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Research Article

RESEARCH ARTICLE

Interaction between two invasive organisms on the European chestnut: does the chestnut blight fungus benefit from the presence of the gall wasp?

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One sentence summary: The ecological interaction between two of the most important invasive chestnut pathogens, the fungus *Cryphonectria parasitica* and the gall wasp *Dryocosmus kuriphilus*.

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ABSTRACT

The impact of invasive fungal pathogens and pests on trees is often studied individually, thereby omitting possible interactions. In this study the ecological interaction between the chestnut blight fungus *Cryphonectria parasitica* and the chestnut gall wasp *Dryocosmus kuriphilus* was investigated. We determined if abandoned galls could be colonized by *C. parasitica* and thereby act as an entry point and a source of pathogen inoculum. Moreover we assessed the identity and diversity of other gall-colonizing fungal species. A total of 1973 galls were randomly sampled from 200 chestnut trees in eight Swiss stands. In a stand *C. parasitica* was isolated from 0.4–19.2% of the galls. The incidence of *C. parasitica* on the galls and the fungal diversity significantly increased with the residence time of *D. kuriphilus* in a stand. All but one *C. parasitica* cultures were virulent. The predominant fungus isolated from galls was *Gnomoniopsis castanea* whose abundance influenced negatively that of *C. parasitica*. This study shows that *D. kuriphilus* galls can be colonized by virulent strains of the chestnut blight fungus *C. parasitica*. This can have effects on the chestnut blight incidence even in chestnut stands where the disease is successfully controlled by hypovirulence. The gall wasp presence influences also the fungal species composition on chestnut trees.

Keywords: biological invasions; *Castanea sativa*; diversity; fungal community; gall-inducing insect; *Gnomoniopsis castanea*; interactions

INTRODUCTION

During their lifespans trees may simultaneously face multiple biotic and abiotic stresses, including infections by different pathogens and pests. The intensification of global trade exposes trees to new pathogenic organisms with which they do not naturally coexist, and thus, against which they have never evolved defensive systems (Anderson *et al.* 2004; Wingfield, Slippers and Wingfield 2010; Hulcr and Dunn 2011). Distribution ranges of species may also change because of changing climatic conditions (Dukes *et al.* 2009). Exotic pathogens and pests in new environments may become invasive and have a dramatic impact on their new host trees. While predicting their invasiveness is crucial in order to limit damage, this is a difficult task. For example, traits which might favor the spread of fungal pathogens include long-term dispersal (aerial pathogens), sexual reproduction and suitable temperature (Philibert *et al.* 2011). Unfortunately, the ecological impact of each invasive organism is often analysed separately, thereby overlooking possible interactions and joint

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effects, which may considerably hinder the determination of the real ecological consequences. Interactions among pathogenic organisms are specific to the tripartite insect–fungus–tree system (Hatcher 1995; Rostás, Bennett and Hilker 2002) and can be extremely complex, making their study a difficult task.

In this study the interaction between the chestnut blight fungus *Cryphonectria parasitica* (Murill) Barr and the chestnut gall wasp *Dryocosmus kuriphilus* (Yasumatsu) (Hymenoptera, Cynipidae) on the European chestnut *Castanea sativa* (Mill.) was investigated. *C. parasitica* (Ascomycota) is a wound parasite that causes lethal lesions on the bark of chestnut species (Prospero and Rigling 2013). The fungus is native to Asia and was introduced to Europe at the end of the 1930s. The European chestnut is highly susceptible to the pathogen and severe tree mortality was observed at the beginning of the epidemic. However, in most European chestnut regions, the disease was successively contained by the appearance and natural spread in the pathogen's population of a hypovirus (*Cryphonectria hypovirus-1*, CHV-1) that reduces fungal virulence and sporulation (Prospero and Rigling 2013). In Switzerland, *C. parasitica* was first reported in 1948, south of the Alps (Ticino), and about 40 years later, north of the Alps (Heiniger and Stadler 1990). In Ticino, natural hypovirulence is currently widespread and successfully controls the disease (Heiniger and Rigling 1994). In contrast, in northern Switzerland no natural hypovirulence has appeared and the hypovirus has been artificially introduced into the main chestnut stands (Heiniger and Rigling 2009). *Dryocosmus kuriphilus* represents the most significant pest for the genus *Castanea* worldwide. The female of the wasp lays eggs from June to August into newly formed buds (Forster *et al.* 2009) and in the spring, wasp larvae stimulate plant tissue to produce galls on young twigs, leaf petioles or leaf midribs. In summer, adult wasps abandon the galls and perform oviposition on the new buds. After adult emergence, the abandoned galls become necrotic and remain attached to trees until decay. Chestnut plant organs carrying galls cannot develop properly and are weakened, which leads to a reduction in leaf and fruit production. Originally coming from China, *D. kuriphilus* has become a major issue in Europe since its first observation in 2002 in Italy (Brussino *et al.* 2002; Graziosi and Santi 2008). In 2009, the pest was found for the first time in Ticino, most likely as a result of invasion from nearby Italy (Forster *et al.* 2009). North of the Swiss Alps, *D. kuriphilus* appeared in 2011.

Recently, an unusual twig dieback caused by *C. parasitica* has been observed in the crown of chestnut trees heavily affected by *D. kuriphilus* in Ticino (Prospero and Forster 2011). It appears that young twigs formed in the current or previous year carrying abandoned galls are more likely to have chestnut blight symptoms (i.e. bark cankers, presence of stromata; Fig. 1) than twigs without galls. Such lethal twig cankers may be caused by endophytic *C. parasitica* strains present in the bark tissue (Bissegger and Sieber 1994) after weakening of the twigs by a massive infestation of the chestnut gall wasp. Alternatively, the chestnut blight fungus may be able to colonize the moribund tissue of abandoned *D. kuriphilus* galls and potentially spread to the adjacent twig, inducing the formation of cankers. Consequently, abandoned galls could act as an entry point and a source of inoculum for *C. parasitica*. In this study, we aimed at determining (i) the incidence of *C. parasitica* on abandoned *D. kuriphilus* galls, (ii) whether the galls are preferentially colonized by virulent or hypovirulent strains of *C. parasitica* and whether these strains are local, and (iii) the fungal community colonizing the abandoned galls.

