Assessing the phytosanitary risk posed by an intraspecific invasion of *Cryphonectria parasitica* in Europe

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Abstract

Intraspecific cryptic invasions may occur when new strains of an invasive species are introduced into an area where this species had already been introduced previously. In plant pathogens, such invasions are not well studied, even if potentially they can have severe consequences. Here, we investigated the effects of a potential intraspecific invasion in Europe of *Cryphonectria parasitica*, the causal agent of chestnut blight. Specifically, we tested the hypotheses that (1) non-European strains are more virulent on *Castanea sativa* than those already present in Europe because they have never encountered this new host, and (2) the variation in virulence among strains is higher within native than within introduced populations. In a greenhouse, two-year old *C. sativa* seedlings were inoculated with *C. parasitica* strains from South Korea, the USA and Switzerland and lesion development and seedling mortality weekly recorded. Additionally, growth and sporulation

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of the strains were measured *in vitro* on agar medium at 15 and 24 °C. While lesion growth was similar for all strains, seedlings inoculated with strains from South Korea and Switzerland died faster than seedlings inoculated with strains from the USA. Moreover*, in vitro* strains from South Korea grew faster and produced more spores at both temperatures than the strains from the other two countries. In conclusion, our results did not support the two hypotheses. All strains, regardless of their origin, were found to be highly virulent on the inoculated chestnut seedlings. Nevertheless, current phytosanitary measures to avoid the introduction of new genotypes of *C. parasitica* into Europe should be further implemented.

Keywords

Chestnut blight, *Castanea* spp., cryptic invasion, virulence assay, avirulence theory

Introduction

Invasive plant pathogens represent a major threat for forest ecosystems as they can cause dramatic and persistent ecological changes (Ellison *et al.* 2005, Loo 2009). Well-known examples include the white pine blister rust (*Cronartium ribicola* J.C. Fisch) in North America (Maloy 1997) and the Dutch elm disease (*Ophiostoma ulmi* (Buism.) Melin & Nannf. and *O. novo-ulmi* Brasier) in North America and Europe (Gibbs 1978). Once an invasive pathogen has become established in a new area, its successful eradication is very difficult to achieve and complex containment measures have to be adopted (Picard *et al.* 2018). Hence, prevention of introduction and establishment (proactive capacity, Early *et al.* 2016), although difficult to implement, is the best way to combat invasive pathogens. This applies

not only to new species, but also to additional genotypes of already introduced species (Schrader and Unger 2003).

In most biological invasions only a small fraction of the genotypic and phenotypic diversity of a source population is, at least initially, introduced in a new area leading to a demographic bottleneck. For example, the epidemic of ash dieback caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya in Europe probably resulted from the introduction of only two fungal genotypes (Gross *et al.* 2014, Schoebel *et al.* 2017). Similarly, one or two genotypes of the ambrosia fungus *Raffaela lauricola* T.C. Harr, Fraedrich, and Aghayeva possibly introduced from Asia are responsible for the invasive laurel wilt disease in southeastern United States (Wuest *et al.* 2016). However, even when an invasive pathogen has already become established, invasions by new genotypes (so-called intraspecific cryptic invasions; Morais and Reichard 2018) can potentially have significant consequences. First, the new genotypes may modify the population biology of the species in the introduced range, for example by allowing or increasing sexual reproduction. This was the case when the A2 mating type of the potato late blight pathogen *Phytophthora infestans* (Mont.) de Bary was introduced to Europe (Hohl and Iselin 1984, Mariette *et al.* 2016). While in some European regions clonal populations remained dominant, in other regions mating type frequencies rapidly changed, and the pathogen started to reproduce sexually (Montarry *et al.* 2010, Kiiker *et al.* 2018). Sexual reproduction itself has the potential to generate new genotypes, which may be better adapted to local conditions than the old ones. Second, newly introduced genotypes may be more virulent toward the new host than the previously introduced ones, especially when a time lag exists between introduction events. According to the "avirulent hypothesis", parasites should co-evolve with their hosts to become avirulent for avoiding extinction of the host and of themselves (May and Anderson 1983).

However, as some degree of virulence is unavoidable for pathogen reproduction and transmission (transmission-virulence trade-off), co-evolution will most likely not result in avirulent genotypes, but rather in genotypes with an intermediate virulence to maximize their transmission and avoid at the same time host extinction (Alizon *et al.* 2009). Therefore, we may expect that genotypes from old introductions are better adapted, i.e. less virulent, to the new host than newly introduced genotypes, which could cause increased damage.

In this study, we assessed the risk that invasion of new strains of the chestnut blight fungus *Cryphonectria parasitica* in Europe would represent using greenhouse and *in vitro* inoculation experiments. *C. parasitica* infects chestnut (*Castanea* sp.) trees through wounds in the bark and induces the formation of necrotic lesions (so-called cankers) that may girdle and eventually kill the affected tree part (Rigling and Prospero 2018). In its native range in Asia (China, Japan, Korea), the fungus is a weak pathogen on native chestnut species (*C. mollissima* Blume and *C. crenata* Siebold & Zucc.). In the 20th century it was accidentally introduced first into North America and then into Europe, causing severe damages on the American (*C. dentata* (Marsh.) Borkh.) and European (*C. sativa* Mill.) chestnuts, respectively. Previous studies (Liu and Milgroom 2007, Dutech *et al.* 2012) showed that, as expected, genetic diversity of *C. parasitica* is higher in Asia than in North America and Europe, which likely is also reflected in variation of phenotypic traits, including virulence.

Chestnut blight was first detected in Europe in 1938 in northern Italy (Biraghi 1946) and nowadays it is present in most European chestnut growing areas (Robin and Heiniger 2001). Because an eradication of the disease in continental Europe it not realistic anymore, *C. parasitica* has recently been recommended for listing as a “regulated non-quarantine pest” by the European Food Safety Authority (EFSA) (Jeger *et al.* 2016). Population genetic studies showed that the pathogen was introduced to Europe several times (Dutech *et al.* 2010,

Dutech *et al.* 2012). One introduction occurred from the United States, from where the fungus was accidentally introduced to Italy (Anagnostakis 1987). Another introduction directly from Asia targeted south-western France. While in the USA chestnut blight virtually eliminated the native American chestnut (Shackleton *et al.* 2018), in Europe the epidemics followed a milder course thanks to the appearance and spontaneous spread of a mycovirus (CHV-1) that reduces virulence and sporulation of infected *C. parasitica* strains (Grente 1965, Heiniger and Rigling 1994). Additionally, the European chestnut is slightly less susceptible to *C. parasitica* than the American chestnut, but it is unclear how much this influenced the *C. parasitica* epidemic in Europe.

