Phytopathologia Mediterranea (2015) 54, 2, 199–211
DOI: 10.14601/Phytopathol_Mediterr-14712

RESEARCH PAPERS

Gnomoniopsis castanea is the main agent of chestnut nut rot in Switzerland

Francesca G. DENNERT[1], Giovanni A.L. BROGGINI[1,3], Cesare GESSLER[1] and Michelangelo STORARI[1,2]

[1] ETH Zürich, Institute of Integrative Biology, Plant Pathology Group, Universitätsstrasse 2, 8092 Zürich
[2] Institute for Food Sciences, Agroscope, Bern, Switzerland
[3] Agroscope Wädenswil, Schloss 1, CH-8820 Wädenswil

Summary. Nuts of sweet chestnut have been an important food source for the alpine population in Switzerland since the Middle Ages and are still valued today for the preparation of traditional food commodities. Nut quality is reduced by insect damage and by various pathogenic fungi. In the last few years, producers and consumers perceived an increase of brown nut rot; while the nut rot agent *Gnomoniopsis castanea* was reported locally in southern Switzerland, its presence has not been investigated over large areas until now. This study assessed the incidence of brown nut rot and identified the causal agent present in Switzerland. Fully ripened nuts were collected from the main sweet chestnut growing areas of Switzerland. A filamentous fungus morphologically identified as *G. castanea* was isolated from 10 to 91% of the sampled nuts, despite only 3 to 21% of the sampled nuts showing brown rot symptoms. This fungus was isolated from symptomatic chestnuts as well as from apparently healthy chestnuts. Our results suggest a possible endophytic lifestyle in ripened nuts as well as in branches, leaves and unripe nuts as previously found. Species identity of 45 isolates was confirmed by *EF-1alpha*, *beta-tubulin* and ITS sequencing. Concatenation of β-*tubulin* and *calmodulin* sequences showed that several haplotypes were present at each sampling locality. No other nut rot pathogens could be isolated in this study, suggesting that *G. castanea* is the main causal agent of nut rot in Switzerland. The presence of this species is reported for the first time in a site in northern Switzerland. Further studies are needed to assess the influence of meteorological conditions and chestnut varieties on the incidence of *G. castanea* in order to provide prevention strategies for chestnut growers.

Key words: *Gnomoniopsis smithogilvyi*, *Castanea sativa*, endophyte, calmodulin.

Introduction

Sweet chestnut (*Castanea sativa* Mill.) was introduced to the alpine region of Europe by the Romans, and the nuts have been a common food in alpine and pre-alpine valleys from the middle ages to the 20th century (Krebs *et al.*, 2005; 2012; Conedera *et al.*, 2004b). In southern Switzerland, sweet chestnut forests, orchards and abandoned orchards cover an area of approx. 26,000 hectares at an altitude between 300 and 900 m a.s.l. (Krebs *et al.*, 2012). Many different sweet chestnut varieties are present in this area, including early and late ripening varieties, many of which are ancient and were bred through centuries of selection by local farmers (Gobbin *et al.*, 2007). Sweet chestnuts have recently been rediscovered as a specialty food by consumers and producers, with increasing numbers of producers' associations forming to promote the cultivation of sweet chestnuts and the production of traditional sweet chestnut based food commodities. However, market-level quality is difficult to obtain because disease prevention strategies are limited to pruning dead branches and removal of leaves from the ground in autumn (Chestnut Growers Association of Bregaglia Valley, personal communication). Pesticide and fungicide use is only permitted in sweet chestnut orchards and forests under exceptional

Corresponding author: F.G. Dennert
E-mail: francesca.dennert@usys.ethz.ch

ISSN (print): 0031-9465
ISSN (online): 1593-2095

circumstances by Swiss law (Chemikalien Risikoreduktions-Verordnung, 2005).

Knowledge of pest and pathogen life cycles is therefore of primary importance in the development of damage prevention strategies against these organisms. A number of different insects and pathogenic fungi are present in Southern Switzerland that reduce nut quality (Sieber *et al.*, 2007). Fungi colonizing nuts in the post-harvest stage and causing various types of nut rots include *Ciboria batschiana, Amphiporte castanea* and *Penicillium* spp. (Sieber *et al.*, 2007).

Recently, growers and consumers have perceived an increase in brown rot incidence in nuts grown in Switzerland (Chestnut Growers Association of Bregaglia Valley, personal communication); while the causal agent was identified locally in southern Switzerland as *Gnomoniopsis castanea* (Visentin *et al.*, 2012), incidence this fungus was not assessed systematically. Similarly, an increase of nut rot was also recorded in northern Italy in the last 10 years (Gentile *et al.*, 2010; Visentin *et al.*, 2012; Maresi *et al.*, 2013). In these studies, the ascomycete *Gnomoniopsis castanea* was identified as the causal agent (Gentile *et al.*, 2010; Visentin *et al.*, 2012). This pathogen has been isolated from sweet chestnut growing areas in different countries worldwide, including, France, Switzerland (Visentin *et al.*, 2012) and India (Dar and Rai, 2013). A similar disease was reported in New Zealand and Australia but the fungus was described as *G. smithogilviyi* (Shuttleworth *et al.*, 2013). Whether *G. castanea* and *G. smithogilviyi* represent the same species still needs to be determined. These findings indicate that *Gnomoniopsis* is ubiquitous in chestnut populations worldwide (Visentin *et al.*, 2012).

Gnomoniopsis castanea infection causes the endosperm of affected nuts to turn chalky, white and sponge-like, resulting in an alteration of flavour (Visentin *et al.*, 2012; Maresi *et al.*, 2013). Symptoms are very similar to those caused by *Phomopsis castaneae* (Washington *et al.*, 1999), which may cause some confusion in the identification of the main causal agent of brown nut rot (Smith and Ogilvy, 2008; Maresi *et al.*, 2013). *Gnomoniopsis castanea* has been found to have an endophytic lifestyle in branches, leaves and unripe nuts, turning to a symptomatic lifestyle in fully ripened nuts (Visentin *et al.*, 2012). The life cycles of *G. castanea* and *G. smithogilvy* have not been investigated in detail to date. There are indications that overwintering of the pathogens takes place on dead burrs, where perithecia are formed (Crous *et al.*, 2012). Ascospores then infect chestnuts during flowering (Crous *et al.*, 2012; Shuttleworth *et al.*, 2013). Rainfall during flowering can influence disease incidence; varieties flowering during periods of heavy rainfall were found to have a greater incidence of *G. castanea* than those flowering in dry periods (Shuttleworth *et al.*, 2013).

