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Ecology and evolution of virulence in *Cryphonectria hypovirus 1*

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Summary

Summary

Parasites take advantage of their host to meet their own needs. However, this often results in damage to the host (i.e. virulence) and – in turn – may affect the parasite, which depends on the host for survival and fitness. In many cases, host-parasite interactions are of ecological, economical and/or social relevance and a better understanding of the evolution of virulence in parasites is therefore important. The aim of the present PhD thesis was to approach this knowledge gap by studying the host-parasite interaction between the plant pathogenic fungus *Cryphonectria parasitica* and its hyperparasitic virus *Cryphonectria hypovirus 1* (CHV-1).

C. parasitica is a serious tree pathogen. It causes lethal bark cankers (chestnut blight) on susceptible chestnut (*Castanea* spp.) and has destroyed the native chestnut forests after its introduction to North America from Asia. It was also introduced to Europe, but hyperparasitation by the double-stranded RNA virus CHV-1 controls the disease. Infection with CHV-1 inhibits sexual reproduction of *C. parasitica*, attenuates asexual sporulation, limits growth and reduces the pathogenic potential of *C. parasitica* towards the tree. CHV-1 is transmitted to the asexual spores of the fungus and from one fungal individual to another by hyphal anastomoses. Natural dissemination and active biocontrol treatments have lead to a high prevalence of CHV-1 in Europe. Nevertheless, environmental conditions may change, new strains of *C. parasitica* may be introduced and CHV-1 may evolve, which could affect the sustainability of the biological control. CHV-1 is an exception among RNA and among fungal viruses. Unlike most RNA viruses, CHV-1 causes persistent infections and does not solely depend on horizontal (to other individuals) but also on vertical (to offspring, i.e. spores) transmission for spread. Furthermore, in being virulent, CHV-1 differs from most fungal viruses, which do not cause any symptoms in their hosts.

The objective of my PhD thesis was to gain a better understanding of the factors that contributed to the successful establishment of CHV-1 as a biocontrol agent throughout the chestnut growing regions in Europe, to detect and describe drivers that govern the evolution of virulence in CHV-1 and to provide insight into the emergence of CHV-1.

An important factor influencing the outcome of host-parasite interactions is the external environment. In a climate chamber experiment, I investigated the impact of temperature on different fungus-virus combinations (Chapter 2). The finding of highly significant genotype-by-genotype-by-environment interactions indicated that environmental factors may change virulence expression in interactions between particular *C. parasitica* and CHV-1 genotypes. Furthermore, different host and parasite genotypes may be selected

under different environmental conditions, affecting the coevolutionary dynamics of the host-parasite interaction, and thus the course of chestnut blight epidemics.

In *C. parasitica*, horizontal transmission of viruses is restricted by vegetative incompatibility between fungal individuals. It had therefore been hypothesized that high levels of vegetative incompatibility not only hampered virus spread within the population but also selected for lower virulence in CHV-1. Ultimately, this would lead to an erosion of the biological control. I tested this hypothesis by assessing the virulence of CHV-1 in natural *C. parasitica* populations with high and with low levels of vegetative incompatibility (Chapter 3). However, presence or absence of transmission barriers did not seem to affect virus virulence, which is promising for the sustainability of the biological control even under increased levels of vegetative incompatibility.

These results also raised the question if high virulence not only negatively affected virus transmission by limiting the production of asexual fungal spores carrying the virus (and thus making transmission possible). To explore the potential for certain virulence benefits for virus transmission, I investigated CHV-1 strains differing in virulence and assessed their transmission rate per host-to-host contact (transmissibility) (Chapter 4). Indeed, high virulence strongly correlated with high transmissibility. In the context of other recent studies, this result suggests that increased host debilitation may be associated with increased transmissibility in CHV-1. Such coupling of virulence factors could explain why CHV-1 has spread widely and has evolved higher virulence than most other fungal viruses.

To reconstruct the invasion history of CHV-1 in Europe, I analyzed nucleotide sequence variations from CHV-1 isolates sampled across a wide area in Europe (Chapter 5). The results indicated that the evolutionary scenarios for CHV-1 and *C. parasitica* in Europe were spatially congruent. Phylogenetic and coalescent analyses suggested that CHV-1 was introduced together with *C. parasitica* from Asia and that its spread across Europe was not continuous. I demonstrated the geographic, vegetation-related, demographic, economic and political factors may help to explain the correlated invasion pattern of a viral parasite and its host.