New introductions of *C. parasitica* to Europe in recent years are not known. Most likely this was prevented by the implementation of international and national phytosanitary measures (Eschen *et al.* 2015). However, for several reasons (e.g. latent infections with no expression of symptoms, Leclerc *et al.* 2014) prevention and control policies might fail, resulting in new introductions. The aim of this study was to understand how an accidental introduction to Europe of *C. parasitica* genotypes from other continents would affect the European population of *C. sativa*. Specifically, we tested the following two hypotheses: (1) Strains from South Korea and North America are more virulent on *C. sativa* than those already present in Europe because they have never encountered this new host; and (2) the variation in virulence among strains is higher within native (South Korea) than within introduced (North America, Switzerland) *C. parasitica* populations. Here, we define virulence as a quantitative trait, i.e. the degree of damage caused by a pathogen to a specific host (Sacristàn and Garcìa-Arenal 2008). For testing the two hypotheses mentioned above, we inoculated *C. parasitica* strains from South Korea, the USA and Switzerland on two-year old European chestnut seedlings and regularly assessed lesion development and mortality. Since not only

virulence, but also other biological traits (e.g. host range, sporulation and dispersal capacity, growth-temperature interactions) determine the invasiveness of a pathogen (Philibert *et al.* 2011), at the same time we assessed growth and sporulation of the selected *C. parasitica* strains *in vitro* at two different temperatures. We then tested whether *in vitro* characteristics are linked to *in planta* virulence of the *C. parasitica* strains.

Material and Methods

Cryphonectria parasitica strains

A total of 29 *C. parasitica* strains from one native (South Korea) and two introduced (USA, Switzerland) populations were selected for the study (Table S1 and Figure S1). The 12 South Korean strains were recovered at eight different locations from infected *C. crenata* trees and based on microsatellite (SSR) analysis (Prospero and Rigling 2012) they represented 12 different genotypes. The 11 strains from the USA (9 different SSR genotypes) were collected at 5 sites in Maryland, New Hampshire, West Virginia, New York State, and Kentucky from *C. dentata* trees (Table S1). Finally, the 6 Swiss strains were isolated at four different locations in Southern Switzerland (Ticino) from bark cankers on *C. sativa* trees (Table S1). These strains were selected to represent the two most widespread and, thus, most likely successful invasive genotypes in that part of Switzerland (M3963, M3053, M2344: CpMG33; M2452, M3983, M1693: CpMG15; Prospero and Rigling 2012). The genetic relationships among the 29 selected strains were visualized by producing a minimum spanning network (MSN) based on Bruvo's distances calculated with the SSR data (Prospero and Rigling 2012). For this analysis, the function "bruvo.msn" of the R package "poppr" (Kamvar *et al.* 2014) was used. Genetic diversity of the *C. parasitica* populations was assessed by calculating allelic richness per SSR locus standardized by sample size using the function "allel.rich" of the R package

"PopGenReport" (Adamack and Gruber 2014). As Swiss and North American strains grouped together in the MSN (see Results), for this analysis they were considered to belong to the same population.

Before inoculation, the absence of a CHV-1 infection in all *C. parasitica* strains was verified based on culture morphology and molecular analysis. Culture morphology on Potato Dextrose Agar (39 g/L PDA, Difco, Franklin Lakes, USA) was assessed after incubating the plates in the dark at room temperature for 7 days followed by an additional incubation under daylight for another 7 days at room temperature (Bissegger *et al.* 1997). After this treatment, virus-free strains typically show a characteristic orange morphology. For molecular analysis, strains were grown on cellophane PDA, and after 7 days, the mycelium was harvested and lyophilized (Hoegger *et al.* 2000). Total RNA was extracted with the RNEasy Plant mini kit (Qiagen, Hilden Germany) as described in the manufacturer's protocol. cDNA was obtained with the Maxima first strand cDNA synthesis kit (ThermoFisher Scientific, Waltham, USA) and used to amplify a specific region of the ORF-A from CHV-1 with the method described in (Gobbin *et al.* 2003). The absence of the ORF-A fragment was then confirmed by electrophoresis on 1.5% agarose gel.

Castanea sativa population

The virulence of the selected *C. parasitica* strains was assessed on two-year-old *Castanea sativa* seedlings (stem diameter 10-20 mm) issued from a wild *C. sativa* population from Southern Germany. Three months before inoculation, the bare-root seedlings were planted in plastic pots containing a soil substratum composed of bark compost, peat, wood fibers and 14% mineral material and placed in the forest nursery of the Swiss Federal Research Institute WSL and watered as required.

Virulence assay

The virulence assay was performed between July and October 2017 in the biosafety greenhouse facility at WSL with the *C. parasitica* and *C. sativa* populations described above. Inoculations were conducted as described in Hoegger *et al.* (2002). Briefly, the *C. sativa* seedlings were inoculated into the stem with an agar plug of *C. parasitica* culture grown on PDA for seven days at room temperature. A hole of 5 mm diameter was cut into the bark with a cork borer, and filled with a PDA plug of a *C. parasitica* culture. The plug was placed in the wound with the mycelium side facing the cambium and then covered with tape to prevent desiccation. Control seedlings were inoculated with sterile PDA plugs. For each *C. parasitica* strain, five chestnut seedlings were inoculated. The experiment was performed at room temperature and under natural light. Seedlings were watered as needed (1-2 times per week) to keep the soil always moist. The pots containing the seedlings were randomly distributed on nine greenhouse trolleys which were weekly moved around to avoid any influence of their position on the experiment. As proxies for virulence, lesion size and seedling mortality were recorded. Starting at two weeks after inoculation, lesion length and width were measured using a millimeter ruler once per week until 23 weeks after inoculation when all seedlings except four and the controls had died (see Results). Mortality was defined as time until seedling death (wilting of the foliage above the inoculation point) and was recorded in weeks.

Re-isolation of *C. parasitica*

To verify that the dead seedlings were effectively killed by the inoculated *C. parasitica* strain, the bark at the edge of the lesion was removed with a knife and the presence of the

192 characteristic pale brown mycelial fans (Rigling and Prospero 2018) was checked. Moreover,
193 15 dead seedlings were randomly selected and *C. parasitica* was re-isolated from the lesions.
194 For this, small pieces of bark and mycelium from the lesion were plated on 1.5% water agar
195 (Industrial agar no. 2, Pronadisa, Madrid, Spain) and plates were incubated for 7 days at
196 room temperature in the dark. After this period, growing *C. parasitica* colonies were
197 transferred to PDA and incubated for 7 days at room temperature in the dark. A mycelial
198 plug of the re-isolated culture was then paired on PDA with a mycelial plug of the original
199 strain. The plates were successively incubated in the dark at room temperature for 7 days. A
200 merging reaction between the two colonies (Bissegger *et al.* 1997) confirmed that the re-
201 isolated strain corresponded to the inoculated one.
202
203 **Growth and sporulation *in vitro***
204 The growth of the 29 studied *C. parasitica* strains was measured *in vitro* at two different
205 temperatures (15°C and 24°C) under a light intensity of 3330 lx and 14 h photoperiod.
206 Mycelial agar plugs of 5 mm diameter originating from the growing margin of pure cultures
207 were placed upside down on the center of PDA plates (8 cm in diameter). Size of the colony
208 was measured with a millimeter ruler daily for 7 days (after this period the majority of
209 colonies completely colonized the Petri plate). Orthogonal lines were traced on the Petri
210 plate and colony growth was measured along the lines. The two measurements
211 corresponded approximately to the major and minor axis of an ellipsis and were used to
212 calculate the colony size.
213 The colonies were then incubated under the same conditions for 7 more days, after which,
214 the sporulation was assessed. At this time, all *C. parasitica* cultures completely covered the
215 Petri plate. Spores were washed from the surface of the colonies with 5 mL sterile, distilled

water and then quantified using a hemocytometer (Neubauer, 0.1 mm depth, 0.0025 mm^2) under a light microscope at 40x magnification.