Figure 1. Young chestnut twig with abandoned *Dryocosmus kuriphilus* galls. *Cryphonectria parasitica* infection is shown by reddish lesions and orange stromata (Picture: Phytopathology, WSL).

MATERIALS AND METHODS

Selected chestnut stands

The study was conducted in eight chestnut stands located in southern (Ticino: Stabio, Mugena, Robasacco and Biasca) and in western Switzerland (Chablais: Bex-Creux, Bex-Montet, Monthey and Choëx) (Table 1). Local climate in Ticino is characterized by mean annual precipitation of 1600–1700 mm, half of which occurs during summer, and mean annual temperatures of 10–12°C (Spinedi and Isotta 2004). In the Chablais, a similar precipitation pattern is observed (Pythoud 2007), but both mean annual precipitations (1000–1300 mm) and mean annual temperatures (about 9°C) are lower than in Ticino. Chestnut stands in the Chablais are small and scattered. In contrast, in Ticino, chestnut is the dominant tree species up to 900 m a.s.l. where it forms a continuous forest belt of about 30 000 ha. Six of the stands selected were orchards (>30-year-old chestnut trees), whereas the remaining two, in Bex-Creux and Bex-Montet, were coppice forests.

Chestnut blight in Ticino was first observed in 1948 (Heiniger and Rigling 1994). Healing cankers were reported starting in 1975, and natural hypovirulence is well established in all chestnut stands. In the Chablais, the disease appeared at the end of the 1980s (Heiniger, Graf and Rigling 2007) and hypovirulence has been artificially introduced since 2003 (Heiniger and Rigling 2009). In the Chablais, genetic diversity of *C. parasitica* (vegetative compatibility types and microsatellite genotypes) is low, with only a few types in each stand (Hoegger *et al.* 2000; Heiniger, Graf and Rigling 2007; Prospero and Rigling 2012). In Ticino, diversity is higher and shows similar levels to those observed in northern Italy (Robin and Heiniger 2001). The invasive chestnut gall wasp was first reported in the Chablais in 2011 (Meier *et al.* 2012). In Ticino, *D. kuriphilus* was first reported in 2009 near the Italian southern border (Forster *et al.* 2009). The four chestnut stands sampled in this study were infested in different years owing to their geographic location (Stabio in 2009, Mugena and Robasacco in 2010, and Biasca in 2012; Table 1).

Table 1. Incidence of the chestnut blight fungus *Cryphonectria parasitica* on abandoned galls of *Dryokosmus kuriphilus*.

Chestnut stand	Region	Coordinates (WGS 1984)	First *D. kuriphilus* appearance	Trees with *C.p.* on galls[a]	Abandoned galls sampled	Abandoned galls with *C.p.* (%)[b]	Hypovirulent *C. parasitica* (%)		Fungal community	
							Galls	Cankers	Mean diversity (SD)	Mean richness (SD)
Biasca	Ticino	46.353812, 8.976810	2012	5/5	254	26 (10.2)	0.0	57.9	1.1 (0.3)	7.0 (2.5)
Robasacco	Ticino	46.142016, 8.944519	2010	5/5	261	50 (19.2)	0.4	55.0	1.5 (0.2)	8.0 (1.4)
Mugena	Ticino	46.050261, 8.885494	2010	5/5	260	9 (3.5)	0.0	30.0	1.2 (0.3)	8.0 (1.2)
Stabio	Ticino	45.846565, 8.920165	2009	5/5	253	42 (16.6)	0.0	36.8	1.8 (0.3)	8.8 (1.8)
Subtotal Ticino				20/20	1028	127 (12.4)	0.1	44.9	1.4 (0.3)	7.0 (1.7)
Bex-Creux	Chablais	46.239145, 7.012009	2011	1/5	252	1 (0.40)	0.0	31.6	1.0 (0.3)	7.6 (1.5)
Bex-Montet	Chablais	46.257859, 7.014974	2011	2/5	196	5 (2.6)	0.0	17.6	0.5 (0.3)	3.8 (1.6)
Choëx	Chablais	46.241162, 6.971451	2011	2/5	252	3 (1.2)	0.0	47.4	0.8 (0.2)	5.4 (1.1)
Monthey	Chablais	46.258282, 6.940308	2011	2/5	245	6 (2.4)	0.0	85.0	0.7 (0.5)	5.0 (2.9)
Subtotal Chablais				7/20	945	15 (1.6)	0.0	46.7	0.8 (0.3)	5.4 (1.8)

[a]Number of trees with *C. parasitica* infected galls/number of sampled trees per chestnut stand.
[b]Galls from which *C. parasitica* was isolated.

Gall and canker sampling

In the summer of 2014, five chestnut trees per stand at a distance between each other of at least 5 m were randomly selected for gall collection. The galls in Bex-Creux and Bex-Montet and in the stands in Ticino were collected at the end of June, the galls in Monthey and Choëx in July. From the lower tree branches (0.5–2.5 m from the ground), we collected approximately 50 abandoned galls, i.e. necrotic galls from which *D. kuriphilus* already emerged. As *D. kuriphilus* emerges from the galls from mid-June to mid-July (Forster *et al.* 2009), the collected galls were mostly formed in 2013, but might include also some specimens from 2014 or from previous years. In the same chestnut stands we also sampled 20 bark cankers from 20 trees including the same trees from which we collected the galls. Bark samples were removed with a fine bone marrow biopsy needle (Jamshidi gauge, 2 mm diam; Baxter, Deerfield, IL, USA) from the upper, central and lower part of each canker.

