



# 85 years counteracting an invasion: chestnut ecosystems and landscapes survival against ink disease

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**Abstract** A retrospective examination of early pest and disease outbreaks, such as ink disease, offers new insights into their impact on ecosystems and landscapes. Ink disease, caused by *Phytophthora* spp., is one of the most destructive diseases affecting *Castanea sativa* Mill. It first appeared in Europe in the early 18th century, with the initial recorded case in Italy (Piedmont) dating back to 1845. However, its spread instilled significant concerns in several chestnut-growing regions primarily devoted to fruit production. In 1938, a comprehensive assessment

of *Phytophthora xcambivora* outbreaks was conducted in the Bolognese Apennines (Italy), providing a detailed overview of chestnut cultivation status. Thirty-six disease foci were documented, and laboratory tests confirmed the presence of ink disease. To understand the disease's impact on chestnut ecosystem and landscape over the past 85 years, the 1938 sites were revisited to assess chestnut persistence and phytosanitary status, with *Phytophthora* species detected using isolation and molecular techniques. Monitoring data revealed that *C. sativa* still thrives in all but one site, with its wood seemingly able to coexist in dynamic equilibrium with the disease. While *P. xcambivora* was still detected in several foci, the extent of damage appeared limited. The potential for

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natural control, likely influenced by the complexity of soil biota, along with the natural spread of hypovirulence in chestnut blight and biological control of the Asian chestnut gall wasp, could explain the continued presence of chestnut in the investigated area and potentially throughout the Italian chestnut range, despite nearly two centuries of ink disease prevalence. Nevertheless, ongoing monitoring is essential to dynamically comprehend the factors at play and their efficacy, particularly in the context of climate change and the possible spread of other *Phytophthora* species. The survival of chestnut ecosystems amidst ink disease underscores the preservation of the economic, ecological, and landscape values associated with these woodlands.

**Keywords** *Castanea sativa* · *Phytophthora xcambivora* · Historical records · Landscape history · Natural and biological control · Place names

## Introduction

*Castanea sativa* Mill., considered a multipurpose tree in Europe, is still an important resource for the Mediterranean countries: used for fruit and timber production, it also characterizes mountain landscapes and plays an important role in the ecological and biological functions of the forest (Gabrielli 1994; Conedera et al. 2004). Moreover, this species represents the cultural and ecological legacy of more than a millennium of history in which chestnut woods have played a substantial role in the survival of mountain populations (Squatriti 2013). The importance of chestnut orchards was so pervasive as to suggest the name “chestnut civilization” to describe the daily life of Apennine populations (Gabrielli 1994). Chestnut, often referred to as the “bread” tree (Italian: *albero del pane*) due to its significance, was often the “famine” tree, enabling people to survive even in times of poverty and hardship as unique resource; nevertheless, the link between chestnut cultivation and mountain inhabitants survived for centuries with some periods of decline but with substantial persistence of both stands and productions (Arnaud et al. 1997; Pezzi et al. 2011). The appearance in the last two centuries of three invasive diseases (ink disease, chestnut blight and Asian gall wasp) caused much concern about

the survival of chestnut cultivation and severely affected both plant survival and growers’ attitudes toward these woods, especially in the context of upland depopulation after World War II (Murolo et al. 2022). The first disease in order of appearance was ink disease, one of the most destructive diseases of sweet chestnut, because it is able to kill trees and their roots (Vettraino et al. 2001; Vettraino et al. 2005). The oomycete *Phytophthora xcambivora* (Petri) Buism., and the more aggressive *P. cinnamomi* Rands (Rands 1922), are found to be the causal agents of ink disease in Europe; however, while *P. cinnamomi* is the main causal agent of the disease in other European countries, such as Portugal, Spain and Switzerland (Urquijo Landaluze 1947; Martins et al. 1999; Prospero et al. 2023), *P. xcambivora* is still considered the principal pathogen in Italy (Frascella et al. 2022). The disease causes root and/or collar rot of trees, and its most evident symptoms include crown distress, with yellowed and smaller leaves, and often immature husks remaining on the tree after leaf fall (Vannini and Vettraino 2001). Root systems and collars are mainly colonized by the parasite: they produce a characteristic dark exudate, which justifies the name of disease. In the more advanced stages of the disease, typical flame-shaped necrosis is evident at the collar level after debarking. In most cases, the trees die in a short time, leaving the entire canopy desiccated; sometimes the decline process can continue for several years, suggesting some kind of equilibrium between trees and disease (Biraghi 1953; Prospero et al. 2023). Water circulating in the soil is the main factor in spore spread, so climatic factors play an important part in the course of the disease (Erwin and Ribeiro 1996).

With a probable Asian origin and a first introduction in the Azores, ink disease was imported to Europe in the early 18th century and first discovered in its western part, in Portugal, Spain, Italy and then in France (Petri 1917a, b; Allain 1935). In Italy, it was first observed in 1845 in the Piedmont (Selva 1872) and then described as “ink disease” by Puccinelli (1859). Because of the high economic and environmental value of chestnut in Italy, the disease was considered a real plague in the late 19th century. Several studies have tried both to detect the causal agent and developing measures to control the disease (Gibelli 1883), but the identification of the pathogen occurred only when Lionello Petri (1875–1946) first

isolated the pathogen by naming it *Blepharospora cambivora* (Petri 1917a, b; Petri 1925). Buisman (1927) revised the genus *Phytophthora* and renamed this pathogen *P. cambivora* (Waterhouse and Waterston 1966). Recently, the pathogen was newly described as hybrid and renamed *P. xcambivora* (Jung et al. 2017).