Data analysis

Data were analyzed with the statistical software R version 3.4.1 (R Core Team 2014). In the greenhouse assay, the five seedlings inoculated with the same *C. parasitica* strain were considered as biological replicates. Since at the first measurement two weeks after inoculation, lesion width significantly positively correlated with seedlings' diameter (Spearman's correlation, rho=0.308, $p=7.224*10^{-05}$), only lesion length data were used for statistical analysis. Lesion length data were analyzed until the first seedlings died at 7 weeks post inoculation. The time until seedling mortality was recorded in weeks after inoculation, and differences between country of origin of the strains was inferred with a linear model, where the strain was nested within the country, followed by a Tukey post-hoc test (significance threshold: $p<0.05$). The stem diameter of the seedlings was considered as a confounding factor in the model. Differences in variances between the populations were analyzed with Bartlett's test. A survival analysis was performed by plotting Kaplan-Meier survival curves with the function "ggsurvplot" of the package "survminer", and comparing them with the log-rank test.

In the *in vitro* experiment, differences in growth between strains from different countries were inferred 7 days post inoculation, while differences in sporulation 14 days post inoculation were inferred as described above for the greenhouse experiment. Correlations at the isolate level between all analyzed parameters (mortality, lesion length, *in vitro* colony size, *in vitro* sporulation) were shown in a heat map. Spearman's correlation analysis was

used (significance level: $p<0.05$), because of the non-normal distribution of the data and the robustness to outliers.

Results

The minimum spanning network (MSN) showed that the Swiss *C. parasitica* strains used in this study clearly grouped with the strains originating from the USA, whereas the South Korean strains were located on another sector of the network (Fig. 1). Genetic diversity estimated by the allelic richness across loci was considerably higher in the South Korean population (4.6) than in the combined Swiss and North American populations (2.5).

Virulence assay

Five seedlings were inoculated with each *C. parasitica* strain and the size of the lesions was measured weekly until seven weeks after inoculation. Lesion length two weeks and seven weeks after inoculation was not significantly different between strains from different countries (Fig. 2). There were also no significant differences in variation of lesion length between countries.

The first seedlings died seven weeks after inoculation (Fig. 3), and the median mortalities (i.e. when 50% of the seedlings had died) were 11 weeks post-inoculation (strains from South Korea), 12 weeks post-inoculation (strains from the USA), and 10 weeks post-inoculation (strains from Switzerland). The highest variation in time until death was in seedlings inoculated with strains from the USA (standard deviation=3.8 weeks), while the smallest in seedlings inoculated with strains from South Korea (standard deviation=2.7 weeks). The variation of time until seedling death was significantly different between countries (Bartlett's test $p=2.2*10^{-16}$) Overall, the time until the seedlings died was

significantly shorter in trees inoculated with strains from South Korea compared with strains from the USA, but there was no significant difference between strains from South Korea and Switzerland (Fig. 2). Two strains from South Korea (SKo77 and SKo178, Fig. 4) killed all inoculated seedlings within 10 weeks after inoculation. Four seedlings, each inoculated with a different *C. parasitica* strain (Sko134 from South Korea, MD-1-2 and BRU-1 from the USA, and M3963 from Switzerland; Fig. 4) survived until the end of the experiment at 23 weeks after inoculation. Control seedlings inoculated with sterile PDA plugs did not develop any lesion and survived without symptoms until the end of the experiment. Visual inspection of bark lesions that developed on dead seedlings revealed the presence of the typical mycelial fans of *C. parasitica* under the bark. Re-isolations and pairing tests showed that the *C. parasitica* strain present in the lesions was the one originally inoculated.

Growth and sporulation *in vitro*

The *C. parasitica* strains used for the inoculation assay were also grown *in vitro* at 15°C and at 24°C and colony growth and sporulation were assessed. Colony size after 7 days at 15°C (Fig. 5A) was significantly larger in strains from South Korea compared to strains from the USA and Switzerland. Variation in colony size was highest in strains from South Korea, followed by strains from the USA and from Switzerland. At 24°C, similar results were obtained (Fig. 5B). *In vitro* growth at both tested temperatures was significantly positively correlated (Spearman's rho=0.55, p=0.002, see Figs. 6 and 7). While the ranking of strains for colony size was different at the two tested temperatures, these differences were not dependent on the geographic origin of the isolates (Fig. 6).

Sporulation was assessed after 14 days by washing the spores from the plate with sterile distilled water and counting the spores in the suspensions. The median number of spores

per plate at 15°C (Fig. 5C) was slightly higher for strains from South Korea, compared to strains from the USA and Switzerland, but differences between countries were not significant. At 24°C (Fig. 5D), sporulation was significantly different for strains from different countries, with strains from South Korea producing the highest number of spores per plate, followed by Swiss and American strains. The variation within populations was again highest in the South Korean strains. Overall, strains from South Korea grew faster at both tested temperatures and produced more spores per plate than strains from the USA and Switzerland. Moreover, South Korean strains showed a greater variation within the population for colony growth and sporulation, compared to the two other studied populations.

Correlations between *in vivo* and *in vitro* data

Spearman's correlations between all the assessed parameters were inferred and shown in a heat map (Fig. 6). Lesion length and seedling mortality were significantly negatively correlated (rho=-0.42, p=0.01), i.e. larger lesions resulted in a faster seedling death. In the *in vitro* experiment, significant correlations ($p<0.05$) were found between colony size (i.e faster growth *in vitro*) and sporulation at both assessed temperatures. The correlations between parameters from the inoculation assay and from the *in vitro* experiment were not significant. Lesion length on seedlings showed no clear positive or negative correlation with colony growth or sporulation in the *in vitro* experiment (rho approximately 0). Similarly, although seedling mortality was weakly negatively correlated with *in vitro* growth and sporulation at 24°C, correlations were not significant (for colony area, rho=-0.3, p=0.09).

Discussion

In this study, we assessed the risk posed by an intraspecific invasion of *C. parasitica* from South Korea and North America to Europe by performing *in planta* and *in vitro* inoculation experiments. All *C. parasitica* strains regardless of their population of origin, were found to be highly virulent producing large bark lesions and high mortality of the inoculated chestnut seedlings. Mortality (in weeks post-inoculation) was only affected by seedling diameter, i.e. seedlings with smaller diameters died faster. A longer survival of seedlings with a larger diameter was previously reported in two studies in which *C. parasitica* strains were inoculated on *C. sativa*, *C. crenata* and *C. sativa* x *C. crenata* seedlings (Xenopoulos and Papachatzis 1999, Pažitný *et al.* 2018). Our results suggest that an intraspecific invasion of *C. parasitica* from North America and South Korea would not necessarily lead to an increased mortality of *C. sativa* in Europe. As known from the invasion history of chestnut blight (Dutech *et al.* 2012) and confirmed by our minimum spanning network, the Swiss *C. parasitica* population is more closely related to the North American than the South Korean population. Therefore, we would have rather expected the Swiss strains to behave more as the North American ones.