Isolation and cultivation of fungal colonies

Galls and canker bark pieces were dipped in 70% ethanol, immediately flamed for 1–2 s and placed on water agar (PPA, Pronadisa Lab. Conda, Madrid, Spain) (Bissegger, Rigling and Heiniger 1997). Larger galls (>10 mm in diameter) were cut into two halves. After 1 week of incubation in the dark at room temperature (RT), growing hyphae were transferred from the colonies onto potato dextrose agar (PDA; Difco, Voight Global Distribution, Lawrence, MD, USA) contained in Petri dishes and incubated for 1 week in the dark, and then for 2 weeks under light at RT. Thereafter, fungal colonies were grouped into morphotypes based on their size and pigmentation. Pictures of the isolated fungal genera were taken after cultivating the fungi for 19 days on PDA plates sealed with Parafilm, at 25°C, either under complete dark or light conditions (14 h light–10 h dark photoperiod, Illuminance Meter, Minolta, Japan) (Supplementary Fig. S1).

Assessment of hypovirus-infection and vegetative compatibility (VC) type of *C. parasitica*

Hypovirus-infected isolates were identified by their white culture morphology (Bissegger, Rigling and Heiniger 1997). The culture morphology of *C. parasitica* strains on PDA was determined after incubation of the plates in the dark at RT for 7 days followed by an additional incubation under daylight for another 7 days at RT. The VC type of the *C. parasitica* cultures was assessed according to the merging/barrage response (Anagnostakis 1988; Bissegger, Rigling and Heiniger 1997). Cultures were paired on PDA with a tester strain of the most common VC types in Switzerland (EU-1, EU-2, EU-5, EU-6; Robin and Heiniger 2001). Cultures that did not belong to one of these VC types were paired with tester strains of other VC types known to occur in Switzerland (Hoegger *et al.* 2000; Bryner and Rigling 2012). Cultures that again resulted in a negative response were not investigated further.

Species identification

For species identification, at least three specimens, when available, for each morphotype were analysed. Total DNA was extracted from 10–20 mg lyophilized mycelium with the Plant DNeasy Mini kit (Qiagen, Hombrechtikon, Switzerland). Subsequently, the nuclear ribosomal internal transcribed spacer (ITS) region was amplified using the forward primer ITS1 and the reverse primer ITS4 as explained in White *et al.* (1990). Before

sequencing, PCR products were purified using Exostar (Exo Star 1-Step Clean up Kit, Thermo Fisher Scientific, Waltham, MA, USA). For the cycle sequencing, the purified PCR product was diluted to achieve approximately 5 μg/ml. Of this dilution, 3 μl was mixed with 4.5 μl master mix containing 0.75 μl BigDye Sequencing Buffer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Foster City, CA), 1.5 μl ITS 1 forward 10 μl or ITS 4 reverse 10 μl primers, 1.5 μl Read Reaction Premix (BigDye, 2.5×) and 0.75 μl PCR water. Sequencing reactions were performed for 1 min at 96°C initial denaturation, 25 cycles of 10 s at 95°C denaturation, 5 s at 50°C annealing and 1 min at 60°C extension. All cycle sequencing products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems) following the manufacturer's instructions. Sequences were run on an 3130xl DNA Analyser (Applied Biosystems) and edited using the Sequencher package (Gene Codes, Ann Arbor, MI, USA). For species identification, sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Two sequences with a similarity of at least 99% were considered to belong to the same species. Unsequenced isolates were assigned to a species based on their morphological similarity to sequenced cultures.

Statistical analyses

To better understand the presence of *C. parasitica* on the abandoned galls, we tested (1) whether the local abundance of *C. parasitica* varied significantly between the two invaded regions (Ticino vs Chablais) and (2) whether differences in abundance were related to the residence time of the co-invasive pest *D. kuriphilus* (i.e. years since the first official record). Additionally, we tested (3) whether the invaded region and appearance year of the gall wasp also influenced overall fungal community diversity, and (4) whether the most abundant fungal genera affect the presence of *C. parasitica*.

A fungal community on individual chestnut trees was defined by the relative abundance of the fungi isolated from the galls collected from this tree. From the larger-sized galls that were bisected (307 out of 1973 galls, i.e. 16%), 197 (10%) yielded the same fungal isolate per halved gall, and 110 (6%) yielded two different cultures. For all fungi and for the diversity analyses, only one randomly chosen fungus from one gall-half was used. For the total count of *C. parasitica*, when two different fungi were recovered per gall-half, the half with *C. parasitica* was used for analysis (33 isolates out of 110 halved galls, 2% of all galls analysed). Each fungal community was characterized by its richness (i.e. the number of identified fungal species), and its diversity estimated with the Shannon diversity index. This index measures the evenness of different species' abundances, and is estimated as $H' = -\sum_{i=1}^{S} p_i \log(p_i)$, where p_i is the relative abundance of species i, and S is the total number of species in the community (Magurran 2004).

To test whether the local abundance of *C. parasitica* significantly differed among invaded regions, we used a mixed-effects regression model as implemented in the R software package *lme4* (Bates *et al.* 2014). With this model, it is possible to account for the nested structure of the sampling design, where, within each region (Ticino and Chablais, considered as fixed effects), five sites were visited and within each site five trees were sampled (nested random effects). The same test was also used to verify whether the native fungal community richness and diversity significantly differed between the two invaded regions.

The correlation between the local abundance of *C. parasitica* and the residence time of *D. kuriphilus* (as estimated by the year of first detection of the species) was tested using linear regression. In this case, we did not use a mixed-effects model since the year of arrival of *D. kuriphilus* (i.e. the fixed effect we wanted to test) varied among the sampled sites. We also used linear regression to test whether the diversity of the fungal communities changed with the residence time of *D. kuriphilus*.

Finally, to estimate the influence of the seven most abundant fungal genera (i.e. *Gnomoniopsis*, *Fusarium*, *Trichoderma*, *Sordaria*-like, *Botryosphaeria*, *Colletotrichum* and *Alternaria*, see Supplementary Fig. S1) on the presence of *C. parasitica*, we calculated the correlation between their local relative abundances. Since the abundance distributions of the genera were non-normal, we used the non-parametric Spearman rank correlation coefficient. We also tested these relationships using linear regressions with quadratic forms of the response variables. However, for most genera (except *Colletotrichum, Gnomoniopsis* and *Trichoderma*), error distributions were not normally distributed, thus violating the model assumption and consequently limiting the reliability of the models.