The presence of the ink disease in the Bolognese Apennine had already been reported by Gibelli (1883), but it was only in the 1930s that Quattrocchi (1938) produced a detailed monograph collecting original data on active outbreaks or foci, as well as a general overview of the chestnut cultivation status. In his work, the presence of ink disease was confirmed by laboratory tests carried out directly by L. Petri who detected *P. xcambivora* on the collected samples. In this part of Apennines, as in all of Italy, ink disease was disguised for several years by the spread of blight due to another invasive pathogen (*Cryphonectria parasitica* [Murr.] Barr), as well as the loss of interest in cultivation after World War II (Biraghi 1946). The appearance of hypovirulence (Biraghi 1950) and its natural spread and persistence (Turchetti et al. 2008) allowed the survival of these stands and their natural recovery (Pezzi et al. 2011; Pezzi et al. 2020). The Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu arrived in the Bolognese Apennines in 2008, causing new damage and raising significant concerns, as in other regions in Italy (Graziosi and Santi 2008; Seddaiu et al. 2017). Biological control with the parasitoid *Torymus sinensis* Kamijo (Quacchia et al. 2008) proved highly effective in mitigating the impact of damage caused by the Asian gall wasp, reigniting interest in chestnut cultivation and production (Pezzi et al. 2020). Almost simultaneously, after several decades of regression, a re-emergence of ink disease in chestnut woods and evident damage has been observed in Italy (Turchetti 1986; Anselmi et al. 1996) and in several European countries (Prospero et al. 2023), probably due to ongoing climatic changes characterized by repeated dry periods. Summer droughts have weakened the root systems, making the fine roots more susceptible to infections during the following rainy months, which are particularly favorable to the pathogen (Turchetti and Maresi 2008). To understand the ongoing effects of ink disease in the new millennium, it is necessary to understand the impact that this disease has had during the two centuries of coexistence between the

pathogen and chestnut trees. The recovery of the data on early attacks of ink disease contained in the Quattrocchi monograph helps to provide such a long-term perspective of this disease.

In this context, we resurveyed historical foci of ink disease in the Bolognese Apennines aiming to: (1) check whether chestnut stands persist in the investigated sites; (2) assess the current phytosanitary status of these surveyed chestnut stands.

## Materials and methods

### Study area: the chestnut belt in Bolognese Apennine

The study area considered in Quattrocchi's monograph covers approximately 2,110 km<sup>2</sup>, and it is located southwest of the city of Bologna (Lat. 44,492,475; Long. 11,342,970). It is characterized by a marked altitudinal gradient (from 49 to 1945 m a.s.l.). Here, the chestnut belt lies between 300 and 900 (1000) m a.s.l. (Fig. S1), within the deciduous *Quercus*-dominated and *Fagus sylvatica* forest belts.

At the beginning of the 20th century, in the Quattrocchi's investigated area chestnut-dominated formations covered approximately 12,863 ha (Ferretti et al. 2018), of which 10,928 ha were classified as chestnut orchards that provided a staple food for the mountain population. Today, in the same area, there are still 1498 ha of managed orchards plus 9096 ha of chestnut coppices and approximately 2500 ha of irregular woods or plantations with a chestnut prevalence, for a total of 13,104 ha of chestnut forests (Regione Emilia-Romagna 2014).

### Early pattern ink disease: data extraction and spatialization

The early pattern distribution of ink disease foci in the Bolognese area was obtained by Quattrocchi's monograph published in 1938.

We considered the 36 foci, where the presence of ink disease was assessed both in the field and by laboratory tests in the 1930s (Table 1). Furthermore, the monograph provided additional data such as the locations, timing of the very first attacks and surface or size of the disease foci in each Bologna Apennine municipality as well as the extent and condition of chestnut cultivation.

**Table 1** Comparison between the ink disease survey of 1938 in the Bologna Apennines and the situation observed in the same sites in 2017, 2020 and 2022