Although the number of strains tested in our study is most likely not sufficient to infer sound conclusions about the evolution of European *C. parasitica* populations, our findings do not seem to support the avirulence theory (May and Anderson 1983). Based on this theory, the *C. parasitica* population in Southern Switzerland after its establishment in the 1940s should have evolved towards lower virulence to avoid host extinction. If we consider roughly one fungal generation per year (i.e. one sexual reproduction event per year), the coevolution time between the parasite (*C. parasitica*) and the host (*C. sativa*) would be about 80 fungal generations, eventually still too short to allow the selection for less virulent genotypes.

However, other particularities of the European pathosystem could have influenced the evolution of the host-pathogen interaction. First, the presence of the hypovirus, which reduces the virulence of the infected isolates and, therefore, could counterbalance the selection pressure for less virulent genotypes in the *C. parasitica* population. In North American populations, on the other hand, hypovirulence could not become established and strains with a reduced virulence may have effectively been selected to avoid the extinction of the local host (*C. dentata*). This process may have been accelerated by the particularly high susceptibility of *C. dentata* to *C. parasitica*. Even without the effect of hypovirulence, host extinction in Europe would most likely have taken a longer time than in the USA because *C. sativa* trees usually support multiple infections before they die. *C. parasitica* is native to South Korea and in accordance with the avirulence theory a weak pathogen on the local chestnut species (Anagnostakis 1992). The greater genetic diversity of South Korean strains did not lead to a greater variation in lesion length among these strains, compared to the strains from the two invasive populations. Therefore, we could not confirm the hypothesis that a more variable natural population exhibits greater variability in virulence. These results suggest that high virulence on European chestnut is a general phenotypic trait of *C. parasitica* from South Korea and presumably also from other areas in Asia. Following this line, the mechanism behind virulence is likely associated with important fitness trait(s) of the fungus in its native range and therefore under strong selection.

In our inoculation study, we only found minor differences in virulence among *C. parasitica* strains from native and introduced populations. Besides the relatively low number of strains considered, the specific inoculation technique applied might have accounted for this result. First, inoculating a mycelial plug in a wound previously made in the bark of a seedling only allows to assess one aspect of virulence, i.e. parasitic mycelial growth in the bark. Second,

the method is very effective (i.e. always results in a lesion), but is far away from the natural infection process. In nature, an infection is caused by sexual or asexual spores which germinate in fresh wounds (e.g. growth cracks) present in the bark tissue (Rigling and Prospero 2018). Since the inoculum pressure is much lower than in the inoculation experiment, differences in virulence among genotypes may be more evident. Unfortunately, spore inoculations are experimentally more difficult to perform than inoculations with mycelial plugs.

The seedling inoculation trial that we conducted has proven to be very useful to predict the outcome of potential intraspecific invasions in *C. parasitica*. Nevertheless, such experiments are work- and time-intensive, need living plant material, and appropriate greenhouses to fulfill all biosafety requirements. *In vitro* studies without living plant material, conducted under conditions that reflect the environment where the invasion would take place, may represent a valuable alternative or provide complementary information. Here, we assessed, at two different temperatures, two biological traits which may potentially also affect invasiveness of the tested *C. parasitica* strains, i.e. growth and sporulation. All strains, disregarding the population of origin, showed a larger colony area after incubation at 24°C than at 15°C, which is in line with the results of previous studies (e.g. Bazzigher 1981, Bryner and Rigling 2011). However, individual strains appeared to be better adapted to one of the tested temperatures, although this was not linked to their geographic origin. The potential importance of temperature for the selection of specific *C. parasitica* strains, thereby influencing the course of the chestnut blight epidemics, was previously shown by Bryner and Rigling (2011).

As *C. parasitica* strains from South Korea grew faster and sporulated more *in vitro* than those from the other two populations at both tested temperatures, one could imagine a higher

virulence of South Korean strains. However, no significant correlation was found between colony growth and sporulation measured *in vitro* and the virulence toward the seedlings at the single isolate level. Similarly, although variability in colony growth and sporulation on PDA within South Korean isolates was higher than within Swiss and USA isolates, this was not observed in the seedling inoculation experiment. Hence, our results rather suggest that *in vitro* experiments can be used in a limited way to predict the outcome of *in planta* inoculation experiments. However, they can represent a valuable approach to assess biological traits, which are difficult to measure on living plant material, e.g. sporulation. In our specific case, the observed variation in sporulation among *C. parasitica* strains on PDA may yet indicate differences in invasiveness. Even though all tested strains are highly virulent toward chestnut seedlings, only those with a good sporulation capacity may be able to rapidly spread and become successful invaders. Thus, a combination of *in vitro* and *in planta* experiments may allow for a more precise assessment of phytosanitary risks.

In conclusion, our study suggests that an introduction of *C. parasitica* strains from South Korea and the USA to Europe would not necessarily result in an increased mortality of *C. sativa*. However, to definitively confirm this result, strains from additional populations (e.g. China, Japan) should also be tested on different provenances of European chestnut trees. Since Robin et al. (2017) showed a thermal adaptation in *C. parasitica* during range expansion, it would be particularly important to consider non-European strains from all climates where the pathogen occurs. Similarly, testing more strains from Europe would allow to better account for eventual differences in the European population due to the different source populations (Asia and North America). Although the non-European *C. parasitica* strains seem not to be more virulent than the ones already present in Europe, other problems could arise with their introduction. In particular, an invasion by new strains could

result in an increase of the vegetative compatibility (vc) type diversity, which could negatively affect spread of the hypovirus responsible for hypovirulence. In fact, high vc type diversity is considered to be one of the main reasons for the failure of biocontrol of chestnut blight with hypovirulence in North America (Milgroom and Cortesi 2004). It is, thus, mandatory that current phytosanitary measures to avoid the introduction of new *C. parasitica* strains in Europe are further implemented.

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Figure Captions

Figure 1. Minimum Spanning Network based on Bruvo's distances using microsatellite data (Prospero and Rigling 2012) showing the genetic relationships among the 29 *Cryphonectria parasitica* strains used in the study.

Figure 2. Box plots of the length of lesions (mm) caused by *Cryphonectria parasitica* on two-year-old *Castanea sativa* seedlings inoculated with strains from South Korea (12 isolates), USA (11 isolates) and Switzerland (6 isolates) two weeks (first measurement; A) and seven weeks (last measurement; B) after inoculation. Black lines in the boxes show medians. Letters above the graph indicate significant differences in the lesion length between strains from different countries (Nested linear model followed by Tukey's post-hoc test, significance level=0.05).

