) in 1954.

Cryphonectria naterciae is a species that has been found rather rarely in Europe and the Mediterranean Basin, mostly in association with the cork oak (Q. suber). D’Oliveira (1931) pointed out that inoculation experiments showed that the fungus cannot penetrate the cork layer and can infect the tree only after removal of the cork or after violent cuts. But even then, the infection developed slowly and first symptoms appeared only after 3 months or even a year. To prevent the spread of infections, D’Oliveira (1931) recommended disinfecting axes after removing the cork. Until present, C. naterciae is mostly observed in Q. suber as secondary parasite (Pinna et al., 2019; Smahi et al., 2018) or asymptotically as endophyte (Costa et al., 2020). It was found accidentally in Ca. sativa because the trees were additionally infested with C. parasitica (Bragança et al., 2011). The rare findings of this species and the coincident 1931–1932 date of d’Oliveira’s publication and date of culture deposit support the hypothesis that CBS 165.32 represents the same voucher sampled by d’Oliveira in 1929, which came in a roundabout way from CBS via Rodríguez Sardiña and Orsenigo to WSL. Accordingly, CBS 165.32, CBS 250.54, and M289 are considered here as culture replicates (Table 1) of the strain collected in 1929 by d’Oliveira from the province Azambuja in Portugal (Figure 2).
3.2.4 | \textit{Cryphonectria parasitica} M284 from Santander, Spain

The other original isolate from Rodríguez Sardiña preserved in our collection is strain M284, which was involved in canker formation on \textit{Ca. crenata} var. \textit{Tamba} and was named \textit{Endothiella gyrosa} Sacc. form \textit{Tamba} by Rodríguez Sardiña (1943). Santander is in the autonomous community of Cantabria, more than 370 km from Córrego (Figure 3). Orsenigo (1955a) wrote for the sampling the year 1947? (with question marks). According to Darpoux (1949), cankers on \textit{Ca. crenata} were observed as early as 1941 in Santander, and later in Elorrichuela. This is also confirmed by Taveira Fernandes (1946). Unfortunately, we have not found any further information about the Santander No. 2 strain to verify the year of collection and the context in which this specimen was obtained by Rodríguez Sardiña. Therefore, we can only speculate that it may be a culture from one of the first outbreaks of chestnut blight in Spain, possibly from the years around 1943–1948, after Rodríguez Sardiña studied the outbreak in Córrego and until the first report of chestnut blight in Spain (Biraghi, 1948). The genotype of \textit{C. parasitica} M284 was analysed and can be assigned to the rather rare type EU-18 (\textit{vic} alleles: 2211-21) and mating type MAT 1-1.

4 | DISCUSSION

The present study examines the chronology and phylogenetic relationship of historical \textit{C. naterciae}, \textit{C. parasitica} and \textit{C. radicalis} cultures collected more than 80 years ago. From the first discovery of the chestnut blight in Europe, a network of phytopathologists has played a central role in why the strains M282–M289 were preserved and sent on from laboratory to laboratory. Noteworthy is Mario Orsenigo’s clear vision into the future of preserving isolates in a central culture collection (CBS) and d’Oliveira’s and Sardiña’s accurate phytopathological studies. It is also worth mentioning that Juan Rodríguez Sardiña was sent into intellectual exile from Madrid to Galicia, to an institute of inferior category, because he had continued to lecture under the Republican government during the Spanish Civil War (Fernández Prieto, 2012; Fraga Vázquez, 2023). We owe
him an extremely careful pathological study and storage of isolates from plantations of Japanese chestnut in north Spain, preserving an invaluable resource for future scientific study.

4.1 | *Cryphonectria radicalis* as causal agent of bark cankers on Japanese chestnut

The isolates obtained from *Ca. crenata* from La Coruña (M282) and Córrego (M283, M287 and M288) belong to *C. radicalis*, which is well documented for Europe and Japan (Hoeppger et al., 2002; Kobayashi, 1970; Kobayashi & Itô, 1956). Among the many important observations Rodríguez Sardiña made was that the disease resembled, but differed from “American canker” because extensive mycelial fans invading the cambium were missing and the isolates from *Ca. crenata* stained the Richard medium purple—today, a well-known feature of *C. radicalis* also on PDA medium (Cornejo et al., 2021). Apparently, *C. radicalis* is able to cause no cankers on *Ca. crenata* of the Shiba variety but significant symptoms on the Tamba variety, but does not kill these trees even after decades, as observed in Córrego (Rodríguez Sardiña, 1943) and Elorrichueta (Biraghi, 1948). These results are the first documented occurrence of canker on *Ca. crenata* in Spain caused by *C. radicalis*.