Plot no.	Locality	1938 Tree condition <sup>a</sup>	1938 <i>Phytophthora xambivora</i> presence	2017 Chestnut presence	2017 Hypovirulence presence	2017 Asian gall wasps <sup>b</sup>	2017 Ink sign presence <sup>c</sup>	2017 Types of attacks (plants)	2020 New attacks	2022 New attacks
1	Pian d'Atiola	nearly dead	confirmed	yes	yes	yes	yes	old dead	no	no
2	Pian-Maggiolino	only dead apex	not sure	yes	yes	yes	yes	old dead	no	no
3	Ca' di Ceccaia	very bad	not sure	yes	yes	no	yes	old dead	no	no
4	Cà Vecchia	nearly dead	confirmed	yes	yes	yes	yes	old dead	no	no
5	Cà di Costanzo	nearly dead	confirmed	yes	yes	yes	yes	old dead & suffering	no	no
6	Calcinara	nearly dead	confirmed	yes	yes	yes	yes	old dead	no	no
7	Chiapporato	nearly dead	confirmed	yes	yes	no	yes	old dead	no	no
8	Stagno	nearly dead	not sure	yes	yes	no	yes	old dead	no	no
9	Ca' di Romiccia	nearly dead	confirmed	no	yes	no	yes	old & new dead	yes	yes
10	Malsacco	very bad	not sure	yes	yes	no	yes	old dead	no	no
11	Poggio (Piani di sopra)	very bad	not sure	yes	yes	no	yes	old dead	no	no
12	Rilò (Casone Cimotti)	very bad	confirmed	yes	yes	yes	yes	old dead	no	no
13	Terzo	very bad	confirmed	yes	yes	no	yes	old dead	no	no
14	Prato Novello	very bad	confirmed	yes	yes	no	yes	old dead & suffering	no	no
15	Ca' di Faina	fair	confirmed	yes	yes	no	yes	old dead	no	no
16	Ca' di Baruffo (Piastrola)	very bad	confirmed	yes	yes	yes	yes	old dead & suffering	yes	yes
17	Moscaccia	nearly dead	confirmed	yes	yes	no	no	no	no	no
18	Chiesina	nearly dead	not sure	yes	yes	yes	yes	old dead	no	no
19	Casa Poli	very bad	confirmed	yes	yes	yes	yes	new dead	no	no
20	Docciola (Vidiciatico)	very bad	confirmed	yes	yes	no	yes	old dead	no	no
21	Valpiana	dead	not sure	yes	yes	no	yes	old dead	no	no
22	Bolgare	very bad	not sure	yes	yes	yes	yes	old dead	no	no
23	Spondola	dead	not sure	yes	yes	yes	yes	old dead & suffering	no	no
24	Pianacci e Fiorentina	very bad	confirmed	yes	yes	no	no	no	no	no
25	Cà di Giacomone	fair	not sure	yes	yes	yes	yes	old & new dead	yes	yes
26	Casellina (Bombiana)	fair	confirmed	yes	yes	no	yes	old dead & suffering	yes	yes
27	Sabbionetola	very bad	not sure	yes	yes	yes	yes	old & new dead	yes	yes
28	Tolè	very bad	not sure	yes	yes	no	yes	old dead	no	no
29	Chiesa vecchia	nearly dead	confirmed	yes	yes	yes	yes	old & new dead	yes	yes

Table 1 (continued)

Plot no.	Locality	1938 Tree condition <sup>a</sup>	1938 <i>Phytophthora xambivora</i> presence	2017 Chestnut presence	2017 Hypovirulence presence	2017 Asian gall wasp <sup>b</sup>	2017 Ink sign presence <sup>c</sup>	2017 Types of attacks (plants)	2020 New attacks	2022 New attacks
30	Compagnia (Baguucci di sotto)	nearly dead	confirmed	yes	yes	yes	no	no	no	no
31	Rasora	nearly dead	not sure	yes	yes	yes	no	no	no	no
32	Cà di Landino	nearly dead	not sure	yes	yes	yes	no	no	no	no
33	Vizzarete e Roncobilaccio	nearly dead	confirmed	yes	yes	no	no	no	no	no
34	Fornelli	very bad	confirmed	yes	yes	yes	yes	old dead	no	yes
35	Castelnuovo di Bisano	nearly dead	not sure	yes	yes	yes	yes	old dead	no	yes
36	Ceppo (Le Ceppe)	dead	not sure					area not identified		

Plot numbers refer to Figure 1

<sup>a</sup>as described by Quattrocchi; <sup>b</sup>*Torymus* was found in every sites where the wasp was observed; <sup>c</sup>New attacks = trees with symptomatic crowns and evidence at the collar; old attacks = trees already degraded and dead trees without resprouting

We relocated the historical surveys according to the four criteria reported by Kapfer et al. (2017) to minimize relocation error (Lelli et al. 2021).

Since the sites did not have an exact geographic reference, the locality name was mostly used, supported by the Emilia-Romagna forestry map (scale 1:10,000) updated in 2014 (available at <https://ambiente.regione.emilia-romagna.it>), together with a stratified approach using altitude, slope and locals' knowledge, to identify the points where resampling should be performed.

The 36 textual location descriptions were converted to a geographic coordinate pair (i.e., retrospective georeferencing) with as much accuracy as possible. Since the site descriptions included ambiguous place names (giving multiple possible interpretations) or locally used location names, geolocalization problems were solved on a case-by-case basis using different sources: the toponym repositories of the IGMI (Istituto Geografico Militare Italiano) derived by topographic map on a scale of 1:25,000 (available at <http://www.pcn.minambiente.it/mattm>), the Emilia-Romagna Region at a scale of 1:10,000 (available at [www.geoportale.regione.emilia-romagna.it](http://www.geoportale.regione.emilia-romagna.it)), the 1936 Italian Kingdom Forest map, the topographic and cadastral maps, and the interviews with locals. The entire georeferencing process was performed in QGIS 2.18 and later versions ([www.qgis.org](http://www.qgis.org)) using the spatial reference system UTM32N –WGS84. All the geo-localized localities were integrated into the original digitized table.