The widespread presence of *G. castanea* in southern Europe led to the hypothesis that this fungus could also be the predominant species causing sweet chestnut nut rot in Switzerland. The aims of this study were, therefore, to i) assess the incidence of brown nut rot in different regions of Switzerland, ii) to isolate the brown nut rot pathogen present in Switzerland and to verify if it corresponds to *Gnomoniopsis castanea*, the main causal agent of brown nut rot in Italy and Australia, and iii) to make a preliminary investigation of the geographical distribution of different haplotypes of *G. castanea* in Switzerland.

Material and methods

Sampling

Nuts analysed in the present study were collected from eight localities in southern Switzerland and one locality in northern Switzerland (Table 1, Figure 1) during October 2012. Sampling localities were chosen in regions of Switzerland with large numbers of sweet chestnut trees, where at least 10% of trees in forests are *C. sativa* (WSL, 1983–1985) and where *C. sativa* is currently cultivated or was cultivated in the past. For each locality, three trees separated from each other by a maximum distance of 500 m were selected, and for each tree 30 fallen ripened nuts were randomly collected. Exceptions were the sampling sites Murg/Siten, where nuts were collected from trees separated from each other by 1–2 km, as well as for Biasca and Cadenazzo, where nuts were collected randomly from several trees. Nuts from Biasca and Cadenazzo were processed, respectively, in two and three batches. Nuts were stored at 3°C for for a maximum of 7 d before further analysis.

Isolation of fungal strains

A protocol adapted from Visentin *et al.* (2012) was used to recover *Gnomoniopsis* spp. isolates from collected nuts. Nuts were superficially disinfected in a

Table 1. Geographical WGS84 datum coordinates of sampling sites and geographical regions where sweet chestnuts were sampled. Canton of the sampling locations are given in parenthese. Average number of infected nuts (nuts from which *Gnomoiopsis castanea* were be isolated) and diseased nuts (nuts with *G. castanea* symptoms) are given for each location.

Sampling site	Coordinates	Population code	Geographical region	Average infected nuts	Average diseased nuts
Biasca	46.353326N,8.967876E	BIA	Ticino (TI)	71%	5%
Cadenazzo	46.149989N,8.950496E	CAD	Ticino (TI)	80%	10%
Campascio	46.245846N,10.130546E	CAM	Poschiavo (GR)	35%	3%
Castasegna	46.369674N,9.518051E	CAS	Bregaglia (GR)	33%	5%
Faido	46.482378N,8.793304E	FAI	Ticino (TI)	31%	5%
Losone	46.175254N,8.746476E	LOS	Ticino (TI)	82%	4%
Murg/Siten	47.111422N,9.222161E	MUS	Walensee (GL)	91%	4%
Selna	46.175462N,8.676274E	SEL	Ticino (TI)	51%	7%
Taverne	46.068526N,8.93471E	TAV	Ticino (TI)	86%	21%

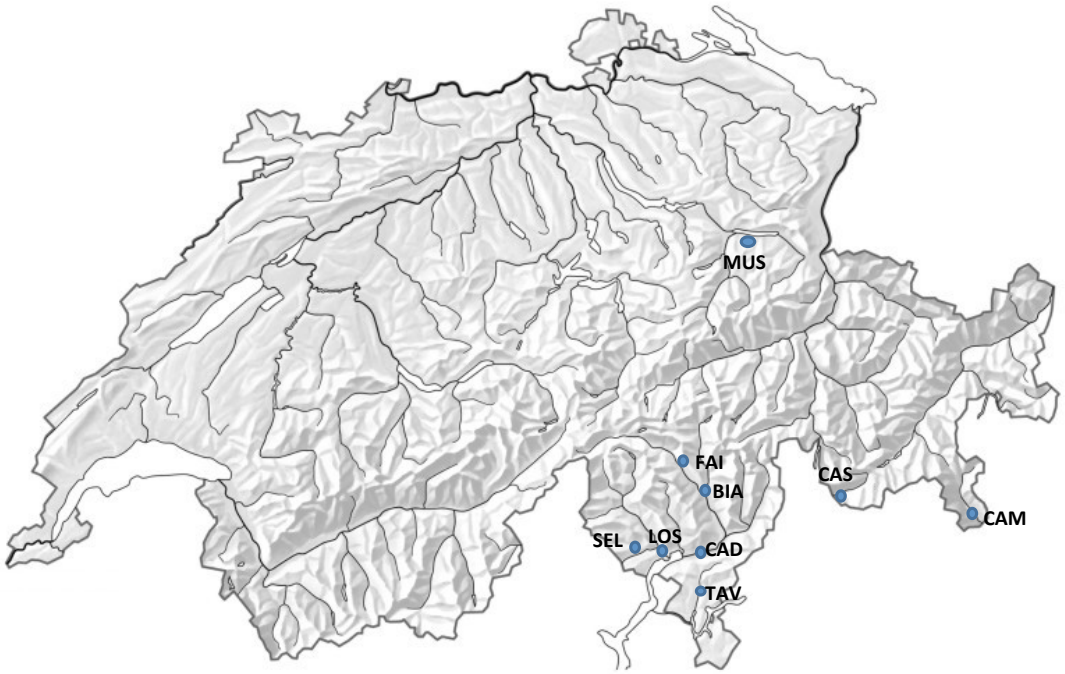

Figure 1. Location of sampling sites in Switzerland (background map: ©Swisstopo, Bern 2005) from which sweet chestnuts were sampled for this study.

1.4% sodium hypochlorite solution for 10 min and then dried in a sterile air flow cabinet. The outer, lignified shell was removed from each nut with a sterile scalpel. Nuts were cut in half and the presence of *Gnomoniopsis* rot symptoms was assessed visually by comparison with descriptions and photographs of symptomatic chestnuts from the literature (Gentile *et al.*, 2010; Visentin *et al.*, 2012; Shuttleworth *et al.*, 2013). One half of each nut was transferred in a 90 mm Petri dish containing malt extract agar (MEA) composed of 30 g L^{-1} malt extract (Oxoid LP0039), 5 g L^{-1} mycological peptone (BD, peptone No. 2) and 15 g L^{-1} bacteriological flake agar (Conda) amended with 100 mg L^{-1} chloramphenicol (Fluka). Plates were incubated at 24°C for 4 d. Emergent fungal colonies were morphologically identified as belonging to the genus *Gnomoniopsis* by light microscope comparison of colony morphology with the three reference isolates of *Gnomoniopsis castanea* ENV_1_2, PVG_ 23 and BSD_9, provided by Dr. P. Gonthier from the University of Turino, Italy. Isolates that corresponded to *Gnomoniopsis spp*. were counted, transferred and purified on MEA plates amended with 100 mg L^{-1} chloramphenicol and grown for 7–14 d at 24°C.

