








Distribution, causal agents, and infection dynamic of emerging ink disease of sweet chestnut in Southern Switzerland

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Abstract

Emerging diseases caused by both native and exotic pathogens represent a main threat to forest ecosystems worldwide. The two invasive soilborne pathogens *Phytophthora cinnamomi* and *Phytophthora × cambivora* are the causal agents of ink disease, which has been threatening *Castanea sativa* in Europe for several centuries and seems to be re-emerging in recent years. Here, we investigated the distribution, causal agents, and infection dynamics of ink disease in southern Switzerland. A total of 25 outbreaks were identified, 19 with only *P. cinnamomi*, 5 with only *P. × cambivora*, and 1 with both species. Dendrochronological analyses showed that the disease emerged in the last 20–30 years. Infected trees either died rapidly within 5–15 years post-infection or showed a prolonged state of general decline until death. Based on a generalized linear model, the local risk of occurrence of ink disease was increased by an S-SE aspect of the chestnut stand, the presence of a pure chestnut stand, management activities, the proximity of roads and buildings, and increasing annual mean temperature and precipitation. The genetic structure of the local *P. cinnamomi* population suggests independent introductions and local spread of the pathogen.

INTRODUCTION

Emerging diseases, that is, diseases that are new or rapidly increasing in incidence or geographic range (Behler McArthur, 2019), represent a main threat to forest ecosystems worldwide (Fisher et al., 2016; Thakur et al., 2019). An emerging disease may develop following the introduction of a pathogen in a new area where hosts are highly susceptible because of the lack of coevolution, and natural enemies (Mack et al., 2000). Humans have been moving living organisms between ecosystems for thousands of years, but invasion rates have increased dramatically in the last century when international trade reached unprecedented levels

(Santini et al., 2013). On the other hand, ongoing climate change is disrupting the equilibrium between pathogens and their native hosts resulting in increased damage (Anderson et al., 2004). For instance, climatic extremes (e.g., drought) may weaken the hosts and increase their susceptibility to weakly pathogenic organisms, whereas a warmer climate may be favourable for the development of thermophilic pathogens. Since both globalization and climate change are expected to further intensify in the coming years, the emergence of both native and introduced pathogenic species will most likely continue to be an important issue in the future.

Ink disease is one of the most serious diseases of sweet chestnut (*Castanea sativa*) in Europe (Vannini &

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Vettrano, 2001). The main symptoms include a thinned crown with smaller and chlorotic leaves, and a reduced nut production (Vannini & Vettrano, 2001). To replace dead branches, trees often produce new branches along the trunk (epicormic shoots), which rapidly die. At the root collar, flame-shaped dark cortical lesions, which can extend to the base of the trunk are visible after removing the bark. Such lesions are often associated with black bark exudates that gave the name to the disease. A tree infection often develops rapidly into mortality centres causing major economic losses in orchards and the abandonment of a millennial-long culture of appreciated biodiversity and loss of landscape value.

Two species of the oomycete genus *Phytophthora* (Stramenopila) are generally considered to be responsible for ink disease of sweet chestnut, namely *Phytophthora* × *cambivora* (Petri) Buism and *P. cinnamomi* Rand (Crandall et al., 1945; Grente, 1961; Milburn & Gravatt, 1932; Petri, 1917). Both species originate from Asia and have been widely dispersed around the world by human activity. As soilborne organisms, *P. × cambivora* and *P. cinnamomi* destroy the root system of a wide range of plant species, including *C. sativa* (Hardman & Blackman, 2018; Rigg et al., 2018; Vettrano et al., 2002). Infections are mainly caused by short-lived asexual spores (zoospores) which, thanks to two flagella, can actively swim through thin films of water in soil pores and reach the roots of susceptible plants. Owing to their thermophilic character, the temperature is the main limiting abiotic factor affecting the occurrence of *P. × cambivora* and *P. cinnamomi* (Erwin & Ribeiro, 1996; Zentmeyer, 1981).

The first official report of symptomatic trees in Europe dates to 1838 in Portugal, but the disease has probably already been present in Spain since 1726 (Vannini & Vettrano, 2001). In the 20th century, the general interest in ink disease decreased, possibly because of the appearance and epidemic spread in Europe of *Cryphonectria parasitica*, the causal agent of chestnut blight (Rigling & Prospero, 2018). However, starting in the 1990s a re-emergence of ink disease has been observed in several European countries, including Italy, France, Greece, Portugal, Spain, and Switzerland (Prospero, 2014; Turchetti & Maresi, 2006; Tziros & Diamandis, 2014; Vettrano et al., 2001). Symptomatic chestnut trees are also increasingly reported in more northern countries such as England, Germany, and the Czech Republic (Černý et al., 2008; Peters et al., 2008; Vannini & Vettrano, 2001). This re-emergence and new appearance of ink disease might be linked to climate warming or related processes, such as increased drought frequency, which might reduce tree vitality and/or increase the chances of survival of the two causal agents. However, despite the economic and ecological impact the re-emerging chestnut ink

disease is having in Europe, still very little is known regarding the causes of this process.

In southern Switzerland, sweet chestnut forms a mainly continuous forest belt that ranges up to 900 m above sea level. Chestnut forests are a traditional component of the landscape and provide basic services (e.g., wood and nut production, and soil erosion protection). Ink disease was first reported in 1943 and was attributed to *P. × cambivora* (Arrigoni, 1950; Gäumann, 1951). However, since then no specific studies targeted this disease, which in the past three decades seems to be re-emerging. In this study, we aimed to determine (1) the current distribution and the causal agents of ink disease in southern Switzerland, (2) the dynamic of infection by *P. cinnamomi* and *P. × cambivora*, and (3) the local population structure of *P. cinnamomi*. For this, we first collaborated with the cantonal forest service to find potentially affected chestnut stands, then we identified the causal agents and performed a dendrochronological analysis to characterize tree pre-disposition to an infection and its dynamic, and finally we assessed the intra-specific diversity of *P. cinnamomi* using microsatellite markers.

MATERIALS AND METHODS

Stand selection and tree sampling

A total of 32 chestnut stands were reported by the local forest service as potentially affected by ink disease. In each stand, except the two in Castasegna, a total of 15 chestnut trees, if available, were sampled (21 trees in Brissago-Ferabò), including 5 symptomatic ($S =$ Symptomatic) and 10 non-symptomatic ($H =$ Healthy) trees, for a total of 431 trees. A tree was considered symptomatic when it displayed chlorotic leaves reduced in size, crown thinning, and/or dark necrotic lesions under the bark at the root collar. The symptomatic trees were randomly selected in mortality centre(s) (diseased sector(s) of a stand), while asymptomatic trees were located both within and outside the diseased sector(s) ($n = 5$ for each category). In the two stands Castasegna-Brentan and Castasegna-Stoll, only symptomatic trees from a diseased sector of the stands were sampled. The minimum distance between sampled trees was set to 10 m. To investigate the presence of the pathogen, soil samples were taken from the rhizosphere (5–20 cm topsoil layer) of each tree at max. 1.5 m from the stem along the four cardinal points using a spade. All samples from the same tree (a total of about 1 kg of soil) were pooled in a plastic bag and brought to the laboratory for further analysis. To avoid contamination, soil samples were first collected from the basis of apparently healthy trees and subsequently from the symptomatic trees. When moving from one

tree to another, the spade was cleaned from soil residues and disinfected with 70% ethanol.