The monograph also included a paper map (scale 1: 200,000) that was a reduction of the 1936 Italian Kingdom Forest map (Ferretti et al. 2018), where the extent of the ink disease was superimposed. Due to the greatly reduced scale, it was not possible to use the related area data.

#### Field survey

We resurveyed the 36 sites assessed in the 1930s during the 2017 growing season (June to August) in a circle plot of 50 m radius centered in each previously defined pair of coordinates.

In each plot, the current presence and management type of chestnut (orchards vs. coppices, managed vs. abandoned) were verified. Both the percentage cover given by chestnut trees, the presence of individuals over 1 m in diameter (i.e., old-growth trees), and the

presence of seedlings and regeneration were also evaluated (calculated as the number of individuals per hectare =  $N/ha$ ).

Ink disease was assessed by (1) counting suffering trees/stumps (early symptoms with rarefied foliage and small, yellowed leaves); (2) counting dead trees/stumps (completely dead crown and brown flames); (3) incidence of ink disease as “single plant/stump” ( $n=1$ ), “small group” ( $n=2-10$ ), and “large area” (more than 10 individuals); and (4) determining the spatial distribution of attacks (“patchy,” “striped,” or “widespread”). Additionally, recent attacks (trees with symptomatic crowns and evidence at the collar) from old ones (already degraded and dead trees without resprouting) have been differentiated.

For a comprehensive view of the phytosanitary status of chestnut stands, chestnut blight and Asian chestnut gall wasp were also monitored. Following the same methodology adopted in Pezzi et al. (2020) and in Murolo et al. (2022), chestnut blight was assessed on a sample of 30 sprouts (in the coppices) or trees (in the orchards), by counting the number of “healings”, “healed”, “virulent”, and “intermediate” cankers (Turchetti et al. 2008). The surveyed trees were selected starting from the center of the plot.

Asian chestnut gall wasp was assessed in at least 10 trees using gall presence, canopy defoliation and developmental arrest levels as proxy parameters (Turchetti et al. 2012). In approximately 5–10 randomly selected galls per site, the presence of *Torymus sinensis* was assessed.

In addition, we detected the drought effect from general signs of sufferance in the crowns and the snow damage by the number of broken branches.

All the investigated sites in 2017 were also resurveyed again in July 2020 and May 2022 with the goal of assessing the presence of new ink disease attacks and the overall sanitary situation. Ink diseased trees were mapped and marked to be identified in the following surveys.

#### Soil and plant sampling

For each resurveyed site (from plot n.1 to 35—see Table 1), soil sampling was performed during the first survey in 2017. Each sample consisted of a mixture of three cylindrical subsamples (approximate size  $30 \times 15$  cm): these were collected according to the vertices of an equilateral triangle with sides 20 m

long, approximately located around the hypothetical center of the plot. Collected soil samples were stored in plastic bags at 5 °C until the DNA extraction and the presence of *Phytophthora* was detected by real time PCR as described below.

In July 2020 and May 2022, new sampling was carried out only at the sites where recent ink disease infections were recorded (Table 1). Six sampling sites have been identified in 2020 and 8 sampling sites in 2022 (Table 1). From each suffering or recently dead tree the following samples were collected: (1) soil, sampled from four cardinal points around the tree; (2) fine roots of the same trees sampled; (3) symptomatic bark/wood by collecting the portion between healthy and necrotic tissue from the basal part of the trunk or collar. All samples were stored in plastic bags at 5 °C until arrival in the laboratory, where the presence of *Phytophthora* was detected after isolation and real-time PCR, as described below.

#### Isolation and baiting from soil and woody tissue

Isolation of *Phytophthora* from soil was performed by using apples and leaves as bait (Erwin and Ribeiro 1996). For each fruit (apple), four holes were drilled using a cork-borer, and the soil samples were placed in the hole and then closed using scotch tape. Inoculated apples were stored in the dark at room temperature. When rot symptoms were visible around the hole of the apples, they were processed for isolation on selective media (PARNPH) (Erwin and Ribeiro 1996).

Baiting with leaves was carried out by placing the soil to cover the bottom of a plastic box ( $20 \times 30$  cm) for 3 cm of thickness, adding distilled water and incubating at room temperature for 12 h. Then, leaves of *Sambucus nigra* L. floated on the water surface (Bregant et al 2020). After 3–4 days, the leaves with necrotic spots were processed (washed and surface sterilized as suggested by Bregant et al 2020) for isolation on selective media (PARNPH) (Erwin and Ribeiro 1996). To recognize the presence of *Phytophthora* from collar and roots tissue, isolation was carried out by placing small woody fragments on selective media (PARNPH) (Erwin and Ribeiro 1996). From the same collected samples, isolation on potato dextrose agar (PDAS) containing 50 mg/l streptomycin sulfate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was carried out after surface

sterilization to detect the possible presence of other fungal species. All plates were incubated at 24 °C in the dark, and after 2–3 days, the colony growing in the media was replaced in new Petri dishes containing PDA.

Based on their morphological characteristics, all the colonies (*Phytophthora*-like and other fungal species) obtained from soil and woody plant tissue were clustered into different morphotypes, and only one representative strain (of each morphotype) was identified after DNA sequencing. All isolates were stored at Fondazione Edmund Mach (FEM, Trento) and Institute for Sustainable Plant Protection (CNR-IPSP, Florence) collections.