Assessment of incidence of *Gnomoniopsis castanea*

Nuts showing visual symptoms of *G. castanea* infection were defined as diseased, while those that showed emergent fungal colonies were defined as infected. Average frequencies of diseased and infected nuts from each sampling locality were calculated. Statistically significant differences in numbers of infected or diseased nuts between sampling localities were determined by analysis of variance (ANOVA) and Tukey's Honest Significant Difference post-hoc test, using a confidence interval of 95% with R version 2.15.2 (R Core Team 2013). Correlation between numbers of diseased and infected nuts was tested with Sperarman's rho rank correlation method using the function cor.test of R version 2.15.2 (R Core Team 2013).The confidence interval used was 95%.

Molecular identification

For a subset of recovered isolates, species identity was confirmed by sequencing fragments of the β-tubulin and the elongation factor 1-α (EF 1-α) genes, as well as the internal transcribed spacer (ITS) following the indications of Visentin *et al.* (2012) and Walker *et al.* (2010). Moreover, a fragment of the calmodulin gene was also sequenced (see Table 2 for more details). For each sampling site, five isolates were randomly chosen and incubated on MEA without chloramphenicol for 7 d at 24°C. Pieces of mycelium of approximately 0.1–0.5 g were scraped from each plate and lyophilized. DNA was extracted from the lyophilized material with the GenElute kit (Sigma-Aldrich) or with the DNAeasy plant mini kit (Qiagen) following the manufacturers' instructions. DNA concentration was assessed by gel electrophoresis using standards for different concentrations (Thermo Fisher Bio) and comparing the intensity of the bands from the samples with the bands from the standards under UV light. DNA was diluted 1:5 to reach a concentration range of 5–20 ng μL^{-1}. Amplification of EF 1-α, calmodulin, ITS and β-tubulin was done using the primers specified in Table 2. For each PCR reaction, the master mix consisted of 0.10 mM dNTPs, 1.5 mM Mg^{2+}, 0.50 μM of each primer and 0.070 U μL^{-1} of TAQ-polymerase (DreamTAQ, Fermentas) and approximately 5–20 ng DNA in a final reaction volume of 25 μL. Cycling conditions consisted of an initial denaturation of 10 min at 95°C, followed by 35 cycles consisting of 30 sec. at 94°C, 30 sec at the respective annealing temperature (see Table 2) and 1 min at 72°C, followed by a final elongation of 5–10 min at 72°C. Amplicons were purified on 0.2 μm filter plates (Corning Inc.). Sequencing reaction was performed with 2 μL of BigDye Terminator Kit (Applied Biosystems), 1 μL of primer and 5 μL of PCR product in a final reaction volume of 15 μL. Sequencing conditions consisted of an initial denaturation of 1 min at 96°C, followed by 27 cycles of 10 sec at 96°C, 5 sec at 50°C and 1 min at 60°C. Sequencing products were purified with sephadex (GE Healthcare) prior to sequencing on an ABI Prism 3100x sequencer (Applied Biosystems).

Forward and reverse sequences were assembled and edited using Sequencher version 4.6 (Gene Codes Corporation). Consensus sequences were aligned with ClustalW using BioEdit (Ibis Bioscience) and Neighbor joining phylogenetic trees were inferred with MEGA4 (Tamura *et al.*, 2007) using the Jukes-Cantor model and 1000 bootstrap replicates. Sequences of the other known species of *Gnomoniopsis* (Walker *et al.*, 2010) were included in the alignments as well as EF-1α sequences obtained previously for *G. castanea* isolates (Visentin et al., 2012). Reference sequences and respective GenBank accession numbers are listed in Table 3.

Table 2. Primers and annealing temperatures for PCR reactions used in this study.

Gene	Primer name	Primer sequence	Annealing temperature	Reference
Elongation factor 1-α	EF1-728F	5′-CATCGAGAAGTTCGAGAAGG-3′	58°C	Carbone and Kohn (1999)
	EF1-1199R	5-′GGGAAGTACCMGTGATCATGT-3′		Walker *et al*. (2010) Diaporthales
Calmodulin	CL1	5′-GA(GA)T(AT)CAAGGAGGCCTTCTC-3′	55°C	O′Donnell *et al*. (2000)
	CL2A	5′-TTTTTGCATCATGAGTTGGAC-3′		O′Donnell *et al*. (2000)
β-tubulin	Bt2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	58°C	Glass and Donaldson (1995)
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′		Glass and Donaldson (1995)
ITS	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	55°C	White *et al*. (1989)
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′		White *et al*. (1989)

Table 3. Reference strains and GenBank accession numbers for fungi assessed in this study.

Isolate name	Collection	EF-1α[c]	B-tubulin[c]	ITS	Reference
Gnomoniopsis castanea CH2	MUT 810[a]	JQ791210	-	JQ898295	Visentin *et al*. 2012
Gnomoniopsis castanea CH4	MUT 814	JQ791199	-	JQ898297	Visentin *et al*. 2012
Gnomoniopsis castanea FR	MUT 815	JQ791212	-	JQ898298	Visentin *et al*. 2012
Gnomoniopsis castanea Rb7	MUT 401	JQ791198	-	HM1142946	Visentin *et al*. 2012
Gnomoniopsis castanea NZ11	MUT 411	JQ791201	-	HM1142948	Visentin *et al*. 2012
Apiognomonia veneta	CBS[b] 342.86	DQ318036	EU219235	DQ313531	Walker *et al*. 2010
Gnomoniopsis alderdunense	CBS 125680	GU320801	GU320787	GU320825	Walker *et al*. 2010
Gnomoniopsis calvulata	CBS 121255	GU320807	EU219211	EU254818	Walker *et al*. 2010
Gnomoniopsis comari	CBS 806.79	GU320810	EU219156	EU254821	Walker *et al*. 2010
Gnomoniopsis fructicola	CBS 125671	GU320793	GU320776	GU320816	Walker *et al*. 2010
Gnomoniopsis guttulata	na	na	na	EU254812	Walker *et al*. 2010
Gnomoniopsis idaeicola	CBS 125675	GU320799	GU320783	GU320822	Walker *et al*. 2010
Gnomoniopsis macounii	CBS 121468	GU320804	EU219126	EU254762	Walker *et al*. 2010
Gnomoniopsis occulta	CBS 125677	GU320812	GU320785	GU320828	Walker *et al*. 2010
Gnomoniopsis paraclavulata	CBS 123202	GU320815	GU320775	GU320830	Walker *et al*. 2010
Gnomoniopsis racemula	CBS 121469	GU320803	EU219125	EU254841	Walker *et al*. 2010
Gnomoniopsis sanguisorbae	CBS 858.79	GU320805	GU320790	GU320818	Walker *et al*. 2010
Gnomoniopsis tomentillae	CBS 904.79	GU320795	EU219165	EU254856	Walker *et al*. 2010
Plagiostoma euphorbiae	CBS 340.78	GU354016	GU367034	EU199198	Walker *et al*. 2010

[a] MUT= Mycotheca Universitatis Taurinensis, University of Turino, Italy; [b]CBS= Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; [c]GenBank accession number

Haplotype analysis

Sequences of β-tubulin and calmodulin were used to infer haplotype networks with TCS version 1.21 (Clement *et al.*, 2000). In a second step, sequences of β-tubulin and calmodulin were concatenated to infer a combined haplotype network with TCS version 1.21 (Clement *et al.*, 2000). Graphs of haplotype networks were edited with Inkscape version 0.48 (www.inkscape.org). EF 1-alpha and ITS1 sequences were not used for haplotype analysis because of the lack of diversity at these loci in our samples (see Results section for more details).