Isolation of *Phytophthora* species

In the laboratory, *Phytophthora* was isolated from the collected soil samples as follows. First, samples were sieved through a 9 mm square mesh to remove eventual coarse roots and small stones. Afterward, about 300 g of soil was transferred into a plastic container and flooded with 500 mL of deionized water. After soil sedimentation, two freshly picked, healthy leaves of *Rhododendron catawbiense* were placed on the water surface (Themann & Werres, 1998). The containers with the soil samples were then incubated in a greenhouse at 22°C. After 5 days, one leaf was removed from the water surface, washed with deionized water, and blotted on filter paper. Following surface sterilization for 1 min in sodium hypochlorite solution (0.5% active chlorine; Chemische Fabrik Schweizerhalle, Basel, Switzerland), rinsing twice in sterile deionized water, and blotting dry between paper towels, five small pieces (~4 mm × 4 mm) were cut from the margin of spots and discolorations which developed on the leaves and placed on CARP selective agar medium (Hansen et al., 2008). The second leaf was processed after 10 days of incubation of the soil samples using the same method. The plates containing the pieces of leaves were incubated in the dark at room temperature. Potential *Phytophthora* hyphae (i.e., hyphae without cross-walls) growing out of the leaf pieces were then transferred after 24–48 h to Petri plates containing Potato Dextrose Agar (39 g L⁻¹, Difco, Voight Global Distribution, Lawrence, Maryland) and incubated in the dark at room temperature.

Identification of *Phytophthora* species

For DNA extraction, an agar plug of each *Phytophthora* culture was transferred onto liquid V8 agar (Werres et al., 2001) and incubated at room temperature under daylight. After 3–8 days, the mycelium was harvested and subsequently lyophilized. DNA was then extracted from the mycelium using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, California) following the manufacturers' instructions. *Phytophthora* species were identified by sequencing the ribosomal internal transcribed spacer region with an ABI 3130 automatic sequencer (Applied Biosystems, Forster City, California) as described in Prospero et al. (2013). All sequences obtained were analysed with CLC Main Workbench 7.9.1 (CLC bio) and compared with reference sequences in the publicly available National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) and Phytophthora-ID.org (<http://phytophthoradb.org>) databases with a

BLAST search for species identification. Sequences showing at least 99% similarity were considered to belong to the same species with the reference sequence.

Pre-disposing factors for ink disease

To explain the probability of occurrence of ink disease in a chestnut stand we considered a total of 15 predictor variables, with the categorical variables comprising several categories, grouped in site variables (altitude, slope, aspect [N, S, W, E, SE, SW, NW, NE], soil pH at 30–60 cm depth as retrieved from ISRIC SoilGrids; Poggio et al., 2021), anthropogenic variables (tree species [pure chestnut, mixed], stand type [coppice, traditional orchard, high forest], stand management [yes, no], minimal distance to a road, minimal distance to a hiking path, minimal distance to houses/buildings), and bioclimatic variables (annual mean temperature, temperature seasonality, the maximal temperature of the warmest month, minimal temperature of the coldest month, annual mean precipitation, as derived for 1981–2010 average from the CHELSA V2.1 dataset; Karger et al., 2017). The selected bioclimatic variables were found relevant for the occurrence of *P. cinnamomi* and *P. × cambivora* in Switzerland (Borsien, 2022). The control sites (21) with no occurrence of ink disease were randomly selected using the 'select random point' tool in ArcGIS within the distribution area of sweet chestnut in southern Switzerland and validated in the field (i.e., verification of the absence of symptoms) by the local forest service.

The importance of each predictor variable was assessed using generalized linear models (glm function of the R Stats package, version 4.1.1; R Core Team, 2021). All but one of the variables from each collinearity group were removed by computing a correlation matrix (of mixed datatypes) for all pairs using the function *model.matrix* from the R package stats. Finally, a standard glm was run with *ink disease* as a dependent variable and with the remaining predictor variables as independent variables. Within the categorical variables of exposure, tree species, stand type, and stand management, each level was tested against a fixed level of the variables.

Infection dynamic and tree pre-disposition

Dendrochronological analyses were performed in the chestnut stands Arbedo, Biasca, Brissago-Motto della Croce, Castasegna (trees from both outbreaks), and Orselina-Eco. At each site, we extracted an increment core from 10 pairs of symptomatic and asymptomatic adult chestnut trees (>40–100 years old) at about 1.3 m height using an increment borer (5 mm in

diameter). After careful preparation (fixation of the partially broken cores on wooden rails and even sanding of the core surface), the cores were imaged, and their tree ring widths were measured and merged into site chronologies using *CooRecorder/Cdendro* (Larsson, 2013). Chronologies were built by cross-dating healthy trees and by progressively adding the symptomatic ones with continuous dating validation using *COFECHA* (Grissino-Mayer, 2001). The ring widths after the death of a tree were set to zero.

To assess when symptomatic trees were infected, we compared the growth pattern of symptomatic trees and the asymptomatic trees at each site. The time of infection was roughly defined as the moment when the annual growth of the currently symptomatic and asymptomatic trees started to differ significantly. We determined it by performing 5 years of moving Wilcoxon rank-sum (with 1 year step) testing for statistically significant growth level differences ($p < 0.05$) between the two groups, similar to the studies of Cherubini et al. (2002) and Colangelo et al. (2018).

The pre-disposition of a tree to infection was investigated considering both differences in growth level and resilience capacity between symptomatic and asymptomatic trees. To compare growth levels, we calculated the Wilcoxon test for the 40 years preceding the disease onset (time of infection) at each site to test for growth rate differences among the two groups of trees. For differences in resilience, we tested resilience capacity in response to a drought extreme that occurred in 2003. Analyses were performed using the *sea* (superposed epoch analysis) function of the *SEA dplR* package (e.g., Gillner et al., 2014; Martín-Benito et al., 2008). A time lag of 4 years was used and the departure from the mean ring width was calculated. Additionally, the resilience components (Resistance, Recovery, and Resilience; Lloret et al., 2011) were calculated and compared among the groups with the Wilcoxon test to identify significant differences. The components were calculated as the ratio between (i) the ring width during and before the drought event (Resistance), (ii) the ring width after and during the event (Recovery), and (iii) the ring width after and before the event (Resilience). Values of 1 indicate no effect of the key event (again the drought in 2003), whereas values < 1 indicate (i) lower resistance, (ii) decline in growth after the event, and (iii) lowered capacity to reach pre-disturbance levels.