#### DNA extraction from mycelium, soil and plant tissue

Each morphotype was grown on PDA on 90 mm Petri dishes covered by cellophane discs (Celsa, Varese, Italy). After 10 days, the mycelium was scraped and placed in a 1.5 ml Eppendorf tube for DNA extraction. DNA from mycelium and woody tissue was extracted by using the EZNA Plant DNA Kit (Omega Biotek) following the manufacturer's protocol.

The soil samples were sieved with a 0.2-mm mesh size, freeze-dried and stored at –80 °C until DNA extraction. Total genomic DNA was extracted from 0.25 g of each soil sample using a FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The quality and quantity of the extracted DNA from soil and plant tissue were evaluated using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Cleveland, OH, United States).

#### Molecular identification of axenic cultures

Molecular identification of different morphotypes was carried out by amplifying and sequencing the internal transcribed spacer of ribosomal DNA, according to White et al. (1990). The ITS sequences were then queried against the GenBank database using Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the most similar available sequences.

The occurrence of each identified species in soil and woody tissue was determined by calculating the relative abundance (RA %), as described by Peters et al. (2020).

#### Real-time PCR (qPCR) analysis

The presence of *Phytophthora* DNA from soil and woody samples was detected by using the MGB TaqMan quantitative real-time PCR (TaqMan qPCR) assay for the genus *Phytophthora*, as described by Migliorini et al. (2015).

The TaqMan qPCR assay was performed in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) in a final volume of 25 µl containing 12.5 µl TaqMan® Universal Master Mix (Applied Biosystems), 300 nM forward primer (Eurofins Genomics, Ebersberg, Germany), 300 nM reverse primer (Eurofins Genomics) and 200 nM TaqMan® MGB probe (Applied Biosystems). For each tube, 5 µl of genomic DNA template was used. Each DNA sample was assayed in three replicates. Two wells containing 5 µl of sterile water were used as the no-template control (NTC). All DNA samples were assayed in MicroAmp Fast 96-well reaction plates (0.1 mL) closed with Optical Adhesive by means of the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Science, Foster City, CA). The amplification protocol was as follows: 50 °C (2 min), 95 °C (10 min), 50 cycles of 95 °C (30 s), and 60 °C (1 min).

Measurements of *Phytophthora* DNA in unknown samples were made by interpolation from a standard curve generated with a DNA standard, which was amplified in the same PCR run. The standard curve was generated from fivefold serial dilutions in sterile water (ranging from 100 ng/tube to 0.25 pg/tube) of a known concentration of *Phytophthora* standard DNA (Ph32SA) according to Migliorini et al. (2015). Each assay was analyzed in triplicate. The amount of pathogen DNA was expressed as pg *Phytophthora* DNA/µg total DNA extracted. The results from the TaqMan assay were analyzed using StepOne™ Software (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold.

## Results

### Early pattern of ink disease

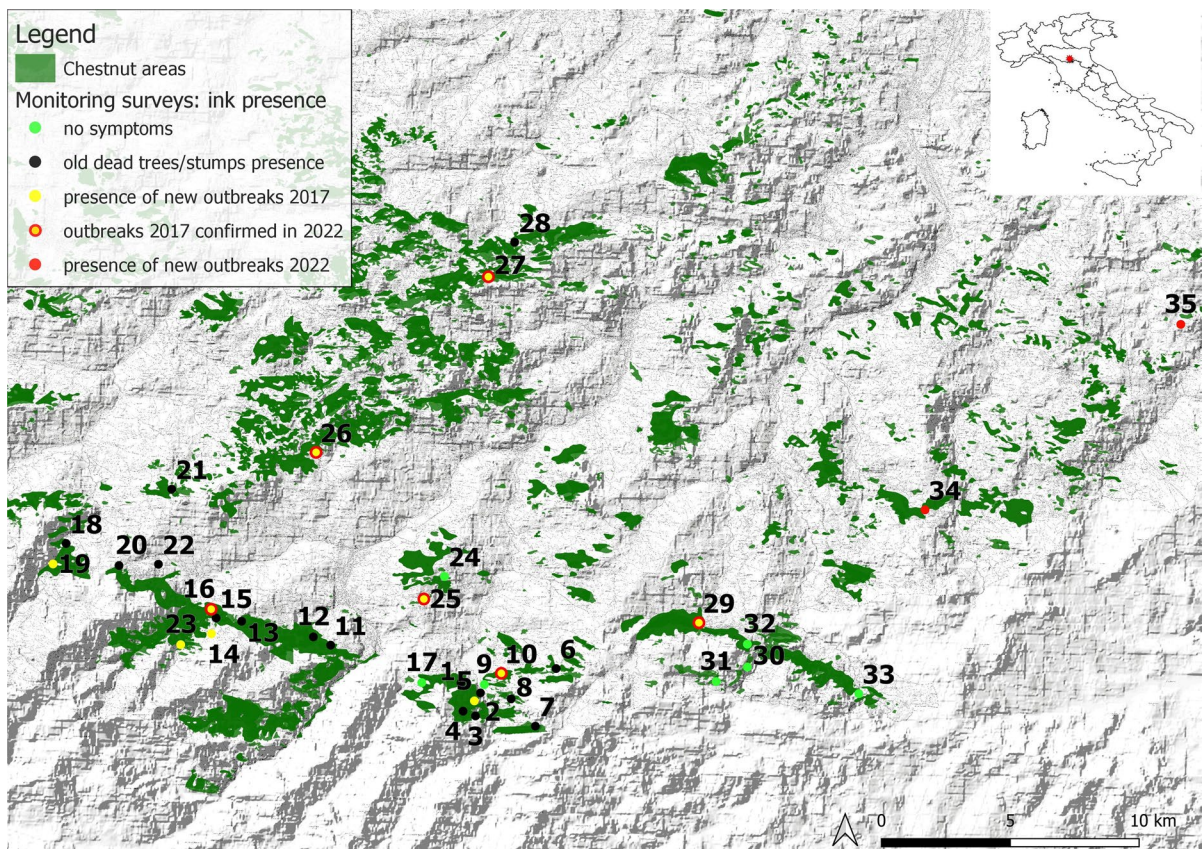
In the 1930s, in the province of Bologna, there were 12,482 ha of chestnut groves (Ferretti et al. 2018). They represented approximately 1/5 of the wooded surface under chestnut cultivation in the