To visualize the relative influence of fungal species on the community structure, and how this changed between sampled sites, we performed a between-stand principal component analysis (hereafter called 'between-PCA'; Dolédec and Chessel (1991). A between-PCA is similar to a traditional PCA, but it uses the correlation matrix based on sites' means (weighted by their sample sizes) to find linear combinations of variables that maximize the between-site variance instead of overall variance. The proportion of variance explained by each axis of the between-PCA represents the part of total variance due to differences between sites. In order to remove the overwhelming influence of rare species (i.e. the many zeros contained in the abundance matrix), we corrected the relative abundance of species by performing a Hellinger transformation, as suggested in Legendre and Gallagher (2001).

RESULTS

Incidence of *C. parasitica* on galls

Fungal cultures were recovered from 1869 out of 1973 (94.7%) analysed abandoned galls. A total of 48 fungal colonies were contaminated by bacteria and could not be analysed further, whereas 56 galls yielded no microbial cultures. *C. parasitica* was isolated from 142 out of 1973 (7.2%) galls. The majority of the *C. parasitica*-infected galls (127, 89.4%) originated from the four chestnut stands in Ticino (Table 1), where the chestnut blight fungus colonized significantly ($P < 0.001$) more galls than in the Chablais (15, 10.6%) (Fig. 2, Table 1). In Ticino, all 20 analysed chestnut trees carried at least one gall that was colonized by *C. parasitica* (Table 1). Chestnut blight infection in galls in individual stands ranged from 3.5% (Mugena) to 19.2% (Robasacco). In Chablais, only 7 out of 20 analysed chestnut trees (i.e. one to two trees per stand) carried galls with *C. parasitica*. Gall colonization ranged in individual stands from 0.4% (Bex-Creux) to 2.6% (Bex-Montet) (Table 1). Linear regression analysis showed that the abundance of *C. parasitica* on the galls significantly correlated with the year of first appearance of *D. kuriphilus* in the chestnut stand (Fig. 2). The longer the gall wasp had been present in the stand, the higher the *C. parasitica*'s abundance on the galls ($P = 0.014$, $R^2 = 0.15$).

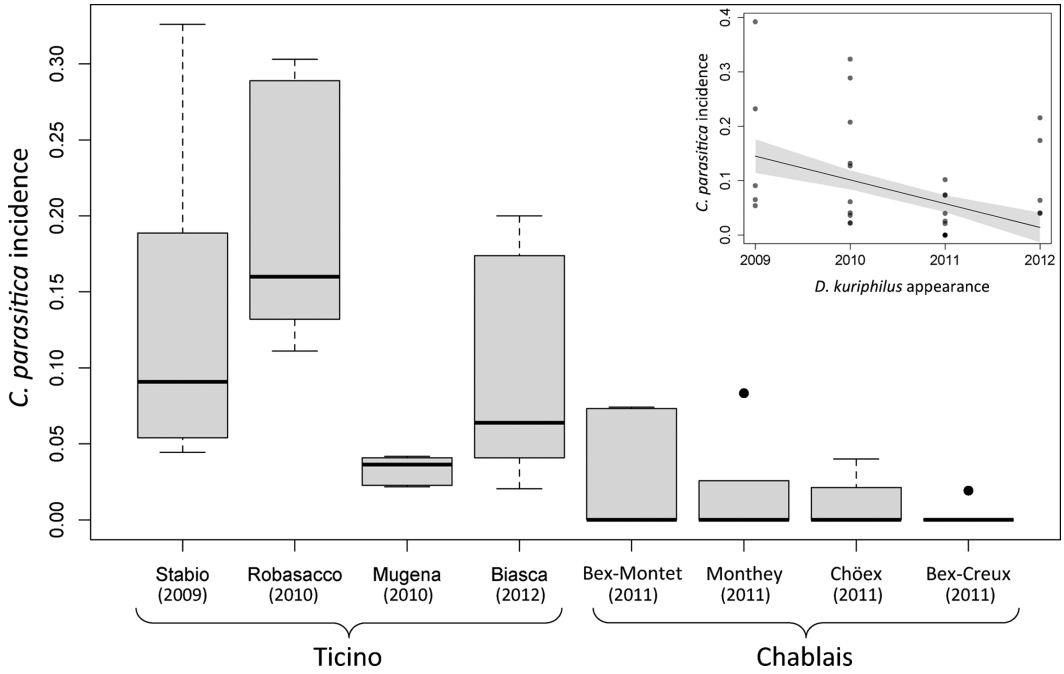

Figure 2. Relative abundance of *Cryphonectria parasitica* on abandoned galls in eight Swiss chestnut stands. In the main panel, the distribution of relative abundance of *C. parasitica* is shown in boxplots, where bold lines indicate the median abundance, box edges indicate quartiles, and whiskers indicate the 0.01 and 0.99 quantiles. In brackets under each site, the year of appearance of the gall wasp *Dryocosmus kuriphilus* is indicated. The upper-right panel shows the relationship between the relative abundance of *C. parasitica* and *D. kuriphilus* residence time, with a black regression line, and gray shade indicating confidence interval around the fitted values.

Hypovirus-infection and VC type of *C. parasitica* on galls and in cankers

Cryphonectria parasitica was obtained as pure culture from 135 out of the 140 colonized galls. Only one *C. parasitica* isolate recovered from a gall collected in Robasacco (Ticino) showed the typical morphological characteristics of a hypovirus-infected culture (i.e. white pigmentation and absence of sporulation). The remaining 134 isolates were orange and sporulated, indicating no hypovirus infection (Table 1). Hypovirus-infected cankers on chestnut trees were detected in all stands, with an incidence ranging from 30% in Mugena (Ticino) to 85% in Monthey (Chablais). The isolation success of *C. parasitica* from cankers in each chestnut stand was high (93–100%).