It is possible that these *C. radicalis* strains came from Japan along with the imported seeds. Morphological examination of specimens from different origins in Japan and a customs interception of symptomatic *Ca. crenata* seedlings imported from Japan to San Francisco in 1915 confirmed the presence of *C. radicalis* in Japan at an early stage after the outbreak of chestnut blight in the USA (Shear & Stevens, 1916). From today’s perspective, however, it is not possible to determine whether the Japanese chestnuts from Córrego were infected locally in France (nursery) or Spain (plantation) or whether the seeds came already infected from Japan.

4.2 | The oldest living isolate of *Cryphonectria natteriae*

The history of isolate M289 could be traced back to CBS 165.32, which proved to be the oldest preserved isolate of the rare
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain no.</th>
<th>Replicates (year of deposition)</th>
<th>Other vouchers</th>
<th>GenBank</th>
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<td>MUCL 7954</td>
<td>MH688842 (LSU)</td>
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<td></td>
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<td>E76</td>
<td>AF368328 (ITS)</td>
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<td>MYA-2673 b</td>
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<td></td>
<td>670</td>
<td>MCU 56</td>
<td>OR840578</td>
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<td>n.a.</td>
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<td>N.58</td>
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</table>

a According to hard copy documentation from 1954 archived in WSL and to Orsenigo (1955a).
b MCU: Numbers used by Orsenigo and deposited in the Swiss Federal Research Institute WSL. N: Numbers used by Orsenigo and deposited in the Westerdijk Fungal Biodiversity Institute, CBS. MCU: Fungal collection of the Université catholique de Louvain. E: Vouchers of Roland J. Stipes collection deposited in the culture collection of the Forestry and Agricultural Biotechnology Institute, FABI. CMW: Numbers used in culture collection of the Forestry and Agricultural Biotechnology Institute, FABI. MYA: Number used in the American Type Culture Collection, ATCC.

c CBS 238.54 was sent directly by Antonio Biraghi to CBS in 1952.
d CBS 165.32 was sent directly by Branquinho d’Oliveira to CBS in 1932.

Cryphonectria naterciae, confirming the presence of this fungus in Europe for almost 100 years. Our literature search also followed another interesting path of the d’Oliveira specimen. Phytopathologist Roland J. Stipes obtained strain No. 165.32 of *Endothia gyrosa* (*Q. suber*, Portugal, 1932)” from CBS, which Stipes designated as E14 (Stipes et al., 1982). Stipes later transferred his Cryphonectria collection to the South African culture collection CMW of the Forestry and Agricultural Biotechnology Institute (FABI), where E14 was deposited as CMW 10436 (Myburg et al., 2004; Venter et al., 2002) (Figure 2c). This is interesting in that strain CMW 10436 was one of two isolates that were positioned in a clade Cryphonectria radialis sensu lato in a phylogeny by Myburg et al. (2004). The second Stipes voucher E83 (CMW 10484) presented in that study came from Italy, collected by Antonio Biraghi from a *Ca. sativa* tree. Unfortunately, voucher CMW 10484 was deposited as CBS 112918 only after its first publication in Venter et al. (2002) and we have been unable to find any primary source to trace the origin of this isolate. However, *C. naterciae* is well known from *Q. suber* in Italy at present (Pinna et al., 2019), and it has also been found on *Ca. sativa* in Portugal (Bragança et al., 2011).