Population structure of *Phytophthora cinnamomi*

The population structure was only analysed for *P. cinnamomi* because the number of isolates available for *P. × cambivora* (7) was too low. A total of 190 *P. cinnamomi* isolates from 16 outbreaks of ink disease were

genotyped at 15 microsatellites (simple sequence repeat) loci as described in Engelbrecht et al. (2017). Briefly, the loci were amplified in two different multiplex reactions, and the PCR products obtained were separated using an ABI PRISM 3100 Genetic Analyser with the GeneScan LIZ-500 size standard for fragment analysis. Allele sizes were determined using Genemapper v4.0 (Applied Biosystems). Loci *Pc_SSR12*, *Pc_SSR13*, and *Pc_SSR22* showed three alleles in several isolates. If at these loci only two peaks were observed, peak height ratios were used to assess allele copy numbers. Multilocus genotypes (MLGs) were defined as unique combinations of alleles at the 15 loci. Basic population statistics, including Stoddart and Taylor's index, $G = 1/\sum Pi^2$, where Pi is the observed frequency of i th genotype and evenness index $E_5 = (1/\lambda) - 1/e^H - 1$, where λ is Simpson's index and H' is Shannon–Wiener's index, was calculated using the R package *poppr* (Kamvar et al., 2014). The G index describes genotypic diversity in a population, whereas the E_5 index is a measure of the distribution of genotype abundances ($E_5 = 1$, population with equally abundant genotypes; $E_5 = 0$, population dominated by a single genotype). Given the low genotypic diversity and small sample size of most populations (disease outbreaks), analyses were only conducted for the overall population in southern Switzerland. To infer population structure, we conducted a principal component analysis (PCA) based on Bruvo's genetic distance (Bruvo et al., 2004) using the R package *POLYSAT v1.3.3* (Pritchard et al., 2000). Only one representative of each MLG was included in the analysis. The genetic relationships among MLGs were visualized by constructing a Minimum Spanning Network based on Bruvo's genetic distance using *poppr*. For this, a clone-corrected data set (i.e., only one representative of each MLG per population) was used.

RESULTS

Occurrence of ink disease

The forest service reported a total of 32 chestnut stands as potentially affected by ink disease, which were distributed across the entire altitudinal range of sweet chestnut (~250–900 m a.s.l.) in southern Switzerland. In two cases (Origlio and Brione), single symptomatic trees were reported. The first symptoms, specifically microphyllly and rarefaction of the crown, were observed between the 1990s and 2010.

Isolates of *P. cinnamomi* or *P. × cambivora*, the two known causal agents of ink disease, were recovered from the rhizosphere of chestnut trees in 25 out of the 32 (78.1%) symptomatic chestnut stands reported by the forest service (Table 1 and Figure 1). According to a glm, annual mean temperature, NE aspect, and

TABLE 1 The main characteristics of the 25 chestnut stands in southern Switzerland in which *Phytophthora cinnamomi* and/or *Phytophthora × cambivora* were detected.

Site	Local name	Coordinates (WGS 84)	Altitude (m a.s.l.)	First symptoms ^a	Sampled trees (N)
Arbedo	I Muntagnett	46.2173, 9.054717	365	u.	15
Arcegno	Pestalozzi	46.16628, 8.73683	420	u.	15
Biasca	S. Petronilla	46.35488, 8.97606	380	1990s	15
Brione	Via Masné	46.18655, 8.8186	530	2010	6
Brissago	Ferabò	46.13386, 8.712056	580	u.	21
Brissago	Motto della Croce	46.130833, 8.7104	505	u.	15
Cademario	Lisone	46.02392, 8.89168	850	u.	15
Camignolo	—	46.107, 8.92988	460	u.	13
Castasegna	Brentan	46.33755, 9.5227	800	1990s	8
Castasegna	Stoll	46.339117, 9.52995	870	1990s	9
Claro	Moncrino	46.27945, 9.01894	800	2000	15
Locarno	Pureta	46.174033, 8.778483	455	u.	11
Locarno	Verigana	46.178267, 8.786533	520	u.	8
Losone	Arbigo	46.1744, 8.7457	270	u.	15
Malcantone	Mugena	46.05007, 8.88211	840	u.	15
Origlio	Via Roagina	46.0561, 8.952117			2
Orselina	Eco	46.18064, 8.79258	800	1990s	15
Orselina	Motto della Fassa 1	46.18261, 8.78896	720	2005	15
Orselina	Motto della Fassa 2	46.18255, 8.796067	670	u.	15
Ronco	Puran	46.15287, 8.73498	590	2000	15
Solduno	Traverse	46.17483, 8.77282	450	1995	15
Taverne	Pian del Maggio	46.06005, 8.934017	420	u.	15
Tegna	Selvapiana	46.18897, 8.74075	500	2003	15
Tenero	Contra	46.19365, 8.82735	785	u.	9
Verscio	Bartegna	46.19006, 8.73404	560	2011	15

^au., unknown.

distance to buildings were the only variables significantly associated with the incidence of ink disease in southern Switzerland (Table 2).

Causal agents of ink disease

The 157 *Phytophthora* isolates recovered belonged to 5 different species. *Phytophthora cinnamomi*, one of the two known agents of ink disease, was the most common species (86.6% of the isolates) and was present in 20 stands (Figure 1). The other causal agent of ink disease, *P. × cambivora*, was found in six stands for a total of seven isolates (4.5%). The *Phytophthora* community also included *P. plurivora* (11 isolates, 6 stands), *P. cactorum* (2 isolates, 2 stands), and *P. cryptogea* (one isolate). In most stands (18 out of 25), only one species of *Phytophthora* was detected, usually *P. cinnamomi* (16 cases). Four stands (Arcegno, Biasca, Cademario, and Orselina-Eco) harboured two species, whereas in three other stands (Camignolo, Castasegna-Brentan, and Castasegna-Stoll) we

identified three species. The co-occurrence of *P. cinnamomi* and *P. × cambivora* was only observed in Biasca.