Among the 135 *C. parasitica* isolates recovered from the galls, a total of 21 VC types were identified. The four most frequent types in Switzerland, EU-1, EU-2, EU-5 and EU-6, accounted for 57.8% of the isolates (Table 2). The other VC types included one to eight isolates each, and were frequently specific to one or more sites. Thirteen isolates did not belong to one of the VC types known to occur in Switzerland. Per site, from five to 15 VC types were detected on galls in Ticino and from two to five in the Chablais. The 139 *C. parasitica* isolates obtained from the cankers belonged to 14 different VC types (Table 2). A total of 89.2% of them were either EU-1, EU-2, EU-5, or EU-6. In all four chestnut stands in the Ticino, VC type diversity of *C. parasitica* was higher on the galls than in the cankers (Table 2). The opposite situation was observed in the Chablais, where, however, only 15 *C. parasitica* isolates were recovered from the galls.

Other gall colonizing fungi

A total of 1746 fungal colonies obtained from galls other than *C. parasitica* were identified. The cultures were first grouped into morphotypes based on their appearance in cultures. For each morphotype, a subset of 1–10 cultures was then randomly chosen for ITS sequencing. Therewith, it was possible to taxonomically identify a total of 1702 fungal cultures (Supplementary Table S1). The remaining 44 cultures (25 from Ticino and 19 from Chablais) were not assigned to species because they were mixed cultures. *Gnomoniopsis* (Diaporthales, Ascomycota) was by far the most frequently isolated genus from the galls in seven out of eight chestnut stands. About 95% of the *Gnomoniopsis* isolates belonged to the species *G. castanea* (synonym *Amphiporte castanea* and *G. smithogilyi*, Supplementary Fig. S1). In total, 53.8% of the sampled galls (or 60.8% of the total fungal isolates) were colonized by this species (Fig. 3). In the Chablais, *G. castanea* was recovered from 60.3% (Bex-Creux) to 74.6% (Choëx) galls per chestnut stand and in Ticino from 10.7% (Stabio) to 61.1% (Mugena). A second, as yet officially undescribed *Gnomoniopsis* species (i.e. *Gnomoniopsis* sp. ICMP 14082; GenBank accession number KC145849.1, Supplementary Fig. S1) was also isolated from the galls, but at a significantly lower incidence (<1% of galls in Ticino), except in three chestnut stands in the Chablais (Monthey: 4.9%, Bex-Montet: 17.3% and Bex-Creux: 6.7%). A Spearman rank correlation test showed that the abundance of *Gnomoniopsis* spp. negatively correlated with that of *C. parasitica* (correlation coefficient = −0.61) (Fig. 5).

All other fungal species isolated from the galls occurred at very low frequencies (<1.1% for each species). Therefore, for statistical analyses we pooled these species according to their genera. Among morphotypes that we visually associated with the genus *Sordaria*, sequence analysis showed the presence of six isolates belonging to other genera of the order Sordariales. For this reason, we preferred to create the category *Sordaria*-like, which included the *Sordaria* species (>95% of the morphotypes) and the three additional genera (Supplementary Table S1). Besides *C. parasitica* and the two *Gnomoniopsis* species, the other fungal genera present on at least 3% of the analysed galls

Table 2. *Cryphonectria parasitica* VC-type in cankers and galls.

		Biasca (TI[a])	Mugena (TI)	Robasacco (TI)	Stabio (TI)	Total Ticino	Bex-Montet (VD[a])	Bex-Creux (VD)	Choëx (VS[a])	Monthey (VS)	Total Chablais	Total
EU-1[b]	C[c]	3	5	6	1	15	8	17	14	4	43	58
	G[c]	1	1	10	6	18		2	3	2	7	25
EU-2[b]	C	8	7	7	5	27		2	2	13	17	44
	G	14	2	10	4	30				4	4	34
EU-3	C				1	1						1
	G											
EU-4	C	1				1						1
	G				2	2						2
EU-5[b]	C	3	2	2	1	8	9		3	1	13	21
	G	4	1	1	3	9	5				5	14
EU-6[b]	C									1	1	1
	G			4	1	5						5
EU-7	C											
	G				1	1						1
EU-8	C											
	G				3	3						3
EU-9	C											
	G			7	1	8						8
EU-12	C			1		1						1
	G	1			2	3						3
EU-13	C		2			2						2
	G	2		4	1	7						7
EU-14	C			1		1						1
	G	2			3	5						5
EU-17	C											
	G		1			1						1
EU-18	C											
	G			1		1						1
EU-19	C				1	1						1
	G				1	1						1
EU-22	C				4	4						4
	G		1		1	2						2
EU-23	C											
	G			2		2						2
EU-24	C											
	G			1		1						1
EU-25	C			1		1						1
	G			1		1						1
EU-26	C											
	G			1	3	4						4
EU-27	C				1	1						1
	G											
EU-30	C											
	G			1		1						1
EU-31	C				1	1						1
	G				1	1						1
EU-65	C									1	1	1
	G											
nd[d]	C											
	G	2	1	7	3	13						13
N[e]	C	15	16	18	15	64	17	19	19	20	75	139
	G	26	7	50	36	119	5	1	3	6	15	135
S[f]	C	1.2	1.2	1.5	1.8	1.8	0.7	0.3	0.8	1.0	1.1	1.5
	G	1.3	1.6	2.1	2.5	2.4	0.0	0.0	0.0	0.6	0.4	2.4
	C+G	2.5	2.8	3.5	4.3	4.2	0.7	0.3	0.8	1.7	1.5	3.8

[a]TI, Ticino; VD, Vaud; VS, Valais.
[b]Predominant VC-type in Switzerland.
[c]C, cankers; G, galls.
[d]nd, not determined.
[e]Number of *C. parasitica* isolates.
[f]Shannon diversity index.