In conclusion, the four studies conducted as part of this PhD thesis have increased our understanding of the *Cryphonectria*-hypovirus pathosystem and provided insight into its complexity. Changes in the virus, the fungus and/or the environment may interact and affect the host-parasite interaction. Overall, this thesis highlights the fact that the biological details of host-parasite interactions and the cost-benefit relationships involved are keys for understanding the ecology and evolution of parasite virulence.

Zusammenfassung

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Parasiten beuten ihren Wirt aus, um ihre eigenen Bedürfnisse zu befriedigen. Die Schädigung des Wirtes kann aber auch den Parasiten selbst beeinträchtigen, dessen Überleben und Fitness vom Wirt abhängig sind. Viele Wirt-Parasit Beziehungen sind ökologisch, ökonomisch und/oder sozial von Bedeutung. Zu verstehen, welche Faktoren die Virulenz (d.h. Schädigung des Wirtes) von Parasiten beeinflussen, ist deshalb sehr wichtig. Das Ziel der vorliegenden Dissertation war, einen Einblick in die Evolution der Virulenz zu erlangen und die Wirt-Parasit Beziehung zwischen dem pflanzenpathogenen Pilz *Cryphonectria parasitica* und seinem hyperparasitischen Virus *Cryphonectria hypovirus 1* (CHV-1) zu untersuchen.

C. parasitica ist ein gefährlicher Krankheitserreger. Er verursacht letale Rindenkrebse (Kastanienrindenkrebs) auf anfälligen Edelkastanien (*Castanea* spp.) und hat nach seiner Einschleppung aus Asien nach Nordamerika die dort heimischen Edelkastanienwälder zerstört. Der Pilz wurde auch nach Europa verschleppt, wo er allerdings vom Doppelstrang-RNA Virus CHV-1 hyperparasitiert und unter Kontrolle gehalten wird. Eine Infektion von *C. parasitica* mit CHV-1 verhindert die sexuelle Reproduktion des Pilzes, verringert die asexuelle Sporulation, reduziert das Wachstum und limitiert die schädigende Wirkung des Pilzes auf den Baum. CHV-1 wird in die asexuellen Pilzsporen übertragen und über Anastomosen von Hyphen von einem Pilz zum anderen weitergegeben. Seine natürliche Ausbreitung und die aktive Ausbringung zur biologischen Kontrolle haben zu einer starken Verbreitung des Virus in Europa geführt. Ob allerdings die biologische Kontrolle des Kastanienrindenkrebses mit CHV-1 auch langfristig nachhaltig ist, bleibt ungewiss. Sich ändernde Umweltbedingungen, neue Einschleppungen von *C. parasitica* und/oder Evolution von CHV-1 könnten das Biokontrollsystem beeinträchtigen. CHV-1 ist eine Ausnahme unter RNA Viren wie auch unter Pilzviren. Anders als die meisten RNA Viren verursacht CHV-1 dauerhafte Infektionen im Wirt. Für seine Ausbreitung ist er nicht nur auf die horizontale Übertragung (von Pilz zu Pilz) sondern zusätzlich auch auf die vertikale Übertragung (auf Nachkommen, d.h. in die Sporen) angewiesen. Im Gegensatz zu den meisten anderen Pilzviren ist CHV-1 virulent und ruft in seinem Wirt starke Symptome hervor.

Mit meiner Dissertation wollte ich ein besseres Verständnis der Einflussgrössen, die zur erfolgreichen Etablierung von CHV-1 als Biokontrollorganismus in Europa geführt haben, erlangen. Mein Ziel war es, Faktoren zu identifizieren, welche die Virulenz von CHV-1 massgeblich beeinflussen und einen Einblick in die Evolution von CHV-1 in Europa zu erhalten.

Die Umwelt übt einen starken Einfluss auf die Ausprägung einer Wirt-Parasit Beziehung aus. In einem Klimakammerexperiment habe ich untersucht, welche Auswirkungen die Temperatur auf die Pilz-Virus Wechselwirkung hat (Kapitel 2). Ich konnte eine hoch signifikante Genotyp x Genotyp x Umwelt Interaktion nachweisen. Dies bedeutet, dass Umweltfaktoren die Virulenz auch in Wechselwirkungen zwischen spezifischen Genotypen von *C. parasitica* und CHV-1 verändern können. Überdies legen diese Resultate nahe, dass die klimatischen Bedingungen eine Auswirkung auf die Selektion von interagierenden Pilz- und Virusgenotypen und somit auch auf deren Koevolution haben. Dadurch könnte der Verlauf von Kastanienrindenkrebsepidemien beeinflusst werden.