Results and discussion

Nuts showing brown rot symptoms were recovered at all sampling sites and frequency of diseased nuts varied between 3% from Campascio and 21% from Taverne. There was large variation among the trees from particular localities. For example from Taverne, 3% of nuts from one tree and 46% of nuts from the second tree showed disease symptoms. After 4 d of incubation, colonies identified morphologically as *G. castanea* were present from all sampling sites. No other species commonly linked to chestnut rot, such as *Phomopsis castanea* (Wadia *et al.*, 1999; Washington *et al.*, 1999) or *Ciboria batsciana* (Sieber *et al.*, 2007) could be detected. However, moulds morphologically identified as belonging to the genera *Penicillium* and *Rhizopus* were occasionally recovered, mainly from insect damaged nuts. In approx. 10 to 60% of the nuts, depending on the sampling site, no fungal growth of could be detected.The nuts from which no fungal growth occurredwere asymptomatic. The number of nuts infected by *G. castanea* was greater than the number of diseased nuts in all populations, ranging from 31% of the nuts from Faido to 91% of the nuts from Murg/Siten. The number of infected nuts was significantly different (TukeyHSD post-hoc test $P<0.05$) between Murg/Siten and Campascio, and between Castasegna and Faido. No significant differences were found in the number of diseased nuts between sampling localities. To confirm differences in infection between localities, studies with a larger number of replicates should be conducted (Figure 2 and Supplementary Material Table 5). *Gnomoniopsis castanea* was isolated from all diseased nuts.

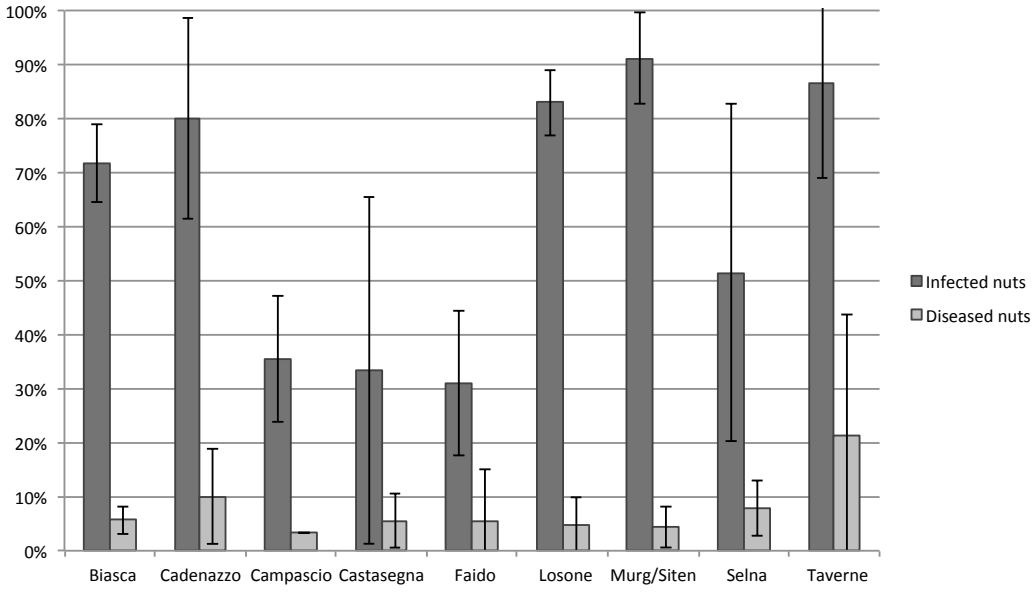

Figure 2. Average percentage of infected nuts (from which *Gnomoniopsis castanea* was isolated) and diseased nuts (showing *G. castanea* rot symptoms) of the three sampled trees in each sampling location. Black bars represent standard errors. For localities Biasca and Cadenazzo, standard error between batches is shown (see Table 5 in Supplementary Material for percentage of infected nuts from each tree). Capital letters represent significant differences of proportions of infected nuts between localities (ANOVA, $P < 0.05$). No significant differences between numbers of diseased nuts were found between localities.

A weak positive correlation between percentages of diseased nuts and infected nuts was found, but was not statistically significant (rho = 0.25, *P*=0.21), indicating a weak tendency for greater numbers of nuts with rot symptoms from trees with large numbers of infected nuts. However, there was no a clear link between these two parameters.

The high proportion of infected nuts showing no disease symptoms indicate that *G. castanea* can have an endophytic lifestyle in ripened nuts. However, whether these infected nuts could develop disease symptoms if incubated for a longer period at ambient temperatures was not tested. *Gnomoniopsis castanea* was previously found to be an endophyte in branches, leaves and unripe nuts of chestnut trees (Visentin *et al*., 2012; Maresi *et al*., 2013), but not in ripened nuts. . In the present study, there were considerable differences in incidence of *G. castanea* between nuts of trees growing in the same locality (Table 5, Supplementary Material). This could be due to differences in the tolerance of *G. castanea* between sweet chestnut varieties. Although *G. smithogilvyi* has been reported to cause rot symptoms in at least three different cultivated varieties (Shuttleworth *et al*., 2010; Shuttleworth *et al*., 2013) no systematic studies of resistance have been undertaken to date. In the present study, chestnuts were almost exclusively collected from wild sweet chestnut trees in abandoned orchards or in forests, making it not possible to determine the ancestor varieties of the sampled trees. A systematic investigation of *G. castanea* infection of different commercially planted sweet chestnut varieties would be of great utility for chestnut growers, since fungicide treatments of sweet chestnut trees for nut rot control are economically not sustainable in many cultivation systems.