Emilia-Romagna region. The chestnut groves with the greatest extension were distributed at the highest altitudes on the border with Tuscany, where they formed an unbroken belt with an east–west direction.

The first appearance of ink disease in the study area was recorded in 1921 in two municipalities (Quattrocchi 1938). Approximately a decade later, eight municipalities were affected. Then, in 1938, it was spread in 10 municipalities (approximately 1024 ha; i.e., 8.2% of the chestnut area).

The altitudinal range of the 36 surveyed points by Quattrocchi (1938) was 500–940 m a.s.l. (average  $761.4 \pm 94$  a.s.l.) and more than 50% of affected trees were located between 700 and 800 m a.s.l., with prevailing northern and eastern exposure (approximately 80%). Sites were unevenly distributed in the 10 municipalities, predominantly bordering the Tuscany region and the province of Modena (Fig. 1).

Symptomatic trees were aged between 30 and 105 years (average  $73.11 \pm 23.37$ ). Plant conditions, obtained from personal inspections, were reported as follows: “completely dead plant” (8%), “almost dead plant” (42%), “plant in bad condition” (39%), “plant dries at the top” (3%), and “discrete plant condition” (8%). In more than 55% of the affected foci, the presence of *P. x cambivora* mycelium was confirmed on sampled wood tissues by laboratory tests (microscopic inspection). Although ink disease in these chestnut groves was certainly the most troubling problem, there were other problems facing chestnut cultivation at the time. Out of a total of 12,482 ha of chestnut groves, only 23.2% enjoyed good conditions, 63% were in bad conditions due to a lack of proper cultivation techniques, and 5.6% were degraded.



**Fig. 1** Location of the study area and distribution of the surveyed historical ink disease affected points in the chestnut range in the Bologna Apennines



## Field survey

Of the 36 locations (Table 1) in the monograph, only one locality (Ceppo Le Ceppe-36) was not identified and geo-localized with the sources used.

In another location (Ca' di Romiccia-9), there was no chestnut (neither old-growth trees nor seedlings): the identified area was covered by other species, mainly *Quercus pubescens* woods.

In all the other 34 plots (Table 1), chestnut was found in different forest systems (high forest, coppices and orchards). In 50% of the cases, only a single forest system was present, while in the others, 50% coppices, orchards and mixed wood constituted the chestnut cover in different percentages. We detected 19 plots with chestnut orchards, 13 still actively managed and the remaining in clear abandonment; in 19 plots, chestnut coppices were present, while in seven areas, mixed wood with chestnut presence was observed. Chestnut orchards were the only forest system in seven (20%) of the surveyed points, while coppices were the only type in eight (23%) of the plots. At three sites, chestnut was present only in mixed woods; at the remaining four points, mixed woods were near chestnut orchards.

In five plots, chestnut canopy cover was estimated to be less than 30%; in 16 plots, it was valued between 30–75%; and the remaining 13 plots showed an almost continuous chestnut cover ranging between

75–100%. Abandonment was the prevailing status in the coppices and mixed woods, where no clear silvicultural management was observed.

Chestnut seedlings were observed in 20 plots, while young chestnut regeneration was evident in 16 plots. In some of these, healthy chestnut regeneration was also observed near or under the ink-affected trees. In 25 plots old growth trees were assayed, while in most of the plots they were truly a residual presence with single or few sporadic subjects in the surveyed area, in two plots they were prevalent suggesting the persistence of almost intact historical orchards.

In 2017, symptoms of ink disease attacks were detected in 28 plots, while the remaining six had no signs at all (Table 1). Old chestnut dead trees (Fig. 2) (20 plots) or stumps (10 plots) were recorded in the affected plots; in 10 cases, both types were present in the surveyed area. Plants with suffering crowns were observed in 8 plots, while recently dead stumps or trees were recorded in only five plots. In two plots, suffering and new dead plants were present simultaneously; new attacks were present together with old ones in all but one of the plots.