Die horizontale Übertragung von Viren wird in *C. parasitica* durch vegetative Inkompatibilität zwischen Pilzen behindert. Deshalb wurde die Hypothese aufgestellt, dass ein hoher Grad an vegetativer Inkompatibilität nicht nur die Verbreitung von Viren innerhalb der Pilzpopulation einschränkt, sondern auch längerfristig eine tiefere Virusvirulenz mittels Selektion herbeiführt. Dies würde schlussendlich zu einer Erosion der biologischen Kontrolle führen. Ich habe diese Hypothese getestet, indem ich die Virulenz von CHV-1 in natürlichen *C. parasitica* Populationen mit hohem und mit tiefem Grad an vegetativer Inkompatibilität untersuchte (Kapitel 3). Das Vorhandensein von Übertragungsbarrieren schien jedoch die Virulenz der Viren nicht zu beeinträchtigen. Diese Erkenntnis ist vielversprechend und legt nahe, dass die Wirksamkeit der biologischen Kontrolle sogar bei steigendem Grad an vegetativer Inkompatibilität in der Pilzpopulation gegeben ist.

Die Resultate warfen allerdings auch die Frage auf, ob hohe Virulenz die Virusübertragung nicht ausschliesslich negativ beeinflusst. Virulente Viren schränken die Produktion asexueller Sporen in ihrem Wirt stark ein und verringern dadurch ihr eigenes Ausbreitungs- und Übertragungspotenzial. Um der Frage nachzugehen, ob hohe Virulenz möglicherweise auch positive Auswirkungen auf die Virusübertragung hat, untersuchte ich Viren unterschiedlicher Virulenz und bestimmte deren Übertragungsrate von einem Pilz auf den anderen (Übertragbarkeit) (Kapitel 4). Es ergab sich eine starke Korrelation zwischen hoher Virulenz und hoher Übertragbarkeit. Vor dem Hintergrund anderer aktueller Studien legt dieses Resultat die Vermutung nahe, dass in CHV-1 eine stärkere Beeinträchtigung des Wirtes mit einer erhöhten Virusübertragbarkeit verbunden ist. Eine derartige Koppelung von Virulenzfaktoren könnte erklären, weshalb CHV-1 eine höhere Virulenz als die meisten anderen Pilzviren entwickelt und sich gleichzeitig erfolgreich weit ausgebreitet hat.

Um die Invasionsgeschichte von CHV-1 in Europa zu rekonstruieren, analysierte ich die Unterschiede in den Nukleotidsequenzen von CHV-1 Isolaten, welche aus einem grossem Gebiet in Europa stammten (Kapitel 5). Meine Resultate wiesen darauf hin, dass die

Evolutionsszenarien für CHV-1 und *C. parasitica* räumlich kongruent sind. Koaleszenz- und phylogenetische Analysen legten nahe, dass CHV-1 zusammen mit *C. parasitica* aus Asien eingeschleppt wurde und dass sich CHV-1 im Folgenden nicht kontinuierlich in Europa ausbreitete. Ich konnte aufzeigen, dass sowohl geographische, Vegetations-bedingte, demographische, wirtschaftliche als auch politische Faktoren berücksichtigt werden müssen, um die korrelierten Invasionsmuster eines Virusparasiten und dessen Wirtes zu erklären.

Zusammengefasst leisten die vier Studien dieser Dissertation einen wesentlichen Beitrag zu einem besseren Verständnis des *Cryphonectria*-hypovirus Pathosystems, beleuchten aber auch dessen Komplexität. Sie zeigen auf, dass sich Veränderungen im Virus, im Pilz und/oder in der Umwelt wechselseitig beeinflussen und somit die Ausprägung der Wirt-Parasit Beziehung bestimmen können. Diese Dissertation macht deutlich, dass die biologischen Details einer Wirt-Parasit Beziehung von grosser Bedeutung sind und dass Kosten-Nutzen Verhältnisse für die Ökologie und die Evolution der Virulenz eine zentrale Rolle spielen.