Identity of *Gnomoniopsis castanea* was confirmed for a subset of isolates randomly taken from all sampling sites. EF-1α sequences from the collected isolates were identical to EF-1 α sequences of *G. castanea* reference strains ENV_1_2, PVG_ 23 and BSD_9 (sequenced in this study), as well as NZ11, FR, and Rb7 (sequenced by Visentin *et al*., 2012). They were not identical but formed one cluster with *G. castanea* isolates CH2 and CH4 (Figure 3). The closest relatives to the isolates used in this study were *G. paraclavulata* and *G. clavulata*, concurring with the findings of Visentin *et al.* (2012). This result was confirmed by the analysis of ITS and β-tubulin sequences (Figures 5 and 6 in Supplementary Material). ITS sequences of

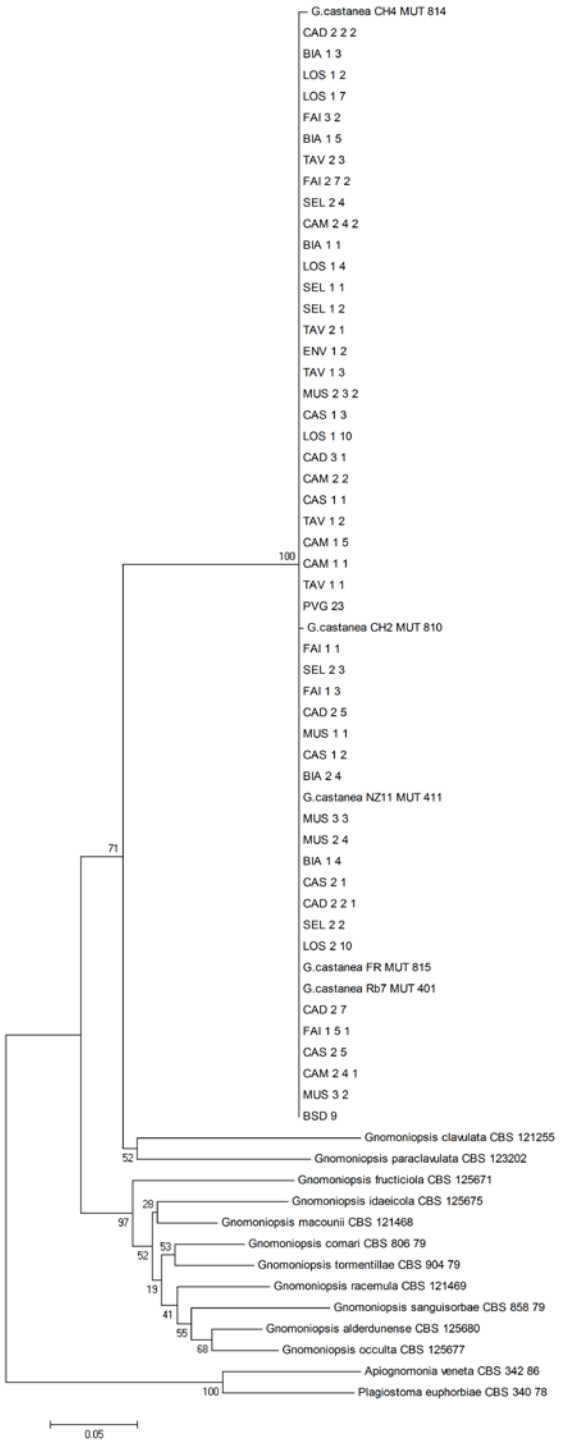

Figure 3. Neighbour-joining tree of EF-1α sequences. No differences were found between the isolates from this study and *Gnomoniopsis castanea* reference isolates. *Gnomoniopsis clavulata* and *G. paraclavulata* are confirmed to be the closest relatives of *G. castanea*.

the isolates from this study formed one single cluster. However, β-tubulin sequences were not identical, subdividing in two clusters; one main cluster containing 86% of the isolates from this study as well as the three references isolates ENV_1_2, PVG_ 23 and BSD_9, while the second cluster contained the remaining 14% of isolates. No phylogenetic tree was inferred from calmodulin sequences, since currently no calmodulin sequences for isolates from the genus *Gnomoniopsis* are available.

Knowing within species diversity is useful for understanding how a pathogen spreads to different locations and colonizes different varieties of the host plants. Therefore, haplotype networks of the isolates sequenced in this study were inferred. A combined haplotype network of the calmodulin and β-tubulin revealed the presence of five haplotypes. The most frequent haplotype, H1, was represented by 30 isolates from this study and by the three reference isolates (Figure 4). Isolates of this haplotype were the most frequent at all sampling sites except Murg/Siten, where H2 was more frequent (Figure 8). Haplotypes H4 and H5 were composed of only one isolate each. H4 differed from H3 by only one SNP of the calmodulin sequence at position 358 of the combined calmodulin and β-tubulin alignment. Positions of the SNP's differentiating haplotypes in the combined alignment of calmodulin and β-tubulin sequences are summarized in Table 4. Sequences of one isolate per haplotype have been deposited in GenBank with accession numbers KM437885 to KM437892 (Table 6, Supplementary Material).

Calmodulin sequences alone revealed the presence of three haplotypes among the collected isolates, while β-tubulin sequences alone revealed two haplotypes, corresponding to the two clusters found in the phylogenetic analysis (Figure 7, Supplementary Material).

At all sampling sites, two to three haplotypes were detected, except in Murg/Siten, where four haplotypes were present (Figure 8, Supplementary Material). Different haplotypes could be found on nuts from the same tree. No evident linkage between haplotypes and geographical location of the sampling site was found. H1 was the most frequent haplotype at all sampling sites located south of the Alps, and all previously isolated strains from Italy were of this haplotype. This suggests that H1 could be more widespread in southern regions than H2, which was the most frequently occurring haplotype

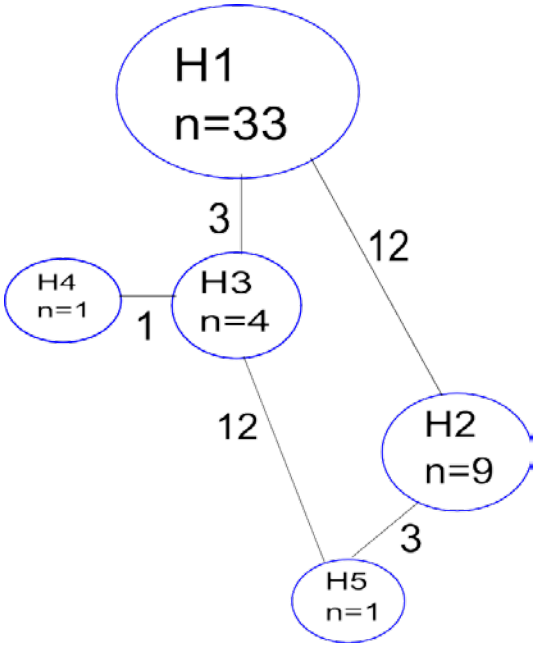

Figure 4. Haplotype network of concatenated calmodulin and β-tubulin sequences. Five haplotypes (H1 to H5) were found. Numbers in circles indicate number of isolates of each haplotype. Numbers of different nucleotides between haplotypes are indicated on the linkage lines between the haplotypes.

in Murg/Siten (Figure 8, Supplementary Material). A study including more sampling sites north of the Alps should be carried out to identify consistent distribution patterns of different haplotypes. Within species diversity of *G. castanea* has yet to be studied in other regions.