Distribution of *Phytophthora* species in a chestnut stand

Overall, *Phytophthora* species were more frequently detected in the rhizosphere of symptomatic trees in a diseased sector of the stands (88.9% of the sampled sectors), followed by healthy trees in a diseased sector (77.3% of the sampled sectors) and healthy trees in a healthy sector (66.7% of the sampled sectors; Figure 2). Similarly, the mean prevalence of *Phytophthora* species was highest in the diseased sector of the chestnut stands, with 64.3% of the sampled symptomatic trees being positive. The percentage dropped to 25% for apparently healthy trees in a healthy sector of the stands. Although *P. cinnamomi* was most frequent in diseased sectors of the stands (55.6% of the sampled symptomatic trees, 46.7% of the sampled

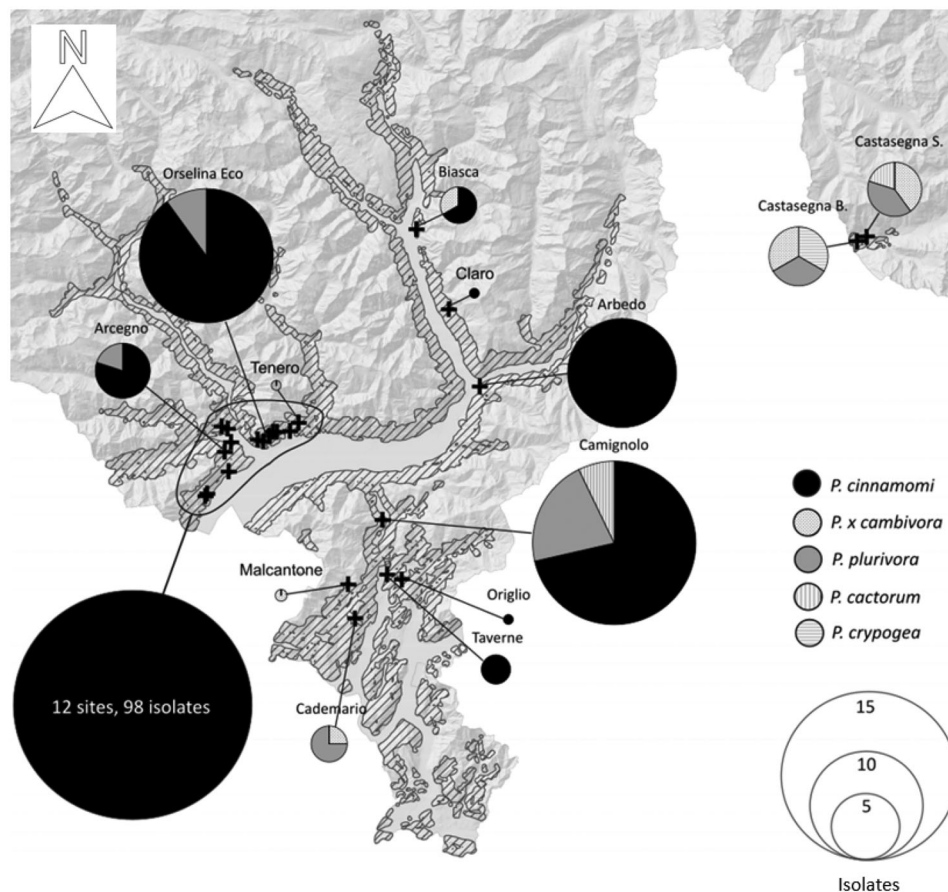


FIGURE 1 Distribution of the chestnut stands with ink disease (*Phytophthora cinnamomi* and/or *Phytophthora × cambivora*) in southern Switzerland and prevalence of the five *Phytophthora* species detected in the rhizosphere of the sampled trees. The hatched area indicates the local distribution of sweet chestnut.

asymptomatic trees), it was also detected in the soil of healthy sectors of the stands (17.7% of the sampled trees; Figure 2). Similarly, all other *Phytophthora* species were isolated both from healthy and diseased sectors of the stands. Only three chestnut trees harboured more than one *Phytophthora* species in their rhizosphere, namely one symptomatic tree in the diseased sector of the stand in Camignolo (*P. cinnamomi* and *P. plurivora*), one healthy tree in the healthy sector of the stand also in Camignolo (*P. cactorum* and *P. plurivora*), and one healthy tree in the symptomatic sector of the stand in Cademario (*P. × cambivora* and *P. plurivora*).

Tree pre-disposition and infection dynamics

The dendrochronological analysis revealed no difference in growth rates and resilience pre-disposition to infection by *P. cinnamomi* or *P. × cambivora* between currently symptomatic and asymptomatic trees at each site. Both the growth rates in the 40 years before the infection (Figure 3) and the resilience capacity after the drought in 2003 (Figure A1) did not differ significantly between the two groups of trees ($p > 0.05$; Table 3). As

visible from the pattern of the group mean chronologies (Table 3), a divergence in growth rates between currently symptomatic and asymptomatic trees started to appear between 2009 (Biasca) and 2021 (the two sites in Castasegna). In Taverne, although annual growth rates of symptomatic trees were not significantly reduced until 2020, 6 of the 10 symptomatic trees investigated died before or around 2020. Similarly, in Castasegna two trees died before the annual growth was significantly impacted in 2021.

Population structure of *P. cinnamomi*

In the overall *P. cinnamomi* population in southern Switzerland, a total of 37 alleles were identified, with two to four alleles per locus (Table A1). Three SSR loci (Pc_SSR12, Pc_SSR13, and Pc_SSR22) showed three alleles per isolate (triploid loci), whereas the other 12 loci only had two alleles per isolate (diploid loci). Locus Pc_SSR16 showed no variation in allele pattern across the entire population.

The 190 *P. cinnamomi* isolates genotyped belonged to 13 different MLGs (Table 4). The Stoddart and Taylor's index $G = 4.63$ showed a low genotypic diversity,

TABLE 2 The output of a standard generalized linear model testing how selected predictor variables affect the risk of occurrence of ink disease (*Phytophthora cinnamomi* or *Phytophthora × cambivora*) in chestnut stands in southern Switzerland.

Predictor variables ^a	Estimate (± SE) ^b	t-Value	p-Value
(Intercept)	−0.03 ± 0.88	−0.03	0.98
Aspect: N	−0.26 ± 0.46	−0.57	0.57
Aspect: NE	−0.61 ± 0.28	−2.18	0.01
Aspect: NW	−0.24 ± 0.26	−0.92	0.37
Aspect: S	0.28 ± 0.29	0.96	0.35
Aspect: SE	0.14 ± 0.23	0.60	0.55
Aspect: SW	−0.18 ± 0.24	−0.73	0.47
Aspect: W	−0.16 ± 0.45	−0.36	0.73
Tree species: mixed	−0.08 ± 0.15	−0.57	0.58
Stand type: High forest	−0.15 ± 0.16	−0.91	0.37
Stand type: Traditional orchard	0.09 ± 0.16	0.54	0.60
Management: yes	0.26 ± 0.17	1.59	0.12
Distance to a road	−5.3e-0.4 ± 0.00	−1.47	0.15
Distance to a building	−1.4e-0.3 ± 0.00	−1.73	0.05
Mean annual temperature	0.08 ± 0.04	1.70	0.05
Mean annual precipitation	8.6e-05 ± 0.00	0.42	0.68
Soil pH	−0.01 ± 0.07	−0.13	0.90
Model information	Observations: 46, dependent variable: ink disease		
Model fit	$\chi^2 = 8.01$, pseudo- $R^2 = 0.92$		

^aPredictor variables significantly affecting the risk of ink disease are in bold. For non-numerical variables, one category within the variable was tested against all other categories (aspect: E vs. N, NW, NE, S, SE, SW, W; Tree species: mixed vs. pure chestnut; Stand type: coppice vs. high forest, traditional orchard; Management: yes vs. no).