Chapter 1

General introduction

Sarah F. Bryner

Introduction

Cryphonectria hypovirus 1, one of a kind

Cryphonectria hypovirus 1 (CHV-1) is a double stranded (ds) RNA virus which infects the ascomycete fungus *Cryphonectria parasitica* (Hillman and Suzuki 2004). Some characteristics of CHV-1 are exceptional among RNA viruses or among fungal viruses and make it a remarkable organism. For example, in contrast to most RNA viruses, which cause acute infections, infections with CHV-1 are persistent (Nuss 2005; Holmes 2008). Furthermore, CHV-1 is virulent and strongly debilitates its fungal host. It inhibits sexual reproduction, strongly attenuates growth and asexual sporulation and reduces pigmentation of the fungus (Milgroom and Cortesi 2004). Throughout this thesis, virulence is referred to as the harm a parasites causes to its host (i.e. mortality as well as reduction in fitness due to morbidity). High virulence is a common characteristic of RNA viruses (Holmes 2004, 2008) but not of fungal viruses. Infection with the vast majority of fungal viruses is asymptomatic in the host, which is consistent with the fact that all fungal viruses completely rely on their host for survival and transmission (Nuss 2005). Fungal viruses lack an extracellular stage outside the host and cannot exit the fungal thallus and reenter as infectious particles (Hillman 2001). Virus transmission between fungi therefore occurs intracellularly. CHV-1 depends on the combination of both horizontal (to other fungal individuals) and vertical (to offspring, i.e. fungal spores) transmission, and thus dispersal of CHV-1 is tightly connected to dispersal of its host. In this respect, CHV-1 also differs from other, virulent RNA viruses, which are exclusively transmitted horizontally (Holmes 2004, 2008), and thus do not require their host to reproduce. CHV-1, however, is transferred into the asexual (but not the sexual) spores of the fungus, dispersed in these spores and then transmitted from the outgrowing spores to other fungal individuals by hyphal fusion (Hoegger et al. 2003; Milgroom and Cortesi 2004). Virulence, thus, compromises the spread of CHV-1 directly, indicating that the cost of high virulence should also be high for CHV-1. Nevertheless, CHV-1 has spread widely throughout the *C. parasitica* populations in Europe (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). This high ecological fitness of CHV-1 is surprising and challenges our notions of virulence and fitness.

Biological control of chestnut blight

Due to the combination of these characteristics, CHV-1 has become a text book example and a well-known biocontrol agent for the serious tree disease chestnut blight. The host of CHV-1, *C. parasitica*, is a tree pathogen, which colonizes the bark of chestnut trees through wounds. *C. parasitica* destroys the cambial tissue of susceptible chestnut

(*Castanea* spp.) and causes lethal bark cankers (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Such cankers are sunken in appearance, typically expand and girdle the stem and eventually kill all parts of the tree distal to the canker (Fig. 1). Infection of *C. parasitica* with CHV-1, however, reduces the pathogenic potential of *C. parasitica*, a phenomenon called hypovirulence (Van Alfen et al. 1975). CHV-1 does not kill *C. parasitica*, but it severely affects the fitness of *C. parasitica.* Virus-infected individuals of *C. parasitica* cause superficial, swollen cankers and do not destroy the cambium of the tree. Such cankers are colloquially known as "healing cankers" because they do little or no harm to the tree (Heiniger and Rigling 1994; Milgroom and Cortesi 2004).

Fig. 1. Chestnut blight symptoms. Highly pathogenic, virus-free *Cryphonectria parasitica* strains destroy the cambium of the tree and cause a sunken cankers (left). Hypovirulent, virus-infected strains cause superficial, "healing" cankers (middle). The fruiting bodies of *C. parasitica* break through the bark of the tree (right). Photographs by Sarah Bryner.

C. parasitica is native to East Asia where chestnut blight is only a minor problem since the Asian chestnuts (primarily *Catanea mollissima* and *Castanea crenata*) are resistant to the disease (Milgroom and Cortesi 2004). However, *C. parasitica* was accidentally introduced to North America around 1904 and to Europe around 1938, where the local chestnut species had not coevolved with *C. parasitica* (Anagnostakis 1982). In a devastating epidemic, *C. parasitica* destroyed the North American chestnut forests and nearly eradicated the American chestnut (*Castanea dentata*). It became a classical example of an introduced pathogen that had encountered a highly susceptible host population (Anagnostakis 1987). In Europe, initially, a similar story unfolded on European chestnut (*Castanea sativa*). *C. parasitica* was first recorded near Genoa (Italy) from where it spread rapidly throughout the chestnut growing regions, evoking a dramatic epidemic. However, a few years later, the appearance of superficial cankers was observed, which did not lead to dieback of the infected trees (Heiniger and Rigling 1994).