4.3 The taxonomic identity of *Cryphonectria decipiens*

Gryzenhout et al. (2009) based their description of the new species *Cryphonectria decipiens* on the observation that different samples within the *C. radialis* species complex have different spore sizes (Myburg et al., 2004) (Table 2). The authors created the new species with the holotype BPI 1112743 for the collections with longer ascospores. Furthermore, they mentioned that the *C. radialis* species complex is phylogenetically divided into two groups (Gryzenhout...
et al., 2009; Myburg et al., 2004). As there was no sequence data of the long-spore specimens of *C. radicais* and no spore measurements for the sequenced isolates, it was not possible for Gryzenhout et al. (2009) and Myburg et al. (2004) to link the two observations. In the present study, we can close this gap by analysing DNA from the holotype of *C. decipiens* BPI 1112743. Due to the severely degraded DNA of the more than 30-year-old specimen, it was only possible to amplify short fragments of the multi-copy ITS1 and ITS2 region with primers developed specifically for this purpose; other frequently used genetic markers are single copy genes and could not be analysed. Nevertheless, the sequence fragments obtained contained sufficient genetic information for a phylogenetic analysis. As the ML tree placed BPI 1112743 monophyletically with *C. radicais* sensu stricto, the holotype of *C. decipiens* is no longer considered a separate species and consequently synonymized with *C. radicais*. The isolates CMW 10436 (E14, CBS 165.32) and CMW 10484 (E83, CBS 112918), which were hypothetically associated with *C. decipiens* (Chen et al., 2013; Cornejo et al., 2021; Gryzenhout et al., 2009; Jiang et al., 2019; Myburg et al., 2004) belong to *C. naterciae* (Figure 1).

*Cryphonectria naterciae* was described based on isolates from *Q. suber* and *C. sativa* from Portugal by Bragança et al. (2011). Therefore, based on molecular phylogenetic data, the *C. radicais* species complex is currently divided into the three species *C. radicais* s. str., *C. naterciae* and *C. carpinicola* (Figure 1). Gryzenhout et al. (2009) list three further specimens that could also belong to *C. decipiens*. These historical specimens could not be examined here as they are no longer on loan from the U.S. National Fungal Collection due to their age. Based on their host trees, Castanea sp. and *Carpinus betulus*, they could belong to other *Cryphonectria* species that have longer ascospores. In particular, the specimen BPI 797698 collected by Michael Woronin in Abkhazia from *Carpinus betulus* could belong to *C. carpinicola*, which has quite long ascospores (Table 2; Cornejo et al., 2023). On the other hand, the example of *C. carpinicola* shows that the size of the ascospores can vary greatly within a species and even within a collection. Cornejo et al. (2023) found spore lengths of 6–11 μm when measuring 40 spores from one sample (Table 2). The collector of BPI 1112743, Françoise Candoussau, also found a large variability in the size of the ascospores in her collection of the holotype of *C. decipiens*, including quite small ones (Figure 1b; Table 2). To summarize, the size of the ascospores alone does not seem to be very useful for the delimitation of *Cryphonectria* species.

The unambiguous application of fungal names is crucial for diagnostics, compliance with laws and regulatory controls, for example, biosafety, food safety, quarantine regulations and industrial applications (Yurkov et al., 2021). This double-lane of a herbarium specimen without DNA data and DNA data without herbarium specimen has led to confusion in various publications over the last decade, where (i) CMW 10436 (E14) and CMW 10484 (E83) continued to be referred to as *C. decipiens* (Chen et al., 2013; Cornejo et al., 2021; Jiang et al., 2019); (ii) *C. decipiens* and *C. naterciae* were assumed to be conspecific (Rigling & Prospero, 2018; Stauber et al., 2020); (iii) the holotype culture CBS 129351 of *C. naterciae* was deliberately renamed to *C. decipiens* (Jiang et al., 2020); (iv) *C. decipiens* was recommended as the holotype species for the anamorph genus *Endothiella* Sacc. (Rossman et al., 2015). In the face of this naming and systematic confusion, our analysis shows once again that morphology alone, independent of DNA data, is not sufficient to infer new fungal species (Aime et al., 2021).

### 4.4 The outbreak of *Cryphonectria parasitica* in Spain

By reconstructing the context of why isolates M282–M289 were collected and from which host, we were able to confirm earlier
findings (Biraghi, 1948) that the chestnut blight epidemic in Spain in the 1940s was due to plantings of imported Asian chestnuts. This is confirmed by the live preserved C. parasitica M284 isolated from Ca. crenata in Santander. Although Ca. crenata and Ca. mollissima developed cankers early in the 1940s in the two experimental plantations in Biscay and in Santander (Darpoux, 1949; Taveira Fernandes, 1946), no bibliography was found on whether these cankers were scientifically studied in Spain. But, on a scientific trip to the plantations in Biscay, Aldo Pavar from the Italian Silvicultural Experimental Station noted that the Japanese trees had large cankers, and described the situation as enigmatic because the disease had not spread from the affected Tamba to the Shiba trees or to the European chestnuts in the same plantation (Pavari, 1949). Today, we can assume that the cankers that did not cause symptoms in nearby Ca. sativa were caused by C. radicalis on Ca. crenata var. Tamba in Biscay and Santander, similar to the outbreak in Córrego.