Figure 3. Main figure: Survival probabilities of two-year-old *Castanea sativa* seedlings after inoculation with *Cryphonectria parasitica* strains from South Korea (12 strains, red), USA (11 strains, blue), and Switzerland (6 strains, green). Curves show median seedling survival probabilities depending on time post-inoculation and shadows show confidence intervals (0.05-0.95). Significant differences between curves at median survival (0.5) are shown with black dotted lines. The shown p-value was obtained by comparing the survival curves with a log-rank test. **Insert:** Boxplots showing the mortality of seedlings by country of origin of the inoculated *C. parasitica* strain. Dots show the mortality of single seedlings (colors indicate replicates inoculated with the same fungal strain). Black lines in the boxes indicate medians. Letters above the graph indicate significant differences in the mortality between strains from different countries (Nested linear model followed by Tukey's post-hoc, significance level=0.05). The experiment ended after 23 weeks, values at this timepoint indicate that the corresponding seedlings survived until the end of the experiment.

Figure 4. Mortality of two-year-old *Castanea sativa* seedlings in weeks after inoculation for each *Cryphonectria parasitica* strain. Five seedlings were inoculated with each strain and seedling mortality was assessed weekly. Red lines indicate the median mortality, i.e. the time point where 3 out of 5 seedlings had died. The two strains highlighted in blue (Sko77 and Sko178) killed all five inoculated seedlings in ten weeks or less. The experiment ended

23 weeks after inoculation. At this time, four seedlings were still alive, all inoculated with different strains (highlighted in red).

Figure 5. Results of the *in vitro* experiment by country of origin of the *Cryphonectria parasitica* strains tested. The cultures were grown on PDA under light at two different temperatures. For each strain, 5 replicates were plated. (A) Colony area 7 days after incubation at 15°C; (B) Colony area 7 days after incubation at 24°C, (C) Sporulation after 14 days at 15°C, (D) Sporulation after 14 days at 24°C. Black lines in the middle of the boxes indicate medians. Letters indicates significant differences between strains from different countries (Nested linear model followed by Tukey's post-hoc test, $p<0.05$).

Figure 6: Scatterplot showing the in-vitro colony size on PDA plates measured after 7 days at 15°C and at 24°C. Each dot represents one tested isolate (average of five technical replicates at each temperature). Growth at the two temperatures was significantly positively correlated (Spearman's correlation, $p=0.0022$, $rho=0.55$), but single isolates were better adapted to one of the tested temperatures (examples highlighted in the plot: isolates MD-2 and SKo126 better adapted to 15°C than 24°C, and isolate SKo210 better adapted to 24°C than 15°C). On average, isolates from South Korea had bigger colony sizes than the isolates from the other two countries at both tested temperatures (red ellipse).

Figure 7. Heat map showing Spearman's correlations between data from the *in vivo* greenhouse experiment and *in vitro* experiments. Significant correlations are indicated with asterisks ($p<0.05$). The bar on the right shows the correlation coefficient (Spearman's rho). Lesion length 7 wpi is the length of the lesions measured 7 weeks after seedling inoculation with *Cryphonectria parasitica* strains; Seedling mortality expressed as number of weeks until the inoculated seedlings died; Colony areas at 15°C and at 24°C is the colony size measured seven days after inoculation on PDA plates; Sporulation at 15° and at 24°C indicates the number of spores produced by a strain on a PDA plate 14 days after inoculation.

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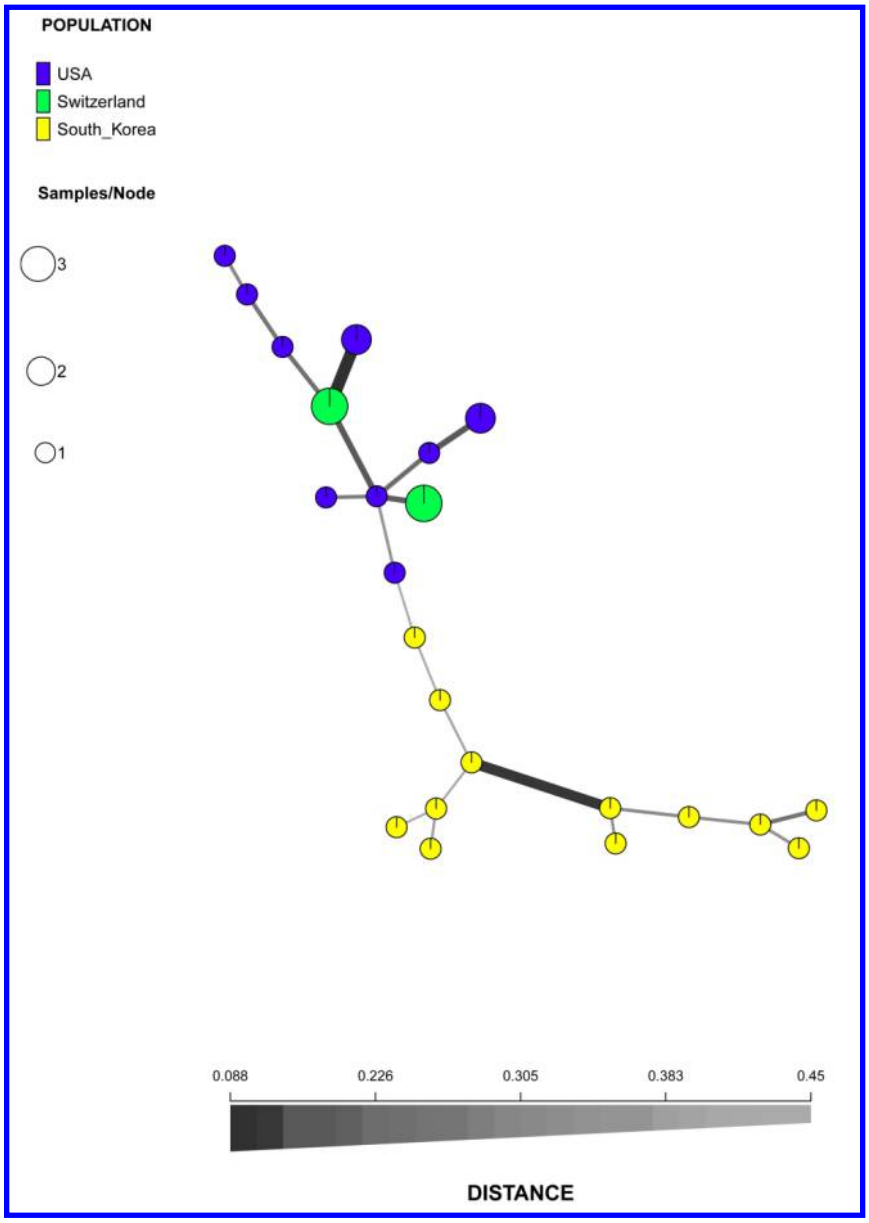