CHV-1 was discovered since healing of these cankers was found to be the result of CHV-1 infection (Choi and Nuss 1992).

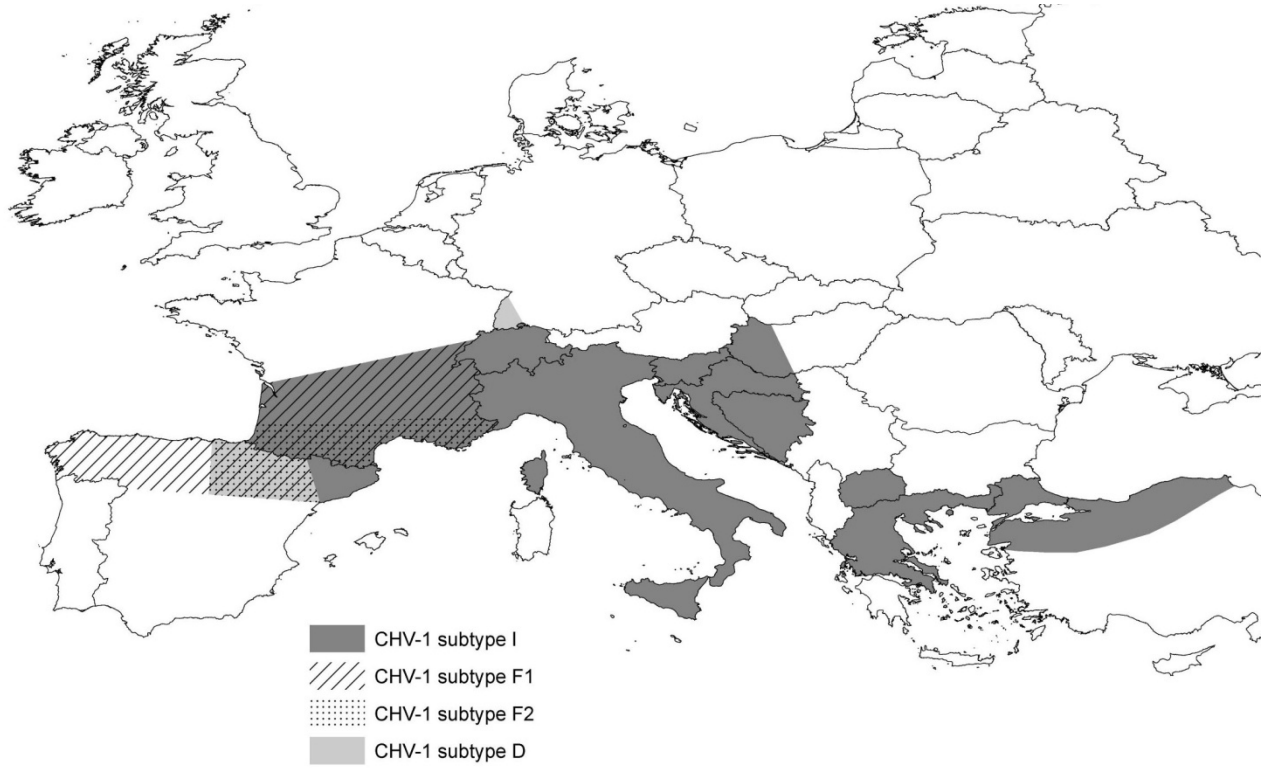

Fig. 2. Distribution of *Cryphonectria hypovirus 1* subtypes in Europe.