^bEstimate ± standard error. A positive value indicates that the specific predictor variable increases the risk of ink disease, whereas a negative value decreases it.

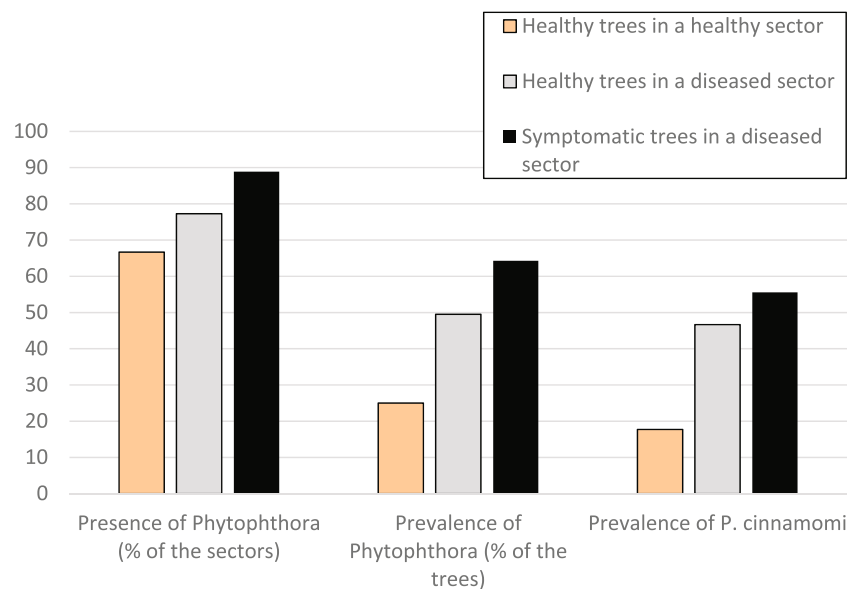


FIGURE 2 Presence and prevalence of *Phytophthora* species in the rhizosphere of healthy and symptomatic chestnut trees in diseased and healthy sectors of the chestnut stands with ink disease (*Phytophthora cinnamomi* or *Phytophthora × cambivora*) in southern Switzerland.

and the evenness index $E_5 = 0.563$ indicated that the MLGs were not evenly distributed in the population. At 12 out of the 16 sites investigated, MLG PcCH1 was predominant with a prevalence of 63.6%–100% of the

isolates. The other MLGs were significantly less frequent (0.8%–8.3% of isolates). From one (Arcegno, Biasca, Brione, Solduno, Tegna) to four (Brissago-Motto della Croce) MLGs were found per site (mean

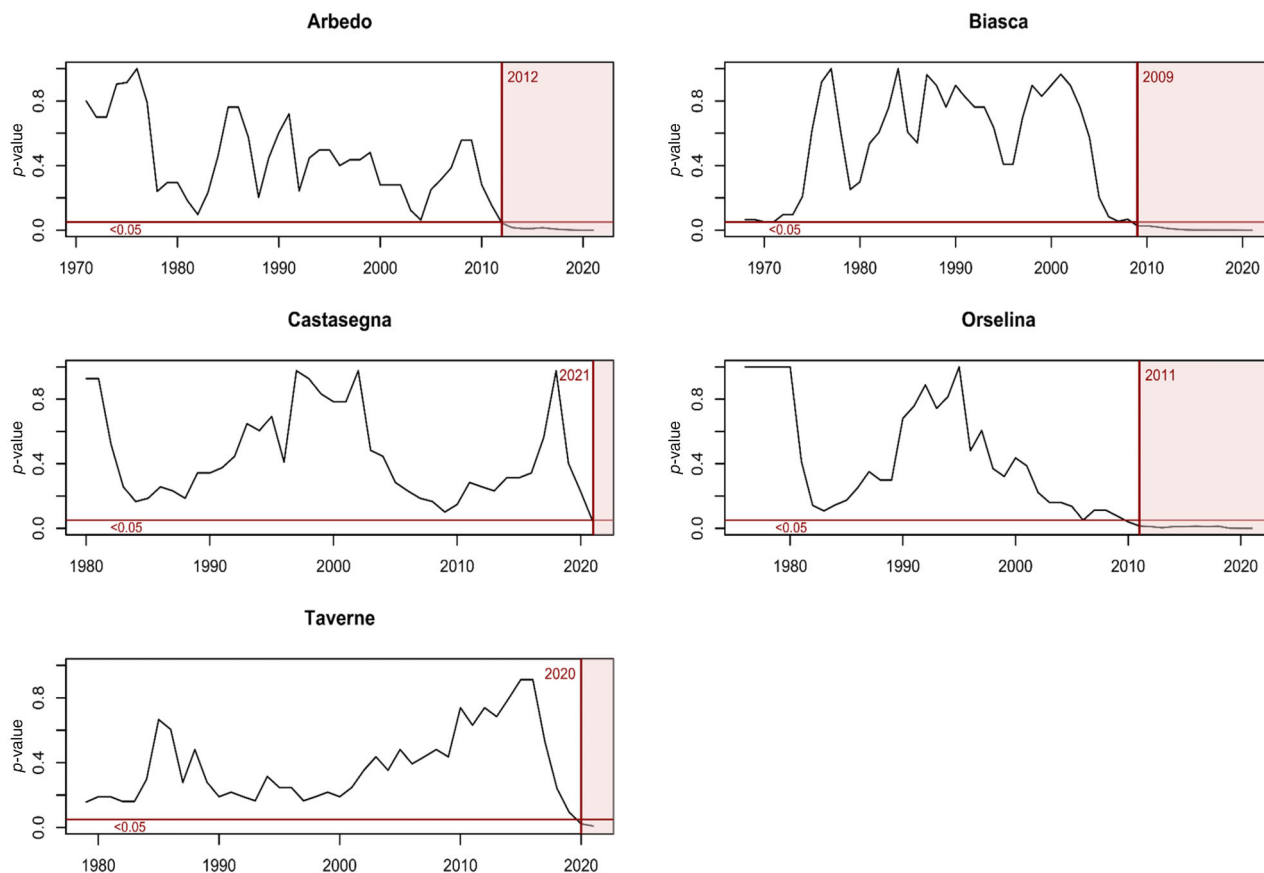


FIGURE 3 Site average growth patterns of currently asymptomatic (apparently healthy) and symptomatic (i.e., infected by *Phytophthora cinnamomi* or *Phytophthora × cambivora*) chestnut trees during the past 40–52 years in Arbedo, Biasca, Castasegna (two outbreaks combined), Orselina-Eco, and Taverne. Red years indicate the year after which the difference in growth between the two groups became significant (p -value of the Wilcoxon test is given).