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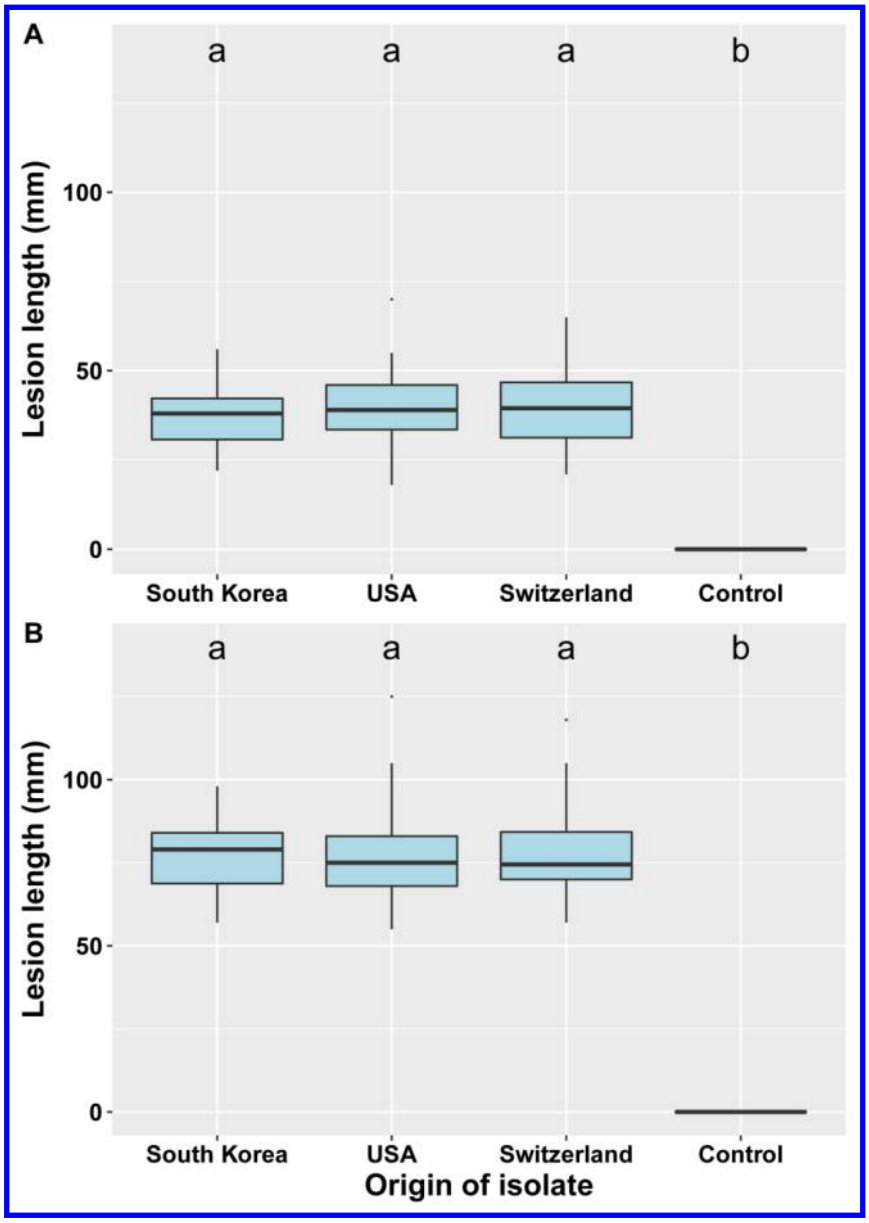

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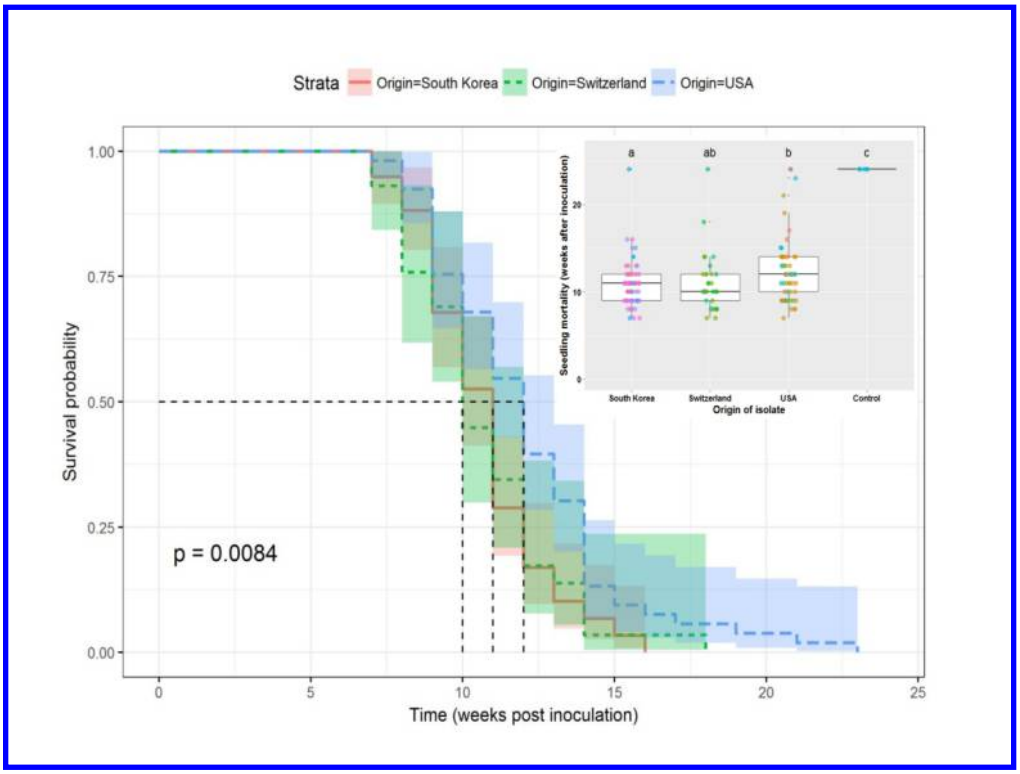