At 20 sites, we recorded only a single tree or coppices dead from ink, while in the other seven sites, small groups of plants (from 2 to 10) were affected. Attack on more than 10 plants occurred in only one area. In 20 areas, the attacks appeared very localized, while in six, there were few stripes or spots. In

**Fig. 2** Basal part of *Castanea sativa* dead tree with still evident sign of a possible ink disease attack: a dark-brown flame shaped lesion under bark and absence of resprouting



two areas, ink damage spread over a larger surface. Interestingly, in most of the foci (17), chestnut natural regeneration was observed (Fig. 3).

As reported in another survey in the same Apennines area (Pezzi et al. 2020; Murolo et al. 2022), chestnut blight was widespread, with a number of cankers ranging from 29 to 85 on the examined trees, but healing and healed infections were clearly predominant, enhancing the role of hypovirulence. Very few recent virulent infections were observed during the survey, even if dead branches due to an old attack were present in several stands. No changes in the observed situation were recorded in the 2022 survey, suggesting a substantial stability of hypovirulence: hypovirulent cankers (healed and healing) ranged from approximately 90–92% in each area.

Asian gall wasp presence was observed in 19 areas, ranging from sporadic (1–10 galls) to frequent (11–100), while in the others, no evidence was

noticed, probably due to the sporadic or scattered presence of galls in the crowns. In all the affected areas, parasitoid larvae were easily detected in the examined galls, suggesting the effectiveness of the biological control.

In 2020, no evident changes were observed in the examined active foci. In the 2022 survey, three new plots showed new ink attacks and joined the previous five with recent damage. Four of the areas with suffering plants instead showed a certain improvement of the crown condition, while in the other three, some of the suffering plants died. In the previously identified foci, new dead or suffering plants were observed but as a single tree or small group; only in one area were the new attacks on a larger surface. Two of the areas where new attacks appeared in 2022 were healthy in the previous survey even if they had old dead plants.

#### Detection of *Phytophthora* and other fungi and oomycetes

The standard curve, used to quantify *Phytophthora* DNA in samples (*Phytophthora*-generic assay) had a slope of  $-3.3$  and a Y-intercept of  $33.0$  ( $R^2=0.99$ ).

The qPCR assay was able to detect and quantify *Phytophthora* DNA from soil samples (collected in 2017) from 9 out of 34 chestnut sampling sites (amount of *Phytophthora* DNA ranged from  $2.8 \times 10^{-3}$  to  $1.5$  pg DNA/ $\mu$ g total DNA extracted). Among these 9 sites, 4 showed only old attacks (plot n. 1, 4, 34, 35), while in other 4 also new infections were present (plot n. 15, 16, 25, 27); one site (n.24) was positive to *Phytophthora* in the soil but had no evidence of symptoms on trees. The qPCR assay was also able to quantify *Phytophthora* DNA from all recent attack sampling sites in 2020 (5 out of 6 sites) and 2022 (7 out 8 sites). The percentages of samples positive for *Phytophthora* were as follows: 80% fine roots (DNA amount ranged from 2.9 to 596 pg DNA/ $\mu$ g total DNA extracted), 71.4% necrotic woody tissue (DNA amount ranged from 10 to 200 pg DNA/ $\mu$ g total DNA), and 30% soil (DNA amount ranged from  $8.8 \times 10^{-3}$  to 10 pg DNA/ $\mu$ g total DNA extracted).

Isolation from collar and root infected tissues by using selective media (PARNPH) confirmed the presence of *Phytophthora xcambivora* from symptomatic chestnut trees (RA% = 6%) positive to qPCR assay. In addition, from the same collar samples, isolation on



**Fig. 3** A recent foci of ink disease but with already growing natural chestnut regeneration

PDAS showed the presence of *Cryphonectria parasitica* (RA%=63%) and *Trichoderma* sp. (31%).

Other species of *Phytophthora*, such as *P. plurivora* (5%) and *P. pseudosyringae* (5%), were isolated from soil samples collected in the plots, both in 2020 and 2022. These samples were positive to qPCR assay. Soil samples also showed the presence of *Trichoderma* (33%), *Pythium* (14%), *Penicillium* sp. (14%) and *Mortierella* (29%).

## Discussion and conclusion

Available historical data about chestnut forest presence and its status represent a valuable source for understanding long-term landscape dynamics over decades. Despite some challenges in using such data types, due mainly to ambiguous and imprecise descriptions of the original plot location and the risk of causing a mismatch in the position of historical and resurveyed plots, the reported description and information can be useful to have an idea of the previous condition of the investigated areas. This methodological approach permits the assessment of the potential of retrospective georeferencing and confirms that the use of historical sources allows an assessment, at a broad time scale, of the landscape evolution in the investigated area.

In this case, a resurvey of the identified plots where ink disease was reported in 1938 confirmed the persistence of chestnut woods and trees, even if the presence of other species in some cases. Therefore, the alarming reports during the 1930s about the spread of ink disease in the Bolognese Apennines did not correspond in the following 85 years to an effective and dramatic impact on chestnut woods. Of the 35 resurveyed sites, only one showed no chestnut presence, while in all the other sites, chestnut was still present, even if some changes in the forest systems (from orchard to coppice or high system) were found. Moreover, the survival of old trees and stands and the recovery of natural chestnut regeneration and seedlings in most of the area enhanced the possibility of establishing an equilibrium between the pathogen (that causes ink disease) and the host (chestnut). This suggestion is consistent with the data about chestnut surface evolution in the whole province: from the 12,482 ha calculated on the 1936 Kingdom Forest map (Ferretti et al. 2018) to the actual 13,104 ha

reported as chestnut forests (Regione Emilia-Romagna 2014). The apparent increase could mainly be due to differences in map scales and mapping criteria (Ferretti et al. 2018), but what we want and can highlight is the substantial persistence of chestnut woods in their historical area.