TABLE 3 Summary statistics of the results of the dendrochronological analysis of healthy and symptomatic (i.e., infected by *Phytophthora cinnamomi* or *Phytophthora × cambivora*) chestnut trees at five different sites in southern Switzerland.

Site	Tree status ^a	Number of trees (dead)	Diameter at breast height (cm)	Age (mean ± SD)	Time from infection to death (years) ^b	Last tree ring formed (year ± SD)
Arbedo	H	10	42.5	48 ± 5.7	—	2021 ± 0.0
	S	11 (3)	35.5	45 ± 11.8	3.7	2018 ± 2.9
Biasca	H	10	83.0	108 ± 39.3	—	2021 ± 0.0
	S	10 (6)	81.5	74 ± 28.2	9.5	2019 ± 1.6
Castasegna ^c	H	10	148.0	106 ± 32.0	—	2021 ± 0.0
	S	12 (2)	161.7	101 ± 23.0	−2.0	2021 ± 1.4
Orselina-Eco	H	10	35.0	42 ± 7.3	—	2021 ± 0.0
	S	10 (8)	33.5	53 ± 17.1	5.4	2017 ± 2.7
Taverne	H	10	72.5	96 ± 26.2	—	2019 ± 3.9
	S	10 (6)	73.0	81 ± 22.7	−1.5	2019 ± 1.4

^aH, Healthy (asymptomatic); S, Symptomatic.

^bYears before the present when annual growth rates of healthy and symptomatic chestnut trees started to differ significantly (see Figure 3). Negative numbers indicate a sudden death.

^cBoth chestnut stands Brentan and Stoll.

2.06). Nine trees (4 in Arbedo: PcCH2 and PcCH3; three in Locarno-Verigana: twice PcCH1 and PcCH12 and once PcCH1 and PcCH7; one in Brissago-Motto

della Croce: PcCH2 and PcCH3; one in Orselina-Eco: PcCH1 and PcCH12) harboured two different MLGs in their rhizosphere and one tree in Losone-Arbigo even

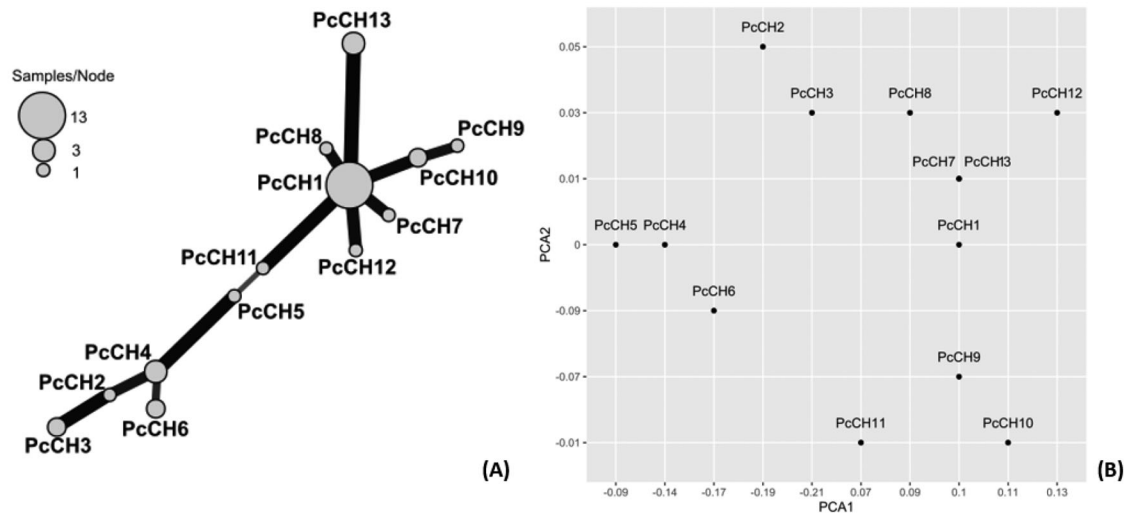


FIGURE 4 Genetic relations among the 13 *Phytophthora cinnamomi* multilocus genotypes (MLGs) detected in chestnut stands with ink disease in southern Switzerland. (A) Minimum spanning network based on Bruvo et al.'s (2004) genetic distances. The size of the nodes is proportional to the number of isolates and the thickness of the lines is inversely proportional to the genetic distance between MLGs (thicker lines indicate smaller genetic distance). (B) Principal component analysis using Bruvo et al.'s (2004) distances.

three MLGs (PcCH1, PcCH4, and PcCH5). The MSN suggests the presence of two groups of MLGs (Figure 4A), one including the dominant PcCH1 and six closely related MLGs (PcCH7–PcCH13) and PcCH2–PcCH6. This structuring of the MLGs is, however, less visible in the PCA (Figure 4B).

DISCUSSION

The diversity of soilborne *Phytophthora* species in chestnut stands with dieback symptoms and mortality in southern Switzerland is low, with only five species found, namely *P. cinnamomi*, *P. plurivora*, *P. cactorum*, *P. × cambivora*, and *P. cryptogea*. A similar community composition and low species diversity was previously reported in chestnut stands with the crown decline in Italy, France, Spain, Greece, and England (Vettrano et al., 2005). In this study, *P. cinnamomi* and *P. × cambivora* were isolated from the soil of 25 out of 32 sampled sites. Based on the observed symptoms (i.e., rarefaction of the crown, small leaves, dark lesions on the stem basis) and the known association of these two species with ink disease (Vannini & Vettrano, 2001), we can assume the stands at the infected site are indeed affected by ink disease. To definitively indicate ink disease as the cause of the observed damage; however, it would be necessary to isolate *P. cinnamomi* and *P. × cambivora* from the symptomatic trees, which is difficult in the absence of fresh lesions. In the five chestnut stands in which *P. cinnamomi* and *P. × cambivora* were not found, tree dieback and mortality may be due to other causes, including abiotic factors like drought, or a combined

effect of chestnut blight (*C. parasitica*) and chestnut gall wasp (*Dryocopus kuriphilus*), both widespread in the study region (Gehring et al., 2020; Prospero & Rigling, 2012). Alternatively, in these stands our baiting method may have failed in catching the two *Phytophthora* species.