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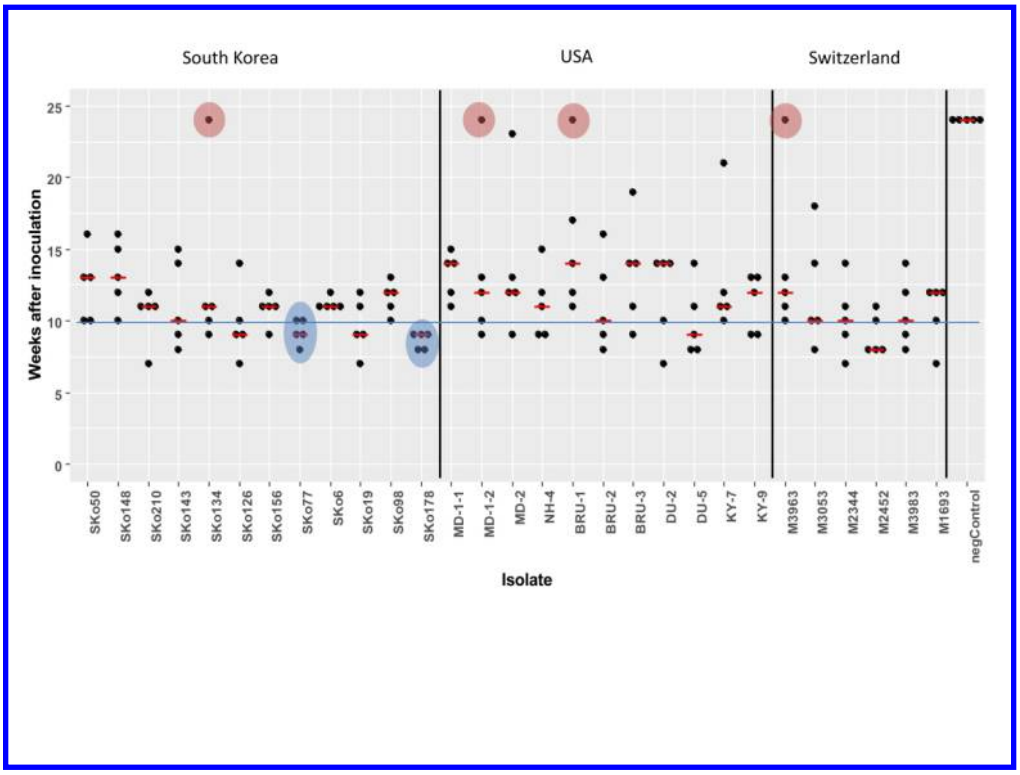

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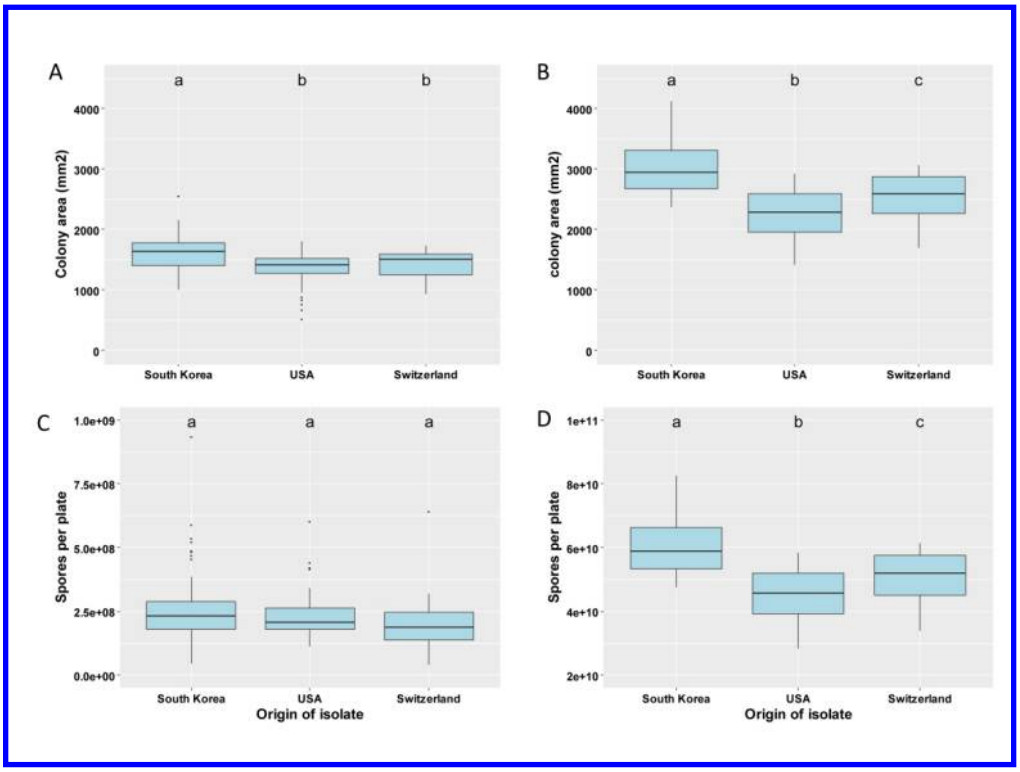

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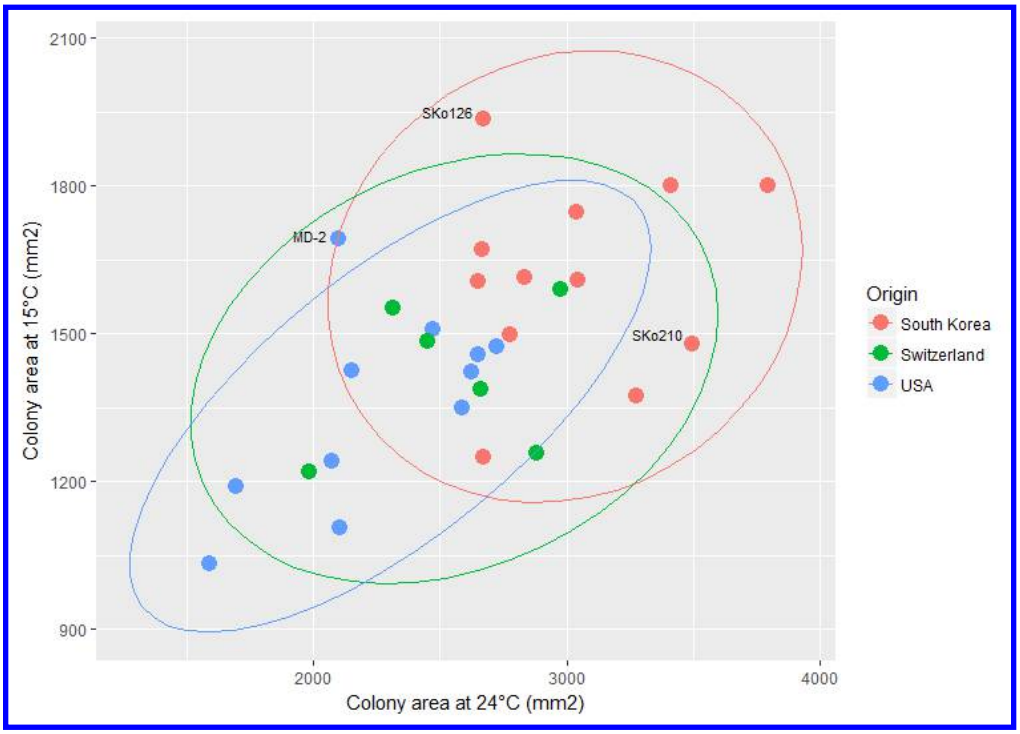