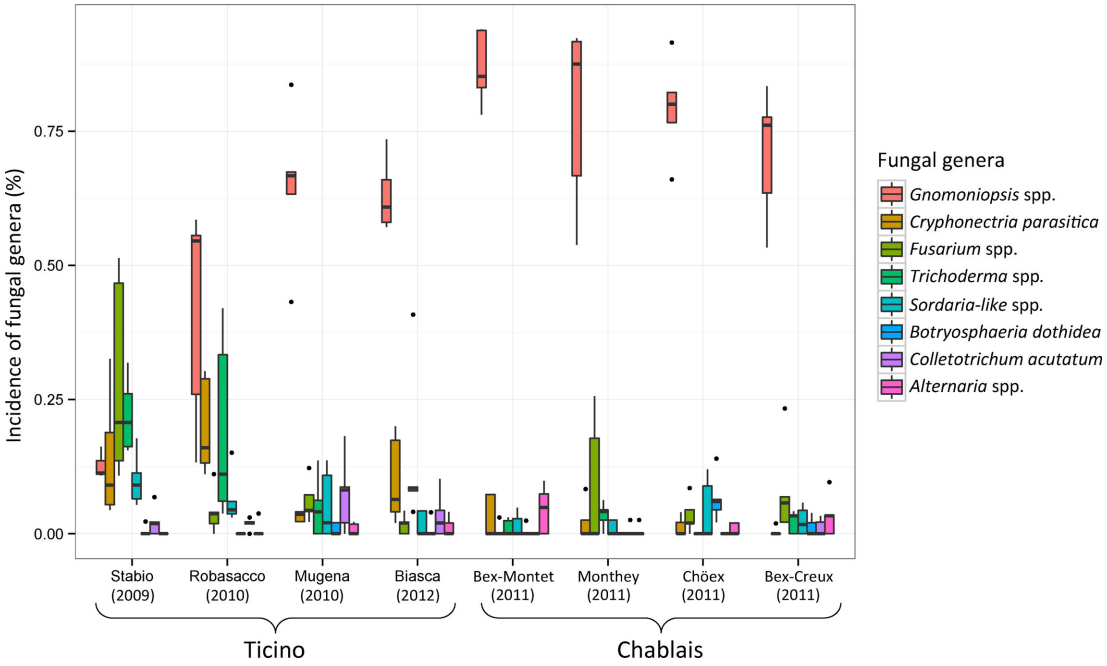

Figure 3. Relative abundance of *Cryphonectria parasitica* and the seven most abundant fungal genera on abandoned galls. In the boxplots, bold lines indicate the median abundance, box edges indicate the quartiles, and the whiskers indicate the 0.01 and 0.99 quantiles.

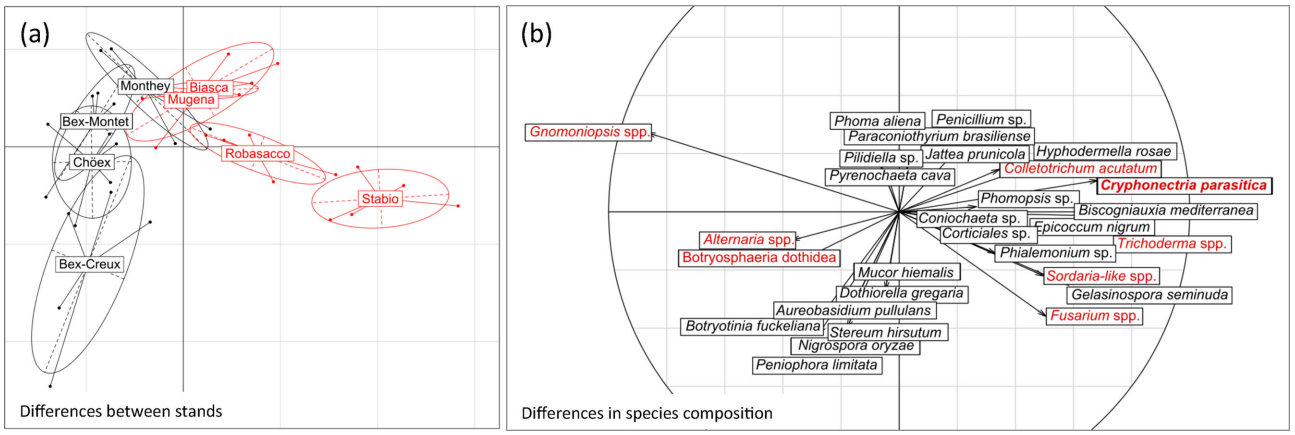

Figure 4. BetweenPCA analysis showing the differences in the fungal community composition on abandoned galls among eight Swiss chestnut stands. (a) The relative abundances of species (for details, see Materials and Methods) are used to distinguish between the different stands. Stands from Ticino are in red from the Chablais region in black. (b) in the panel is shown the contribution of each species to site differences. The most abundant fungal genera/species are marked in red.

were *Trichoderma*, *Fusarium*, *Sordaria*-like (*Sordaria* spp., *Gelasinospora seminuda* and two isolates having the closest match with uncultured Sordariales that are closely related to *Trichocladium asperum* and *Phialemonium* sp.), *Colletotrichum*, *Alternaria* and *Botryosphaeria* (Supplementary Table S1 and Supplementary Fig. S1).

Factors influencing fungal diversity on galls

The region where the chestnut stand is located and the year of first appearance of *D. kuriphilus* in the stand significantly affected the composition of the fungal community on the galls. Based on a mixed-effects regression analysis, communities of gall-associated fungi in Ticino were significantly richer (7.0 vs 5.4 species, $P < 0.007$) and more diverse (H′: 1.4 vs 0.75, $P < 0.001$) than in the Chablais (Table 1). Overall, the fungal diversity in galls significantly increased with the residence time of the gall wasp ($P = 0.011$, $R^2 = 0.31$).