Our results indicate that *G. castanea* is present in chestnut populations under different climatic conditions, in accordance with the hypothesis that it is a ubiquitous pathogen present worldwide (Visentin *et al.*, 2012). In the present study, none of the other chestnut pathogens previously found in southern Switzerland (Sieber *et al.*, 2007) could be isolated, indicating that *G. castanea* is the dominant nut rot pathogen in this region. This could be due to the isolation method used, which is different from methods used in a previous study (Sieber *et al.*, 2007). However, the symptoms of *Phomopsis castanea* , *Phoma endogena* and *Amphiporte castanea* are very similar to those of *G. castanea* (Washington *et al.*, 1999; Conedera *et al.*, 2004a; Maresi *et al.*, 2013), and it has been speculated

Table 4. Position of SNP's differentiating haplotypes in the combined alignment of calmodulin and β-tubulin sequences. Capital letters indicate nucleotides present at the variable position for each haplotype. Positions 136 to 540 are part of the calmodulin sequence and positions 692 to 806 are part of the β-tubulin sequence. The combined alignment is 919 bp. The number of isolates for each haplotype is indicated in parenthesis. To construct the table, a total of 48 isolates was used (five isolates from each sampling site and three reference isolates).

Haplotype	Alignment position																
	136	140	150	173	248	288	358	384	468	499	519	538	540	692	793	799	806
H1 (n=33)	G	T	G	A	T	C	T	A	C	G	A	G	T	G	G	T	G
H2 (n=9)	A	C	A	G	C	A	T	G	G	A	T	A	C	G	G	T	G
H3 (n=4)	G	T	G	A	T	C	T	A	C	G	A	G	T	C	C	C	A
H4 (n=1)	G	T	G	A	T	C	C	A	C	G	A	G	T	C	C	C	A
H5 (n=1)	A	C	A	G	C	A	T	G	G	A	T	A	C	C	C	C	A
calmodulin																	
β-tubulin																	

that in the past *G. castanea* may have been mistakenly identified as one of these three species (Maresi *et al.*, 2013). Within species diversity of *G. castanea* was assessed for the first time in the present study. Further research on diversity of *G. castanea* is particularly important to establish effective prevention strategies for growers in extensive chestnut cultivation systems such as those present in southern Switzerland.

Acknowledgments

We thank Dr. Paolo Gonthier from the University of Torino, Italy, for providing reference isolates of *G. castanea*. Dr. Michele Gusberti, Dr. Mauro Jermini, Marcello Zala and David Lindenmaier provided chestnut samples from Faido, Biasca and Cadenazzo, Campascio, and Murg/Siten respectively. Dr. Stefano F.F. Torriani assisted with the software TCS. The Chestnut Grower Association from Bregaglia Valley gave useful information about traditional chestnut cultivation. We thank two anonymous reviewers for useful comments on the manuscript and Ethan Stewart for proofreading.

Literature cited

Carbone I. and L.M. Kohn, 1999. A Method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91, 553–556.

Chemikalien Risikoreduktions-Verordnung C.A., 2005. www.admin.ch/ch/d/sr/8/814.81.de.pdf.

Clement M., D. Posada and K.A. Crandall, 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9, 1657–1659.

Conedera M., M. Jermini, A. Sassella and T.N. Sieber, 2004a. Ernte, Behandlung und Konservieren von Kastaneienfrüchten. *Merkblatt für die Praxis, eidg. Forschungsanstalt WSL, Birmensdorf* 38.

Conedera M., P. Krebs, W. Tinner, M. Pradella and D. Torriani, 2004b. The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale. *Vegetation History and Arcaeobotany* 13, 161–179.

Crous P.W., B.A. Summerell, R.G. Shivas, T.I. Burgess, C.A. Decock, L.L. Dreyer, L.L. Granke, D.I. Guest, G.E. Hardy, M.K. Hausbeck, D. Huberli, T. Jung, O. Koukol, C.L. Lennox, E.C. Liew, L. Lombard, A.R. McTaggart, J.S. Pryke, F. Roets, C. Saude, L.A. Shuttleworth, M.J. Stukely, K. Vanky, B.J. Webster, S.T. Windstam and J.Z. Groenewald, 2012. Fungal Planet description sheets: 107–127. *Persoonia* 28, 138–182.

Dar M.A. and M. Rai, 2013. Biological and phylogenetic analyses, evidencing the presence of *Gnomoniopsis* sp. in India causing canker of chestnut trees: A new report. *Indian Forester* 139, 37–42.

Gentile S., D. Valentino, I. Visentin and G. Tamietti, 2010. An epidemic of *Gnomonia pascoe* on nuts of *Castanea sativa* in the Cuneo Area. *Acta Horticulturae, ISHS* 866, 363–368.

Glass N.L. and G.C. Donaldson, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, 61, 1323–1330.

Gobbin D., L. Hohl, L. Conza, M. Jermini, C. Gessler and M. Conedera, 2007. Microsatellite-based characterization of

the *Castanea sativa* cultivar heritage of southern Switzerland. *Genome / National Research Council Canada = Genome / Conseil National de Recherches Canada* 50, 1089–1103.

Krebs P., M. Conedera and P. Fonti, 2005. The Inventory of the Giant Chestnut Trees in Southern Switzerland. *Acta Horticulturae, ISHS* 693, 171–178.

Krebs P., N. Koutsias and M. Conedera, 2012. Modelling the eco-cultural niche of giant chestnut trees: new insights into land use history in southern Switzerland through distribution analysis of a living heritage. *Journal of Historical Geography* 38, 372–386.

Maresi G., C.M. Oliveira Longa and T. Turchetti, 2013. Brown rot on nuts of *Castanea sativa* Mill: an emerging disease and its causal agent. *iForest - Biogeosciences and Forestry* 6, 294–301.

O'Donnell K., H.I. Nirenberg, T. Aoki and E. Cigelnik, 2000. A Multigene phylogeny of the *Gibberella fujikuroi* species complex: Detection of additional phylogenetically distinct species. *Mycoscience* 41, 61–78.

R Core Team 2013. R: A language for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN: 3-900051-07-0, URL: http://www.R-project.org

Shuttleworth L.A., D.I. Guest and E.C. Liew, 2010. Survey of the Incidence of Chestnut Rot in South-Eastern Australia. *Acta Horticulturae, ISHS* 866, 477–482.