CHV-1 spread independently throughout the *C. parasitica* populations in Europe by transmission from one fungal individual to another and caused hypovirulence in *C. parasitica*. It was assumed that CHV-1 had been introduced together with *C. parasitica* and that it followed emergence and spread of the fungus (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Evolution of obligate parasites such as viruses is often spatially congruent with that of their host, since their distribution is shaped by host dispersal (Real et al. 2005; Biek et al. 2007; Nadin-Davis et al. 2010; Torres-Pérez et al. 2011). However, no studies have investigated this in CHV-1. Natural dissemination of CHV-1 was aided by active biocontrol efforts, which together have led to a high prevalence of virus infection in Europe (Heiniger and Rigling 1994). To date, chestnut blight incidence in Europe is very high but the disease is maintained at low severity due to hypovirulence. In North America, biocontrol treatments with CHV-1 were also undertaken, but with little success (Milgroom and Cortesi 2004). Answers to the question why efforts to establish hypovirulence in North America had failed have remained inconclusive. Potential reasons might be the higher blight susceptibility of the American chestnut, differences in the fungal host population structure, differences in the virulence

of the viruses applied, environmental differences or an interaction of these factors (MacDonald and Fulbright 1991; Dawe and Nuss 2001; Milgroom and Cortesi 2004). Furthermore, in contrast to the situation in North America, hypovirulence emerged at an early phase of the blight epidemic in Europe (Anagnostakis 1982), and thus may have prevented devastating epidemics.

Four genetically distinguished subtypes of CHV-1 have been described in Europe: subtype I, F1, F2 and D (Allemann et al. 1999; Gobbin et al. 2003). Nucleotide difference among subtypes was estimated to be 11 – 19% (Gobbin et al. 2003). Subtype I is the most widespread and is prevalent all over Italy, France, Switzerland, the Balkans, Greece and Turkey (Fig. 2) (Allemann et al. 1999; Gobbin et al. 2003; Sotirovski et al. 2006; Krstin et al. 2008; Robin et al. 2010; Krstin et al. 2011). Subtypes F1 and F2 are less prevalent and have only been identified in France and northern Spain, while Subtype D has been recorded from southern Germany and northern Spain (Fig. 2) (Allemann et al. 1999; Gobbin et al. 2003; Montenegro et al. 2008; Robin et al. 2010). The four subtypes not only differ in distribution and prevalence but also in virulence (Chen and Nuss 1999; Peever et al. 2000; Robin et al. 2010). Subtype I is the least virulent, subtype D is intermediate and the subtypes F1 and F2 are the most virulent.

Genetic organisation of CHV-1, functions and ecology

CHV-1 belongs to the family *Hypoviridae*, which consists of the single genus *Hypovirus* (Nuss and Hillman 2011; Rigling and Hillman 2012). Three other members of that genus – CHV-2, CHV-3 and CHV-4 – have been described, all of which are parasites of *C. parasitica*. Unlike most other viruses, hypoviruses do not possess a coat protein. Their dsRNA genomes are encapsulated within host-derived vesicles (Hillman 2001). Despite their dsRNA genome, hypoviruses follow more closely the properties of positive-sense RNA viruses than true dsRNA viruses. It is generally thought that the dsRNA is most likely an artefact of the replication cycle during which the nascent and template RNA strands anneal and copurify (Fahima et al. 1993).

The four hypovirus species differ in genome size and organization (Hillman and Suzuki 2004). The genome of CHV-1 is 12.7 kb in size and consists of two large open reading frames (ORFs) designated ORF A and ORF B. Both ORFs encode polyproteins which are autocatalytically cleaved (Dawe and Nuss 2001). ORF A encodes the proteinase p29 and the basic protein p40. ORF B encodes a paralogue of p29, the proteinase p48, and the RNA-dependent RNA polymerase and RNA helicase. The genome of the hypovirus CHV-2 is similar in size (12.5 kb) and also consists of two ORFs (Dawe and Nuss 2001). The organization of ORF B is very similar to the CHV-1 ORF B with a p48 homologue, p52, and polymerase and helicase motifs of high sequence conservation. The organization of

ORF A, however, is different. In fact, ORF A of CHV-2 encodes for just one protein, p50, that contains a basic domain homologous to CHV-1 p40, and thus lacks a p29 homologue (Hillman and Suzuki 2004). The genome of CHV-3 is considerably smaller (9.8 kb) than those of CHV-1 and CHV-2 and consists of a single ORF (Dawe and Nuss 2001). It encodes a homologue of CHV-1 p29, p32, and a glycosyltransferase, a polymerase and a proteinase domain (Hillman and Suzuki 2004). CHV-4 has a 9.1 kb genome also consisting of a single ORF, encoding for a polymerase, a helicase and most likely for a CHV-1 p29 homologue (Linder-Basso et al. 2005). Furthermore, it also encodes a glycosyltransferase domain homologous to the one present in CHV-3.