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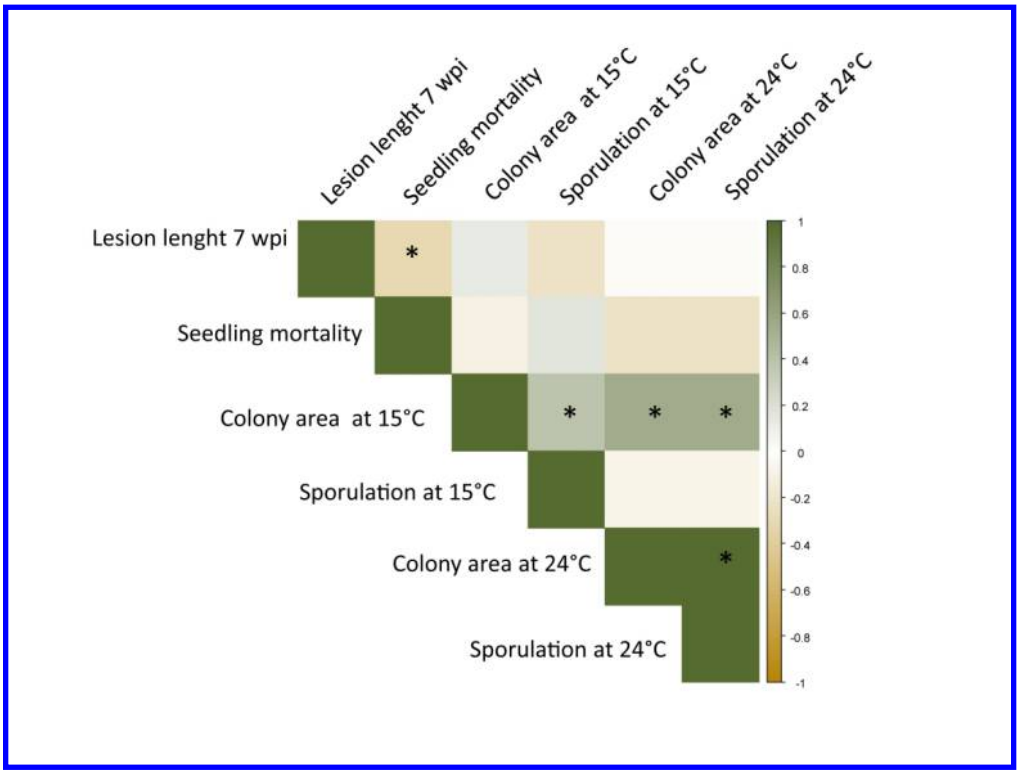

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Table S1: Geographic origin, sampling year, references and allele sizes at 10 microsatellite loci of the 29 *Cryphonectria parasitica* strains used in the study.

Isolate	Country	Province/State	Site	Sampling year	Reference	CPE1[1]	CPE5[1]	CPG14[1]	CPG4[1]	CPE3[1]	CPE4[1]	CPG6[1]	CPE8[1]	CPG3[1]	I07-650[1]
MD-1(1)	US	Maryland	Finzel, Allegany County	1991	Milgroom and Lipari (1995)	148	260	256	207	192	230	265	111	216	274
MD-1(2)	US	Maryland	Finzel, Allegany County	1991	Milgroom and Lipari (1995)	130	255	256	190	194	218	245	111	216	280
MD-2	US	Maryland	Finzel, Allegany County	1991	Milgroom and Lipari (1995)	148	260	256	190	192	230	245	111	216	295
NH-4	US	New Hampshire	Concord, Merrimack County	1993	Milgroom and Lipari (1995)	148	260	256	207	192	230	265	111	216	274
BRU-1	US	West Virgina	Butow, Pocahontas County	1994	Peever *et al.* (1997)	148	255	256	190	192	230	243	111	216	274
BRU-2	US	West Virgina	Butow, Pocahontas County	1994	Peever *et al.* (1997)	148	252	268	207	192	230	245	111	216	274
BRU-3	US	West Virgina	Butow, Pocahontas County	1994	Peever *et al.* (1997)	148	255	256	207	192	230	256	111	216	280
DU-2	US	New York	Depot Hill, Dutchess County	1990	Milgroom and Lipari (1995)	148	252	268	188	192	230	245	105	211	295
DU-5	US	New York	Depot Hill, Dutchess County	1990	Milgroom and Lipari (1995)	148	255	262	207	192	218	243	111	216	280
KY-7	US	Kentucky	Natural Bridge State Park, Powell County	1993	Milgroom and Lipari (1995)	148	260	256	190	192	230	245	111	216	295
KY-9	US	Kentucky	Natural Bridge State Park, Powell County	1993	Milgroom and Lipari (1995)	148	260	268	207	192	230	245	111	197	280
M3963	Switzerland	Ticino	Lattecaldo	2000	Prospero and Rigling (2012)	148	252	256	207	192	230	265	111	216	274
M3053	Switzerland	Ticino	Gnosca	1999	Bissegger *et al.* (1997)	148	252	256	207	192	230	265	111	216	274
M2344	Switzerland	Ticino	Claro	1992	Hoegger *et al.* (2000)	148	252	256	207	192	230	265	111	216	274
M2452	Switzerland	Ticino	Claro	1992	Hoegger *et al.* (2000)	130	252	268	207	192	230	243	111	216	280
M3983	Switzerland	Ticino	Lattecaldo	2000	Prospero and Rigling (2012)	130	252	268	207	192	230	243	111	216	280
M1693	Switzerland	Ticino	Novaggio	1997	Prospero and Rigling (2012)	130	252	268	207	192	230	243	111	216	280
SKo-126	South Korea	Geongnam	Hadong		Sang Hyun Lee, unpubl.	130	258	256	188	187	234	316	105	211	268
SKo-134	South Korea	Geongnam	Hadong		Sang Hyun Lee, unpubl.	130	255	238	188	190	234	241	102	211	328
SKo-50	South Korea	Gyeongbuk	Cheongdo		Sang Hyun Lee, unpubl.	130	252	256	188	192	236	269	105	205	268
SKo-98	South Korea	Jeonnam	Naju		Sang Hyun Lee, unpubl.	146	255	261	176	192	212	245	105	211	268
SKo-148	South Korea	Jeonnam	Naju		Sang Hyun Lee, unpul.	142	255	238	188	196	232	243	105	211	268
SKo-178	South Korea	Jeonnam	Wando		Sang Hyun Lee, unpubl.	144	258	250	190	192	234	308	99	197	268

SKo-143	South Korea	Jeonbuk	Sunchang	Sang Hyun Lee, unpubl.	130	255	250	188	192	230	267	111	197	268
SKo-77	South Korea	Jeonbuk	Imsil	Sang Hyun Lee, unpubl.	132	252	238	188	190	232	314	102	197	268
SKo-156	South Korea	Jeonbuk	Imsil	Sang Hyun Lee, unpubl.	130	258	256	188	192	236	263	105	197	268
SKo-6	South Korea	Chungnam	Gongju	Sang Hyun Lee, unpubl.	142	258	256	188	192	234	310	102	211	268
SKo-19	South Korea	Chungnam	Gongju	Sang Hyun Lee, unpubl.	144	255	256	188	192	234	310	102	211	268
SKo-210	South Korea	Chungbuk	Chungju	Sang Hyun Lee, unpubl.	130	255	238	188	198	290	267	102	211	260

[1]Size (bp) of allele at the specific microsatellite locus (Prospero and Rigling 2012).

Supplementary References

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