In contrast, Antonio Biraghi (who participated in the same trip as Pavar) described a dozen Ca. crenata trees of about 25 years in the village of Elorrichueta, most of which were symptomatic and mixed with some Ca. sativa trees of the same age, one of which was also symptomatic (Biraghi, 1948). To investigate these cankers, Biraghi inoculated European chestnuts in an existing coppice forest in Veroli, central Italy, with isolates from Ca. sativa and Ca. crenata from Elorrichueta suffering from bark canker. The Institute of Experimental Forestry in Madrid sent additional isolates from Spain and a C. parasitica strain from Italy was used as positive control. After about a month and a half, most inoculations showed lesions comparable to those of the positive strain (Biraghi, 1948). Therefore, the 1947 report of bark canker on Ca. sativa in Elorrichueta (Biscay) is considered the first finding of C. parasitica in Spain (Cobos, 1989; Elorrieta y Artaza, 1949; Muñoz & Cobos, 1991).

4.5 The European epidemic of Cryphonectria parasitica

Since the mid-nineteenth century, ink disease (Phytophthora spp.) spread and damaged chestnut populations in Europe, so that in the most affected countries like Italy, France and Spain, all hopes were pinned on the resistant Asian chestnuts (Darpoux, 1949; Elorrieta y Artaza, 1949; Pavari, 1949). While in France from 1920 to 1931, 81,000 seedlings of the two varieties Tamba and Shiba of Ca. crenata imported as seeds directly from Japan were distributed to interested growers (Darpoux, 1949), the state forestry administrations in Italy and Spain initially promoted experimental plantings (Elorrieta y Artaza, 1949). In Italy, the experimental plantings used seeds imported from the main Japanese island Honshu (Pavari, 1949), while the two Spanish stations in Biscay and Santander used seeds of Ca. crenata from Japan and Korea as well as Ca. mollissima from China, initially imported directly from Asia. According to Elorrieta y Artaza (1949), after the first positive results with the Asian chestnut in the control of ink disease, the news spread quickly and plantings were soon made en masse in the northern Spanish provinces by private planters that imported direct from Asia, but also from nurseries in France as proved by C. radicalis M282, M283, M287 and M288 isolated from trees imported from France (Rodríguez Sardiña, 1943). In Italy, between 1926 and 1939, the Silvicultural Experimental Station distributed 50,000 seedlings of Japanese chestnuts to be planted throughout Italy from the Alps to Padua in the east and along the 1200 km long Apennines Mountains (Elorrieta y Artaza, 1949). The result of all these efforts was that in France, Italy and Spain chestnut private plantations of Asian origin were not monitored by either the authorities or the scientific experts from 1920 onwards.

Early studies found that Ca. dentata nuts could be infected with C. parasitica, and superficial sporulation was even observed on the nutshell lying on the ground (Collins, 1913, 1915). A later study confirmed that an average of 14% of the nutshell harvested from a plantation of Ca. dentata in Connecticut (USA) were indeed infected with the pathogen of chestnut blight, which apparently did not affect seed germination (Jaynes & DePalma, 1984). Unfortunately, no recent studies on pathogen transmission through nuts could be found for the European and Asian species. Nevertheless, the European Food Safety Authority does not recommend the regulation of fruits and seeds of Castanea or Quercus from non-European countries and categorizes nuts for human consumption as a minor route of spread for the fungus (Jeger et al., 2016). However, this and other studies have shown that the importation of seeds for tree cultivation in Spain has led to the introduction of C. parasitica into this country. Gravatt (1952) visited the experimental plantings in Biscay in 1951 and reported that the Spanish authorities also believed that the disease had been introduced through the importation of seeds from Japan. It is also well documented that high amounts of nuts of Ca. crenata and Ca. mollissima were introduced in south-western France in the beginning of the twentieth century (Darpoux, 1949). Detailed genetic analyses of C. parasitica populations from Europe have clearly shown that several genotypes have been able to settle and colonize new areas in this country, which can only be attributed to the high number of seeds distributed in the early twentieth century (Darpoux, 1949; Demené et al., 2019; Dutech et al., 2010, 2012). For this reason, and to prevent the further spread of C. parasitica spores, methods for disinfecting the seed surface were tested in France and have been used in the 1950s (Darpoux & Ridé, 1952).