CHV-1 has received the most attention among these hypovirus species because of its important role in biological control and its prevalence in Europe. The ability of CHV-1 to fundamentally alter the phenotype of *C. parasitica* and its ecological fitness (at least in Europe) have provoked efforts in identifying the functional domains in CHV-1 responsible for these effects. Mapping studies using sequence knock-out mutants succeeded in identifying CHV-1 encoded determinants responsible for altering fungal host phenotype and for increasing viral fitness (Dawe and Nuss 2001). It was shown that p29 plays an important role in the virus-fungus interaction. Suppression of pigmentation, of asexual sporulation and of laccase production of the fungus were associated with p29 (Craven et al. 1993). In addition, p29 also increased RNA replication and vertical virus transmission into asexual spores (Suzuki et al. 2003). It was further shown that p40 influenced RNA accumulation (Suzuki and Nuss 2002). Domains in ORF B were identified to be responsible for canker size, canker morphology, growth of the fungus (Chen et al. 2000) and perturbation of cellular signal transduction pathways in the fungus (Parsley et al. 2002).

Differences in genome organization and functions may explain differences in phenotype, ecology and fitness among the four CHV species. CHV-1 is not only prevalent in Europe but also in Asia, which is thought to be its centre of origin (Milgroom and Cortesi 2004). However, CHV-1 has not been found in North America, except in a few places where it has been released for biological control (Peever et al. 1997). In contrast to CHV-1, CHV-2 is more limited in its distribution (Hillman and Suzuki 2004). It has been found in two locations only: one small population in New Jersey and a few independent isolates in China. CHV-2 also induces hypovirulence in *C. parasitica* and debilitates the fungus even stronger than CHV-1 does but it is of very low ecological fitness (Hillman et al. 1994). The situation is again different with CHV-3. CHV-3 occurs naturally in North America, but neither in Asia or Europe. It is prevalent in Michigan and Ontario and also causes hypovirulence but its effect on the fungus is much lower than that of CHV-1 or CHV-2 (Hillman et al. 1994). CHV-4, finally, is widespread throughout North America, however, it is not associated with hypovirulence and has very little or no effect on *C. parasitica*

(Linder-Basso et al. 2005). None of these three hypovirus species have, thus, been very successful as biocontrol agents, either due to a lack of ecological fitness or due to a limited ability to reduce the pathogenic potential of *C. parasitica* (Milgroom and Cortesi 2004). Virulence combined with ecological fitness is a special characteristics of CHV-1, which has led to the successful establishment of CHV-1 as a biocontrol agent of *C. parasitica* in Europe.

Factors that govern virulence of CHV-1

Understanding the factors that govern the evolution of virulence in CHV-1 may be of interest in both a fundamental and an applied context. Parasites live in an intimate relationship with their hosts. From a fundamental perspective, the question why and under which circumstances parasites harm their host when – at the same time – they depend on their host for survival and fitness has been intriguing researchers for several decades (Anderson and May 1982; Bull 1994; Frank 1996; Walther and Ewald 2004; Alizon et al. 2009). Despite the growing understanding of the evolution of virulence in host-parasite systems, answers to this question are still inconclusive. From an applied perspective, virulence evolution is crucial to the sustainability and effectiveness of biological control. Biocontrol agents need to be virulent enough to achieve sufficient disease control but as well, should be able to spread independently and persist in the ecosystem. However, little is known about the factors that drive the evolution of virulence in biocontrol agents (Hufbauer and Roderick 2005).

One important factor influencing virulence and outcome of host-parasite interactions is the external environment. Temperature, for example, has been shown to alter infection-related traits in host-parasite systems (Wolinska and King 2009). Temperature may generally increase or decrease the effect of parasitism (Mitchell et al. 2005; Vale et al. 2008). Furthermore, it may specifically affect the relative resistance of certain host genotypes (Browder 1985; Blanford et al. 2003) or the relative virulence of certain parasite genotypes (genotype-by-environment interaction; Laine 2008; Vale and Little 2009). This raises the question of whether temperature can also alter the interaction between specific host and parasite genotypes (genotype-by-genotype-by-environment interaction). The vast majority of studies has investigated environmental effects on different genotypes in one of the two interacting species and has therefore not enabled statistical testing of genotype-by-genotype-by-environment interactions (Thomas and Blanford 2003; Lazzaro and Little 2009; Wolinska and King 2009). Knowing about the presence of genotype-by-genotype-by-environment interactions would be important since they would not only affect the outcome of host–parasite interactions in different environments but could also govern the direction and strength of natural selection in host–parasite systems. Genotype-by-genotype-by-environment interactions in the