Ink disease is still present in several areas, where however, it seems able to produce localized damage but not to spread and cause extensive outbreaks. Indeed, the presence of old attacks in most of the areas investigated suggests that Quattrocchi's survey was effective and correct in detecting the presence of the pathogen. *Phytophthora* action was not able to produce the disappearance of the chestnut tree in all but probably one spot in the following 85 years. However, field observations have shown that new attacks are still occurring and may reappear even in earlier healthy stands. Thus, a constant presence of the disease can be assumed, but with alternating attacks both in terms of spacing and in time. The examined old foci always seemed very localized, as did the new ones observed during the surveys.

Hypovirulence in chestnut blight (Turchetti et al. 2008) and biological control based on parasitoids in Asian gall wasps (Quacchia et al. 2008) proved to obtain effective and persistent control of these diseases, also in the investigated sites, as confirmed by our survey.

Therefore, neither ink disease more than 100 years after its first detection nor chestnut blight in the last 70–80 years was able to cause the disappearance of chestnut woods in the investigated area. Moreover, neither abandonment in the 1950s nor the more recent Asian gall wasp spread have hampered the survival of chestnut coppices or orchards: chestnut is still a main component of this part of the Apennines as well as of the entire Italian mountains, where it still covers almost 788,408 ha of pure or mixed wood (INFC 2015). Of course, damage due to constraining factors such as disease and changes in the cultivation attitude are still evident: approximately half of the chestnut surface is described in bad condition in the same inventory (INFC 2015).

Laboratory tests confirmed the presence of *Phytophthora* in the soil. Although a small amount of *Phytophthora* DNA was found in soil samples, *P. xambivora* was isolated from new attacks, confirming the persistence of the parasite and the validity of Quattrocchi's data. The difficulty

in isolating *P. xcambivora* from bating could be attributed both to the casual choice of soil samples and probably to the large and rich microbial component of the soils themselves. The latter factor has recently been enhanced by Venice et al. (2021), who strongly suggest the role of soil biodiversity in *P. xcambivora* control in a chestnut orchard in Tuscany. Some of the fungi isolated in our study (e.g. *Mortierella*) have already been reported as factors of higher resistance to soil-borne pathogens or promoters of plant growth (Ozimek et al. 2021). Therefore, their abundant presence in soil and bark could be considered a possible factor of restraints versus *P. xcambivora*.

It should be remembered that traditional Italian chestnut orchards are completely different from intensive orchards, being woods of anthropogenic origin with seminatural undergrowth, assimilated to sparse woodland where management is limited to mowing or grazing and pruning (Bounous 2014); they can be considered forest ecosystems in which natural balances between the soil microbial biome are saved and persistent due to the absence of any heavy chemical or mechanical disturbance (De Feudis et al. 2021).

The chestnut woods in Bolognese Apennines have survived and are still surviving in the presence of *P. xcambivora* and, perhaps, of other *Phytophthora* species, probably thanks to their rich biodiversity and seminatural condition. Although damage is present and can recur at any time, chestnut ecosystems seem to show effective resilience against the disease. Rotation in space and time can be observed for attacks but not the complete disappearance of the chestnut woods. In addition, natural regeneration of chestnut seems to be possible even in affected areas, as observed during the survey.

In other contexts, especially where the resilience of chestnut orchards or coppices is depleted, the damage due to ink disease presence could be completely different. Moreover, this favorable trend could be completely altered or reversed by the appearance and spread of other *Phytophthora* species, such as *P. cinnamoni*, as reported in central Italy (Vettraino et al. 2005; Vannini et al. 2013), where severe damage due to this pathogen was observed. Both microbial richness and *Phytophthora* presence in chestnut stand soils need further and more in-depth investigations for a

better understanding of the dynamics and balances involved.

Another risk factor for the equilibrium “chestnut–*P. xcambivora*–microbial biodiversity” is the marked change in weather and climate behavior: even in the Apennines, a clear increase in mean temperature and more frequent drought periods were observed in recent years, as confirmed by the comparison with data from the last 55 years (Table S1) (Regione Emilia-Romagna 2017). Stress due to these changes could cause an increasing weakness of root interaction between chestnut trees and soil boma, enhancing *Phytophthora* attacks. Therefore, a brutal decrease in chestnut forest resilience against disease and perturbation (Conedera et al. 2010) is still possible, as already observed for temperate forests, due “to increased water limitations and climate variability” (Forzieri et al. 2022).

This work highlights that historical records, going back multiple decades, proved to be a valuable resource to evaluate the impact of disease and could help to better understand past and predict future forestry patterns in the context of global change.

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**Data Availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

**Declarations**

**Competing interests** The authors have no relevant financial or non-financial interests to disclose

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