However, in contrast to Spain and France, where the introduction of chestnut blight through the import of seeds from Japan and China is generally accepted, the situation in Italy is less clear. It has been suggested that C. parasitica may have been introduced with seeds or cuttings of Ca. mollissima from the USA, which were used for grafting, but such activities only began after the Second World War in Italy (Pavari, 1949). Ironically, hundreds of thousands of Chinese chestnut seeds were imported directly from China or the USA, but also from northern Spain, and planted throughout Italy to combat the chestnut blight, because it was assumed that Ca. mollissima was resistant to both Phytophthora sp. and C. parasitica (Gravatt, 1951; Pavari, 1949). Genetic analyses of C. parasitica populations from the USA revealed that most of the gene flow originated in Japan (Milgroom
However, only one population from New Hampshire was found to be closely related to isolates from Ibaraki Prefecture in northern Honshu, while the other eight North American populations were of unknown origin in Japan. As one northern Italian and one southern Swiss population in the same study were closely related to isolates of unknown origin from the USA, it was again hypothesised that the introduction of C. parasitica in Italy may have originated in the USA (Milgroom et al., 1996). The close phylogenetic relationship between the so-called European/North American (E/NA) cluster of C. parasitica has been independently confirmed by whole genome analyses and is undisputed (Demené et al., 2019; Stauber et al., 2021). However, as the American genotypes themselves are of Japanese origin, it is difficult to genetically distinguish whether the Italian outbreak was introduced from the USA or directly from Japan. The genetic reconstruction of the invasion pathway depends crucially on the representativeness of specimens from the native and invaded areas. However, sampling has so far been rather patchy and the source populations in Japan have not been identified for either the USA or Italy.

Therefore, in this study, an alternative hypothesis for this close relationship of the E/NA isolates is considered, suggesting that the random exchange of chestnuts between continents may have led to the worldwide spread of closely related genotypes from the area of origin as mentioned in an earlier study (Dutech et al., 2012). Given the mass importation of Japanese chestnuts to Italy only 15–20 years after the North American outbreak, it seems not unlikely that C. parasitica was introduced with the chestnuts, which were imported exclusively from Honshu and planted throughout Italy. This is also confirmed by the outbreaks of chestnut blight within a short period of time in remote areas of Italy (Bazzigher, 1951; Gravatt, 1952; Pavari, 1949; map in Figure S3). Within a few years, the chestnut blight spread simultaneously, starting from four main foci, three of which developed almost in parallel but were hundreds of kilometres apart (Genoa discovered in 1938; Udine in 1940; Naples in 1943). Another main outbreak in the Alps (Varese discovered in 1946) and countless small foci along the Apennine Mountains, which correspond to the experimental planting areas. According to Gravatt (1952), blight caused by C. parasitica was reported in 1938 in Italy, “but it undoubtedly had been present for many years previously”. This view is also confirmed by a time calibrated genetic analysis, which estimated the age of the common ancestor for isolates from France and Italy representing the E/NA cluster to be the beginning of the twentieth century (mean age 1931; Demené et al., 2019), suggesting an early onset of the epidemic in both countries. Although Japanese chestnuts were imported in large quantities from Honshu, probably only a few genotypes of C. parasitica were able to successfully establish in the new environment in Europe and the USA and colonize new areas, which we now refer to as the E/NA cluster. Similar to France, Ahmad and Baric (2022) identified three separate introduction events of C. parasitica in South Tyrol, a north province of Italy, based on simple sequence repeats (SSR) analyses, which also match genotypes from France, Switzerland, other regions of northern Italy and the USA.

To summarize, it is important to look back in history to learn from past experiences. It is still unclear and it will probably remain unclear in the future where C. parasitica originally came from in Italy. However, it is clear that the mass import of plant material (nuts) from other continents has left its mark in all the countries concerned and, although certainly unintentional, has nevertheless led to an economic, social and ecological catastrophe. In this study, we were able to show an interesting chronology of events closely linked to the epidemic of C. parasitica in Europe based on the history of old living isolates, and we hope that we can learn valuable lessons for the future.

AUTHOR CONTRIBUTIONS

Conceptualization: CC; Formal analysis: CC; Investigation: CC and LB; Methodology: CC; Visualization: CC; Writing original draft: CC and LB; Reviewing and editing: CC and LB.

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DATA AVAILABILITY STATEMENT

The literature data that support the findings of this study are available on request from the corresponding author. The DNA sequences generated for this study are publicly accessible in GenBank (www.ncbi.nlm.nih.gov) under the accession numbers OR840573–OR840580.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.