Shuttleworth L.A., E.C. Liew and D.I. Guest, 2013. Survey of the incidence of chestnut rot in south-eastern Australia. *Australasian Plant Pathology* 42, 63–72.

Sieber T.N., M. Jermini and M. Conedera, 2007. Effects of the Harvest Method on the Infestation of Chestnuts (*Castanea sativa*) by Insects and Moulds. *Journal of Phytopathology*, 155, 497–504.

Smith H. and D. Ogilvy, 2008. Nut rot in chestnuts. *The Australian Nutgrower* 2, 10–15.

Tamura K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24**,** 1596–1599.

Visentin I., S. Gentile, D. Valentino, P. Gonthier, G. Tamietti and F. Cardinale, 2012. *Gnomoniopsis castanea sp. nov. (Gnomoniaceae, Diapothales*) as the causal agent of nut rot in sweet chestnut. *Journal of Plant Pathology* 94**,** 411–419.

Wadia K.D.R., D. Klinac and D.L. McNeil, 1999. Survey of Chestnut nut-rot in New Zealand. *Proceeding of the 52nd New Zealand Plant Protection Conference***,** 136–140.

Walker D.M., L.A. Castlebury, A.Y. Rossman, M.V. Sogonov and J.F. White, 2010. Systematics of genus *Gnomoniopsis (Gnomoniaceae, Diaporthales)* based on a three gene phylogeny, host associations and morphology. *Mycologia* 102**,** 1479–1496.

Washington W.S., S. Stewart-Wade and V. Hood, 1999. *Phomopsis castanea*, a Seed-borne Endophyte in Chestnut Trees. *Australian Journal of Botany* 47**,** 77–84.

White T.J., N. Arnheimand and H.A. Ehrlich, 1989. The polymerase chain reaction. *Trends in Genetics* 5**,** 185–189.

WSL (1983–1985) National Forest Inventory. http://www.lfi.ch/resultate/daten/trees/kastanie1.php.

Accepted for publication: November 24, 2014
Published online: July 10, 2015

Supplementary Material

Table 5. Percent of infected and diseased nuts for each sample

Sample	Infected (%)	Diseased (%)
Losone1	76	10
Losone2	87	0
Losone3	86	3
Selna1	87	7
Selna2	28	3
Selna3	40	13
Castasegna1	70	0
Castasegna2	20	10
Castasegna3	10	7
Faido1	43	17
Faido2	33	0
Faido3	17	0
Campascio1	23	3
Campascio2	37	3
Campascio3	47	3
Taverne1	93	14
Taverne2	100	47
Taverne3	67	3
Murg1	100	0
Murg2	90	7
Murg3	83	7
Cadenazzo1	60	0
Cadenazzo2	83	17
Cadenazzo3	97	13
Biasca1	77	4
Biasca2	67	7

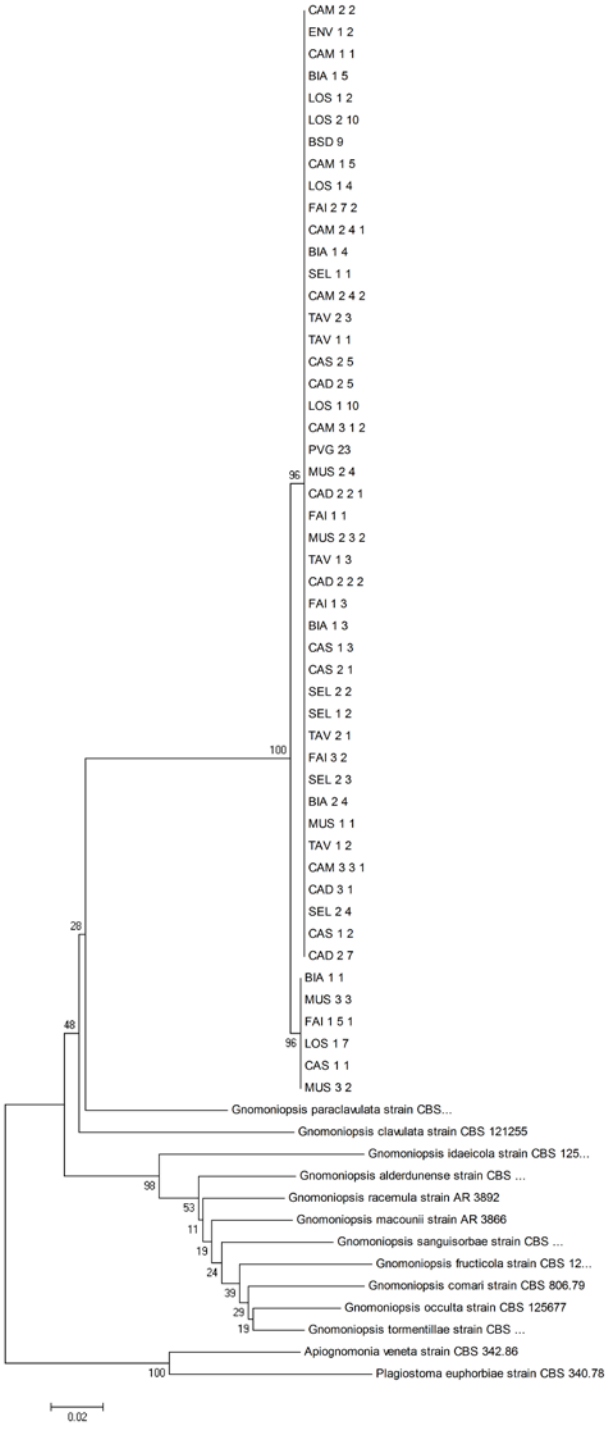

Figure 5. Neighbour-joining tree of β-tubulin sequences.