Ink disease of chestnut in southern Switzerland shows a patchy distribution within the distribution range of the host tree *C. sativa*. Based on a generalized linear model, the local risk of occurrence of ink disease is increased by an S–SE aspect, the presence of a pure chestnut stand, management activities, the proximity of roads and buildings, and increasing annual mean temperature and precipitation. Since *P. cinnamomi* and *P. × cambivora* are two species that do not belong to the native *Phytophthora* community in the study region, the central role played by anthropogenic activities in their introduction and spread is easily understandable. Noteworthy, the highest prevalence of ink disease was observed in a region characterized by a significant presence of human infrastructure, including residential and industrial areas and communication routes. In a previous study, Vannini et al. (2021) showed that in central Italy the occurrence of ink disease is largely explained by the density of roads and drainage networks around infected sites, which facilitate the inoculum dispersal. Other studies conducted in different parts of the world highlighted the importance of human activity for the spread of soilborne invasive *Phytophthora* species in forests (e.g., Bradshaw et al., 2020; Jules et al., 2002; Vélez et al., 2020). Since *C. sativa* seems to be the most susceptible tree species to *P. cinnamomi* and *P. × cambivora* in the study area, having pure chestnut stands increases pathogen

transmission by reducing the distances to which inoculum must move to reach a new susceptible host. Finally, increasing annual mean temperature and precipitation may contribute to creating more favourable conditions for pathogens' survival and sporulation. This could apply especially to the more thermophilic *P. cinnamomi* whose outbreaks are particularly frequent along a lake where the climate is particularly mild.

Within chestnut stands affected by ink disease, the highest presence of *P. cinnamomi* was observed, as expected, in the rhizosphere of symptomatic trees. However, the pathogen was also found associated with asymptomatic trees, both within and outside mortality centres. The absence of symptoms on trees in which *P. cinnamomi* is already present in the rhizosphere may be explained in several ways. First, such chestnuts may be resistant to the pathogen. In an inoculation experiment, Robin et al. (2006) reported a large amount of within-population variation of susceptibility to *P. × cambivora* in *C. sativa*. Similarly, Miranda-Fontaina et al. (2007) found significant differences among chestnut clones in the development of root rot and collar rot after artificial inoculation with *P. cinnamomi*. To test for the presence of heritable resistance to *P. cinnamomi* in the *C. sativa* population in southern Switzerland, it would be worth determining whether seedlings issued from fruits from healthy mother trees and artificially inoculated with *P. cinnamomi* show a lower mortality rate compared with seedlings issued from fruits from diseased mother trees. In a previous study, Camisón et al. (2019) found that mortality due to *P. cinnamomi* was similar in offspring of healthy and *P. cinnamomi*-infected trees, but seedlings germinating from a diseased tree showed increased tolerance to the pathogen. Alternatively, the presence of positive asymptomatic trees may be explained by a recent dispersal of the pathogen via surface water or vectors (e.g., wild animals or cattle) without resulting in an infection yet. Studies on the detailed spatial distribution of the inoculum of pathogenic soilborne *Phytophthora* species in infected stands are still largely missing but would be helpful to better understand the disease dynamics. Finally, some chestnut trees may possess a particular soil microbiome that protects their roots from the pathogen (e.g., Liu et al., 2021; Ruiz-Gómez et al., 2019).

Following infection by *P. cinnamomi*, an adult chestnut tree may die within 5–15 years. Our tree ring analysis revealed a significant growth reduction of currently symptomatic trees starting 2–10 years before our core sampling. If we add roughly a couple of years for a root system to become strongly damaged by the pathogen, we obtain the above-given estimates of 5–15 years. Although concrete data about the speed of the dieback process are not available, scientific literature mentions that infection by ink disease may lead to the death of a tree within a few years or induce a state of general decline lasting for many years (Biraghi, 1953; Černý

et al., 2008; Fenaroli, 1945; Vetraino et al., 2005). Both scenarios seem to be in line with our results. Interestingly, our data do not show a clear correlation between the size of a tree (diameter) and the rapidity of its decline and death. The fastest and slowest dying processes were observed in Castasegna and Biasca, respectively, the two sites with the largest chestnut trees. Hence, additional factors other than tree size play a role in the outcome of the interaction with the two pathogens, possibly including the amount of inoculum present in the soil, virulence of the *P. cinnamomi* or *P. × cambivora* strains involved, tree susceptibility, and specific site conditions (e.g., type of soil, soil water content, or soil depth). Regarding the tree's pre-disposition to become affected by ink disease, our study indicates that vitality is not a pre-disposing factor. Indeed, annual growth rates and resilience capacity of currently symptomatic and asymptomatic trees did not significantly differ before the onset of an infection. This confirms that *P. cinnamomi* or *P. × cambivora* are primary pathogens toward sweet chestnuts, that is, they do not need already weakened trees to cause an infection.

The *P. cinnamomi* population in chestnut stands in southern Switzerland is characterized by an overall low genetic diversity, with most disease outbreaks founded by the same MLG (PcCH1). In some cases, PcCH1 is accompanied by a few additional MLGs which, given their close genetic relatedness to PcCH1, could have been derived from PcCH1 locally through mutations. This situation indicates an asexual spread of the pathogen via zoospores or chlamydospores within outbreaks. Comparable studies on the population structure of *P. cinnamomi* in chestnut forests are missing. However, low diversity of this pathogen was previously reported in South Africa (Engelbrecht et al., 2022, using the same SSRs as in this study; Linde et al., 1997, using isozymes) and Australia (Dobrowolski et al., 2003, using three SSR loci) populations in both wild and cultivated hosts. The low diversity of the overall population of *P. cinnamomi* in southern Switzerland complicates the reconstruction of a potential invasion history of the pathogen. The predominance of a single MLGs (PcCH1) suggests the two following scenarios. First, PcCH1 was independently introduced in the local chestnut stands with infected plant material from the same source (e.g., nursery). Second, only a few introductions occurred, followed by local spread by humans or animals inhabiting the chestnut stands (e.g., wild boars, deer). This second scenario could be true, especially for the continuous and strongly affected chestnut stands above Locarno, which have a very important recreational function for the local population and tourists and are a habitat for various wild animals.

In summary, our study confirms that ink disease is locally present in chestnut forests in southern Switzerland. The causal agent is mainly *P. cinnamomi*, while *P. × cambivora*, which was the first ink disease-associated pathogen officially identified in the region in

1943 (Arrigoni, 1950), is currently rare. The disease seems to have emerged in the last two to three decades and in the future, this process may be further accelerated by the ongoing global warming (*P. cinnamomi* and *P. × cambivora* are thermophilic species). Given the occurrence of most disease outbreaks in regions densely populated with high levels of human infrastructure, it is likely that the two pathogens were spread by human activity. From the urban areas, they have then escaped into the surrounding chestnut stands. Sweet chestnut stands in southern Switzerland provide main ecosystem services (such as erosion control, wood and nut production, and recreation) and are an important cultural heritage. Thus, future research should focus on developing management strategies aiming at reducing the further spread and mitigating the impact of ink disease.