The differences in fungal community composition between the two regions, and the different sites can also be visualized in Fig. 4. The four sites in Ticino were mainly distinguished by the first principal component of the between-PCA (43% of inter-site variance) from those in Chablais (Fig. 4a). These differences were characterized by the higher abundance of *C. parasitica*, *Colletotrichum acutatum*, *Fusarium* spp., *Sordaria*-like spp., and *Trichoderma* spp. in Ticino, while *Alternaria* spp. and *Botryosphaeria dothidea* were more abundant in the Chablais (Fig. 4b).

Influence of other fungi on *C. parasitica*

The abundance of *C. parasitica* on the abandoned galls significantly increased with increasing overall fungal diversity ($P < 0.005$, $R^2 = 0.22$). A Spearman rank correlation test showed that

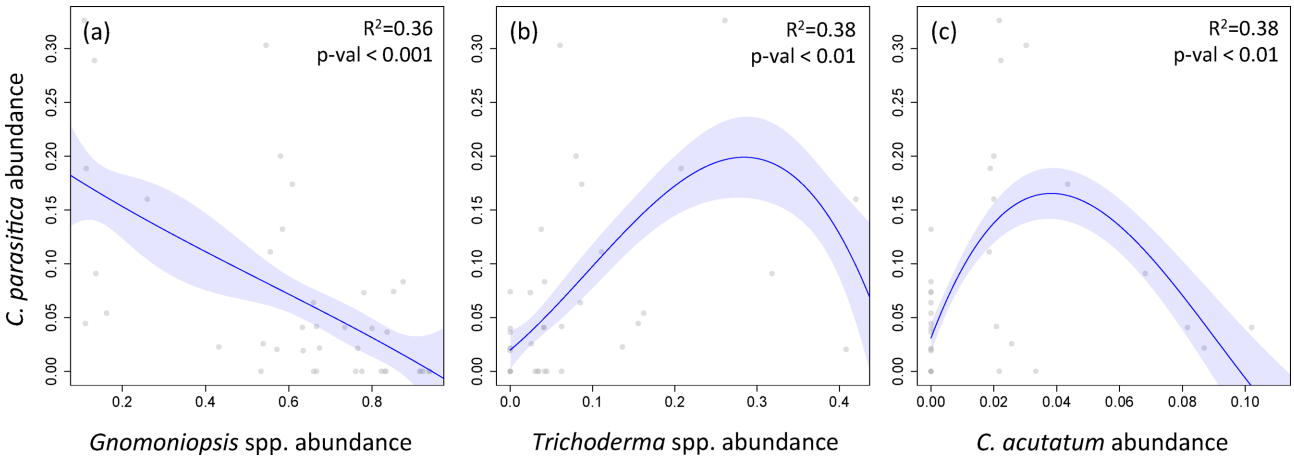

Figure 5. Relationships between the relative abundances of *Cryphonectria parasitica* on abandoned galls and the incidence of (a) Gnomoniopsis spp., (b) Trichoderma spp. and (c) *Colletotrichum acutatum*. The different panels show, for each species, the observed abundance values (grey dots), the values fitted by the linear (a) or quadratic (b-c) regressions (black line), and the model goodness-of-fit score (R^2). Grey shades represent the confidence interval around the fitted values.

the abundance of three fungal groups in particular correlated with that of *C. parasitica*, specifically *Gnomoniopsis* spp. (correlation coefficient = −0.61), *Trichoderma* spp. (0.61) and *C. acutatum* (0.39). *Gnomoniopsis* spp. negatively influenced the abundance of *C. parasitica*, whereas the abundance of *Trichoderma* spp. and *C. acutatum* increased together with that of *C. parasitica* until a determined threshold value was reached and then decreased (Fig. 5). The other fungi had lower correlation coefficients.

DISCUSSION

In the present study we demonstrate the existence of an indirect interaction between two invasive pests on the European chestnut, namely, the blight fungus *C. parasitica* and the chestnut gall wasp *D. kuriphilus*. Abandoned *D. kuriphilus* galls can be colonized by *C. parasitica*, which suggests that dead or dying gall tissue is an adequate substrate for the growth of the fungus. The incidence of *C. parasitica* on abandoned galls was higher in Ticino (10–19% of the galls being infected, except for Mugena with 3.5%) than in the Chablais with only 1–3% of the galls colonized. One reason for this remarkable regional difference could be the higher disease pressure in Ticino, where continuous chestnut forest provides to *C. parasitica* more woody substrate on which to sporulate. In contrast, in the Chablais, chestnut stands are relatively small and isolated from each other. Another reason may be the difference in the galls' ages between the two regions. In the two chestnut stands in Ticino with the highest incidence of *C. parasitica* on galls (Stabio and Robasacco), *D. kuriphilus* has already been present for 5 years (Forster *et al.* 2009). Although we attempted to sample only galls from the previous or present year (2013–2014), it is possible that we also picked galls that were older. The incidence of *C. parasitica* on the galls may increase in a cumulative way with the age of the galls. If this hypothesis holds true, the incidence of *C. parasitica* colonized galls can be expected to increase in the Chablais in the future.

Only one out of the 135 *C. parasitica* isolates recovered from abandoned galls was infected by the hypovirulence virus. This finding is surprising given that hypovirulence is well established in all eight chestnut stands (30–85% of virus-infected cankers). We can, therefore, assume that abandoned galls are mainly colonized by sexual ascospores which, in contrast to the asexual conidia, do not carry the virus (Prospero and Rigling 2013). It is not clear why virus-infected conidia do not colonize galls. One reason could be the different means of dispersal of the two types of spores, i.e. ascospores are predominantly wind-dispersed and conidia are dispersed over short distances mainly through water splash or insects (Heald, Gardner and Studhalter 1915). The *D. kuriphilus* galls are arranged predominantly on young shoots on the outer edge of the tree crown. Hence, they may be more accessible for airborne ascospores than for conidia. Assuming that some galls may, however, be colonized also by conidia, the very low incidence of virus-infected *C. parasitica* strains on the galls is most likely not due to a reduced fitness of virus-infected conidia compared to virus-free conidia. A previous study showed that the presence of the hypovirus does not influence germination capability of the conidia (Peever *et al.* 2000).