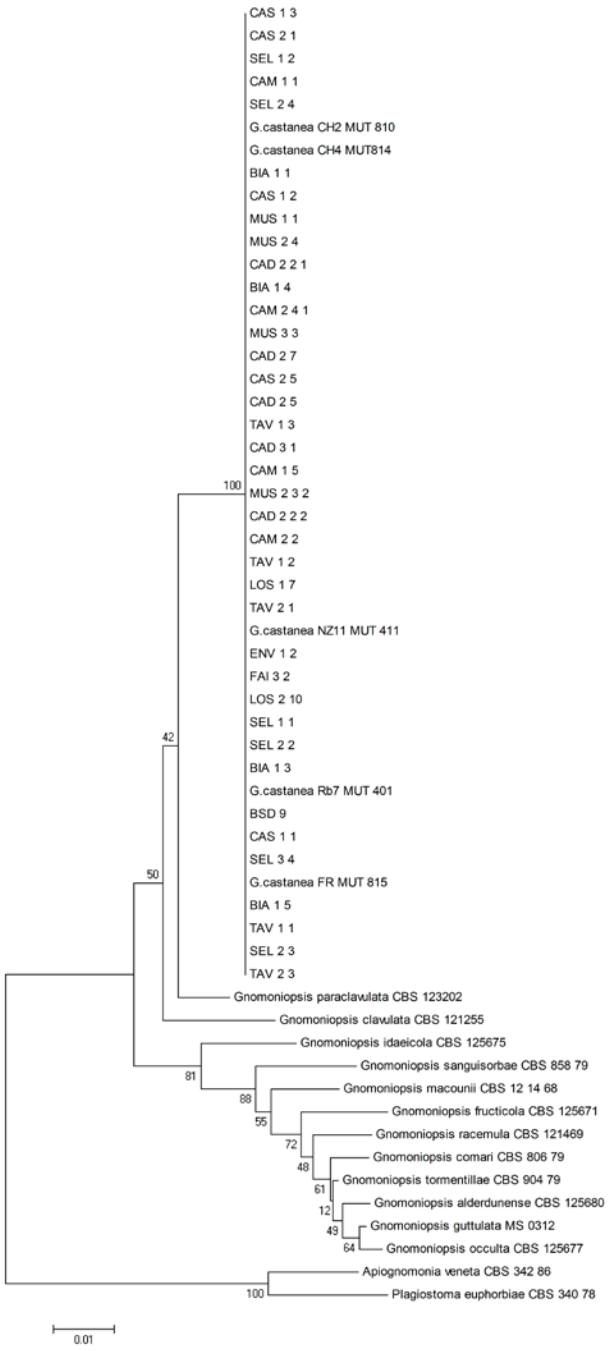

Figure 6. Neighbour-joining tree of ITS sequences.

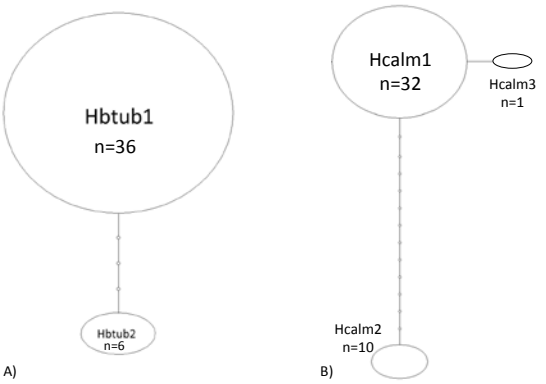

Figure 7. Haplotype networks of A) β-tubulin sequences and B) calmodulin sequences. Five isolates from each sampling site and three reference isolates were used to construct the networks.

Table 6. GenBank accession numbers of deposited sequences. At least one sequence for each β-tubulin and calmodulin haplotype was deposited.

Isolate	Locus	GenBank accession number
BIA_1_1	β-tubulin	KM437889
CAS_1_1	β-tubulin	KM437890
TAV_1_1	β-tubulin	KM437888
FAI_1_1	Calmodulin	KM437885
MUS_1_1	Calmodulin	KM437887
TAV_1_1	Calmodulin	KM437886
TAV_1_1	EF-1 alpha	KM437891
TAV_1_1	ITS1	KM437892

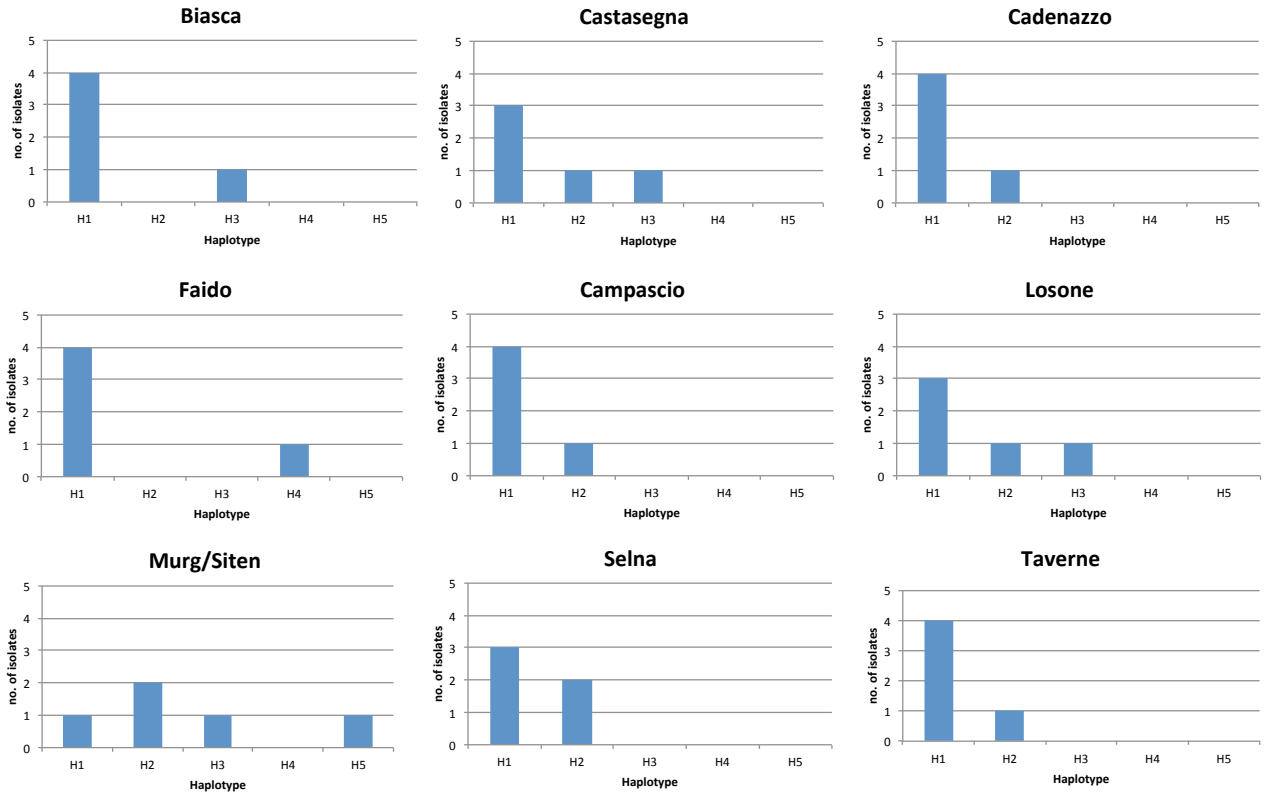

Figure 8. Frequencies of haplotypes obtained with the concatenated calmodulin and β-tubulin sequences at each sampling site. Haplotype H1 was the most frequent at all sampling sites except in Murg/Siten.