AUTHOR CONTRIBUTIONS

Simone Prospero: Conceptualization (lead); funding acquisition (lead); methodology (equal); project administration (lead); supervision (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **Malve Heinz:** Formal analysis (supporting); investigation (equal); software (equal); visualization (equal); writing—original draft (supporting); writing—review and editing (equal). **Eva Augustiny:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Ying-Yu Chen:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Juanita Engelbrecht:** Investigation (equal); software (equal); writing—review and editing (supporting). **Marina Fonti:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Aliona Hoste:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Beat Ruffner:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Romina Sigris:** Investigation (equal); methodology (equal); writing—review and editing (supporting). **Noelani van den Berg:** Funding acquisition (supporting); writing—review and editing (supporting). **Patrick Fonti:** Conceptualization (supporting); methodology (equal); supervision (equal); validation (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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APPENDIX A

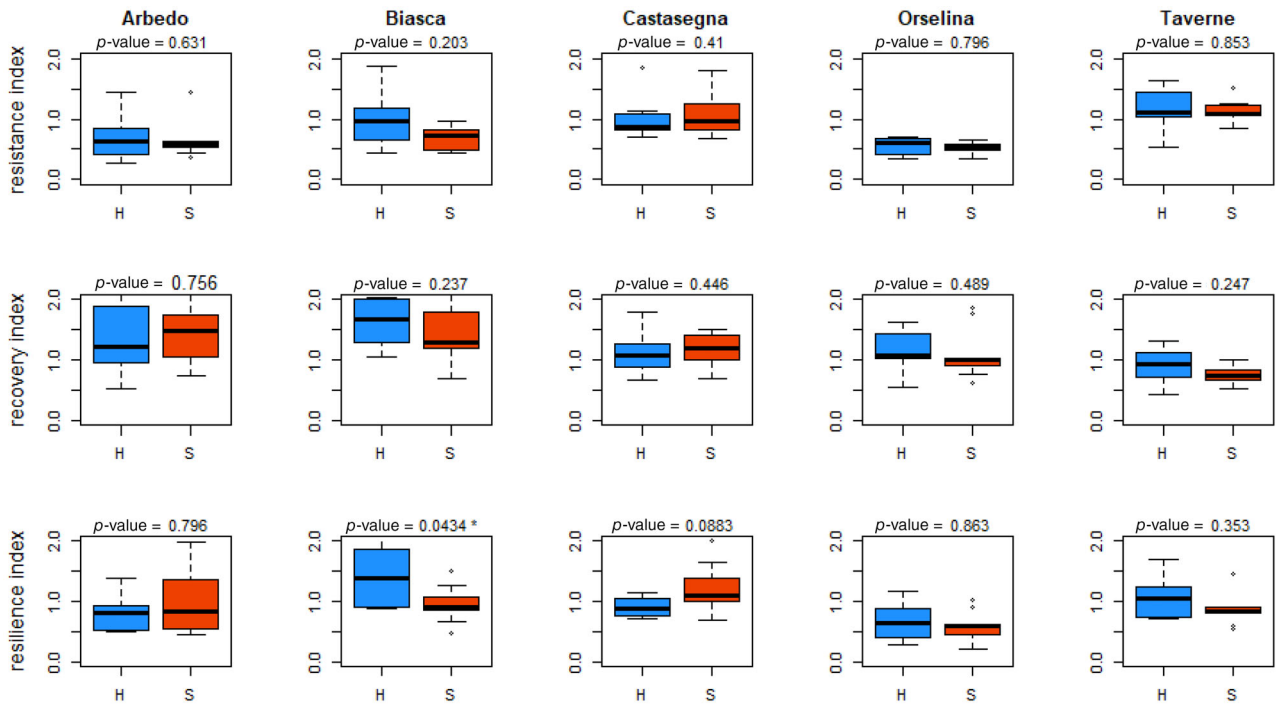


FIGURE A1 Components of tree resilience showing resistance, resilience, and recovery to and after the 2003 drought. Boxplots show the range of tree response at the different sites, asterisks mark significant differences as determined by a Wilcoxon test (p -value < 0.05). H, healthy trees; S, symptomatic trees.

TABLE A 1 Allele patterns (fragment sizes, bp) of the 13 multilocus genotypes (MLG) of *Phytophthora cinnamomi* detected in this study in southern Switzerland.

MLG	Pc_SSR12	Pc_SSR13	Pc_SSR16	Pc_SSR3	Pc_SSR8	Pc_SSR22	Pc_SSR9	Pc_SSR7											
PcCH1	211	214	214	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	266
PcCH2	211	211	211	436	442	451	337	341	374	374	221	221	252	258	258	344	347	268	268
PcCH3	211	211	211	436	442	451	337	341	374	374	221	221	252	258	258	344	347	268	268
PcCH4	211	211	211	436	442	451	337	341	374	377	221	221	252	258	258	344	347	268	262
PcCH5	211	211	211	436	442	451	337	341	374	377	221	221	252	258	258	344	347	268	262
PcCH6	211	211	211	436	442	457	337	341	374	377	221	221	252	252	258	344	347	262	268
PcCH7	211	211	214	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	266
PcCH8	211	214	214	436	442	442	337	341	376	376	218	221	249	252	258	347	347	262	266
PcCH9	211	214	214	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	262
PcCH10	211	214	214	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	262
PcCH11	211	214	214	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	266
PcCH12	211	214	214	436	442	442	337	341	377	383	218	221	249	252	258	347	347	262	266
PcCH13	211	214	217	436	442	442	337	341	377	377	218	221	249	252	258	347	347	262	266
MLG	Pc_SSR14	Pc_SSR15	Pc_SSR19	Pc_SSR2	Pc_SSR4	Pc_SSR20	Pc_SSR23												
PcCH1	214	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			
PcCH2	214	217	217	322	329	170	173	264	264	314	314	172	172	172	332	338			
PcCH3	214	217	217	322	329	173	173	264	264	314	314	172	172	172	332	338			
PcCH4	214	217	217	322	329	170	173	264	264	314	314	172	172	172	332	338			
PcCH5	214	217	217	322	329	170	173	264	264	314	314	172	172	172	335	338			
PcCH6	214	217	217	322	329	173	173	264	264	314	314	172	172	175	332	335			
PcCH7	217	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			
PcCH8	214	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			
PcCH9	214	217	217	329	329	170	173	264	264	314	314	162	172	172	335	338			
PcCH10	214	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			
PcCH11	214	217	217	329	329	170	173	264	264	314	314	172	172	172	335	338			
PcCH12	214	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			
PcCH13	214	217	217	329	329	170	173	264	267	314	314	172	172	172	335	338			