VC tests showed that the VC types that were dominant in the cankers were also most frequently found on galls colonized by *C. parasitica*. Galls are therefore most likely to be colonized by local strains of *C. parasitica*. Noteworthly, in Ticino VC type diversity on galls was higher than in cankers on trees and 13 *C. parasitica* isolates from the galls did not belong to VC types already known to occur in the study area. This suggests that the necrotic tissue of galls can also be colonized by fungal strains which are rare in the local *C. parasitica* population or which are newly generated by sexual recombination. If such strains are able to sporulate on the galls subsequent to gall colonization, we would expect an increase in their incidence in cankers.

In addition to *C. parasitica*, several other genera of mainly ascomycetes were shown to colonize abandoned *D. kuriphilus* galls. Among these, *G. castanea* (syn. *G. smitholgilvy* or *A. castanea*) was clearly the predominant species. This ascomycete is an endophyte in the family *Fagaceae* (Bissegger and Sieber 1994) and in recent years, it has been frequently reported as the causal agent of brown rot in European chestnut fruit and flowers (Maresi, Longa and Turchetti 2013; Shuttleworth, Liew and Guest 2013; Dennert *et al.* 2015). In Italy, *G. castanea* has also been observed to cause lesions on chestnut leaves and to colonize *D. kuriphilus* galls before adults emerge (Magro *et al.* 2010). A second *Gnomoniopsis* species was also found on the galls mostly in the Chablais, namely, *Gnomoniopsis* sp. ICMP 14082 (GenBank accession number KC145849.1). It is worth noting that in Switzerland, these two *Gnomoniopsis* species have been recurrently isolated, together with *C. parasitica*, from healed chestnut blight cankers (D. Rigling, unpublished data).

Gnomoniopsis castanea, as an endophyte, may already be present in green *D. kuriphilus* galls. Thus, it may have a competitive advantage in colonizing the dying gall tissue immediately after gall wasps emerge compared with other non-endophytic fungi, which must first reach the galls. In our chestnut stands, the abundance of *G. castanea* on galls was negatively correlated with that of *C. parasitica*, indicating that the endophyte might effectively outcompete the pathogen, thereby reducing the amount of *C. parasitica* inoculum on the abandoned galls. In Italy, *G. castanea* is able to sporulate on abandoned galls (Ugolini *et al.* 2014). It would, therefore, be interesting to determine whether the invasion of European chestnut stands by *D. kuriphilus* has caused an increase in the incidence of chestnut fruit brown rot.

On older galls (e.g. in Stabio), however, *Gnomoniopsis* spp. seem to be replaced by other fungi, which may have a better saprotrophic ability, including species of *Trichoderma*, *Fusarium*, *Sordaria*, *Alternaria*, *Botryosphaeria* and *C. acutatum*. Species of these genera are known to occur on *D. kuriphilus* galls and on fruits and bark of the European chestnut (Bissegger and Sieber 1994; Akilli, Katircioğlu and Maden 2007; Sieber, Jermini and Conedera 2007; Addario and Turchetti 2011; Visentin *et al.* 2012; Double *et al.* 2014). The community of gall-associated fungi in the Ticino was richer and more diverse than in the Chablais. This might possibly be correlated with the older age of the galls. Additionally, climatic factors may also affect the fungal colonization of galls. The higher mean annual temperature in the south of the Alps may allow a better dispersal and germination of fungal propagules that might then better outcompete the endophytes like *Gnomoniopsis* spp.

Among the other gall-colonizing fungi, a strong presence of *Trichoderma* spp. and *C. acutatum* appears to hinder gall colonization by *C. parasitica* and/or vice versa. However, it is difficult to determine whether this is the outcome of a direct interspecific interaction, with one species influencing gall colonization by the other, or whether the presence of one species rather than the other is dependent on the amount of airborne inoculum available. In contrast to the endophyte *G. castanea*, these two fungi might reach galls through airborne spores. Interestingly, *C. acutatum* was recently reported as an efficient parasite of the gall wasp in Italy and the USA (Magro *et al.* 2010; Addario and Turchetti 2011; Gaffuri *et al.* 2015). Hence, this fungus might parasitize the larvae and may also be present on galls prior to wasp emergence. The saprophyte *Trichoderma* is one of the most frequently isolated fungi from chestnut blight cankers (especially older ones) besides *C. parasitica* (Akilli, Katircioğlu and Maden 2007; Double *et al.* 2014). This might partly explain its prevalence in abandoned galls in chestnut stands in Ticino, which have a longer *C. parasitica* history. *Trichoderma* is also antagonistic to *C. parasitica*, and has been suggested to be involved in canker healing processes (Akilli, Katircioğlu and Maden 2007). Galls might allow *Trichoderma* to enhance their inoculum and this could counteract the negative effect of the increase in *C. parasitica* inoculum.

CONCLUSION

Our study shows that *D. kuriphilus* galls that have become necrotic after the emergence of young adults can be colonized by the virulent (virus-free) form of the chestnut blight fungus *C. parasitica*. This could increase the load of virulent inoculum in forests, which would lead to a recrudescence of the disease even in chestnut stands with well-established hypovirulence.The chestnut gall wasp by modifying the physical environment influences fungal species composition on chestnut trees and, potentially, the whole surrounding ecology. The abandoned galls represent a new ecological niche not only for virulent strains of *C. parasitica*, but also for other fungi (e.g. *G. castanea*) associated with the European chestnut. Apart from fungi, insects and spiders have also been observed to find shelter in galls (Judd 1967; Crawford, Crutsinger and Sanders 2007; Almeida, Santos and Carneiro 2014). The introduction of an alien species like *D. kuriphilus* into a new ecological environment can, therefore, shape the global community of living organisms in forests. Consequently, it is most important to understand these interactions better in order to foresee their effects and, in the case of a pest, to develop effective control measures.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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