Cryphonectria-hypovirus pathosystem may therefore be crucial for the sustainability and effectiveness of the biological control. For example, viruses that successfully control chestnut blight in certain regions might be ineffective in other regions with a different climate. This would require designing adjusted management strategies for each region and evaluating the most suitable biocontrol agent for each case individually. Furthermore, under the presence of genotype-by-genotype-by-environment interactions, climate change could disturb the subtle interaction between CHV-1 and *C. parasitica*. This may turn a formerly hypovirulent fungus more agressive and lead to a reemergence of the chestnut blight epidemic in regions where the disease has been under control.

Another important factor that might affect virulence and biocontrol success of CHV-1 is the host population. As mentioned earlier, CHV-1 depends on the combination of vertical and subsequent horizontal transmission for spread. Horizontal transmission by hyphal fusion between fungal individuals, however, is restricted by vegetative incompatibility in *C. parasitica* (Liu and Milgroom 1996). Vegetative incompatibility in fungi is a self/nonself recognition system (Glass and Kaneko 2003). In *C. parasitica,* vegetative incompatibility is controlled by at least six bi-allelic unlinked vegetative incompatibility (*vic*) loci (Cortesi and Milgroom 1998). Compatible individuals share the same alleles at all six *vic* loci while incompatible individuals differ in their alleles at one or more loci. Hence, the *vic* haplotype defines the vegetative compatibility (vc) types (Cortesi and Milgroom 1998). Fusion between cells of incompatible individuals results in cell death, hampering the transmission of viruses and other cytoplasmic elements. Between compatible individuals of *C. parasitica*, CHV-1 is transmitted in virtually 100% of host-to-host contacts. Among different vc types, transmission can also occur, but at reduced rates (Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). The transmission probability of CHV-1 in *C. parasitica* populations with high vc type diversity is therefore expected to be greatly reduced, thus, limiting the spread of CHV-1 (Cortesi et al. 2001; Milgroom and Cortesi 2004). In addition, high vc type diversity in the host population may also affect the evolution of virulence in CHV-1. Virulent viruses excessively debilitate their host and, in doing so, greatly reduce their own transmission potential. Under the presence of increased transmission barriers imposed by the host population, virulent viruses are therefore expected to be strongly selected against, leading to evolution of low virulence. The existence of these vegetative incompatibility barriers in fungi may be one of the factors that has promoted evolution of low virulence in most fungal viruses (Milgroom 1999). One of the reasons for the failure of biological control with CHV-1 in North America was assumed to be the much higher vc type diversity of American *C. parasitica* populations compared to European populations (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). However, new introductions or sexual reproduction between divergent genotypes of *C. parasitica* (Jezic et al. 2012) may also lead to an increase in vc type

diversity in European populations. A potential erosion of the biological control over time is therefore of great concern.

In addition to the environment and the host population, virus-dependent factors may also influence virus virulence. Current theory suggests that the evolution of virulence depends on a cost-benefit relationship in the parasite: The benefits of host exploitation on parasite multiplication within hosts are traded off against the cost of this exploitation for parasite transmission between hosts (Anderson and May 1982; Bull 1994; Frank 1996). Highly virulent parasites multiply rapidly and exploit their host efficiently. This, however, occurs at the cost of increased host mortality or debilitation, limiting the host's ability to transmit the parasite. Several models were developed to describe the evolution of virulence in host-parasite systems (Levin and Pimentel 1981; Frank 1992; Antia et al. 1994; Day 2001; Koella and Restif 2001; Day 2002) and generally suggest that the optimal degree of virulence maximizes the parasite's fitness, and thus depends on the particular cost-benefit relationship of the host-parasite interaction. Some parasites rely on relatively normal host functions for transmission and, hence, may not be highly virulent, while other parasites may debilitate their host without strongly limiting transmission (Read 1994; Ewald 1995). Vertically (to offspring) transmitted parasites, for example, depend on reproduction of the infected host. Their cost of host debilitation is much higher than for horizontally (to other hosts) transmitted parasites, which do not require the host to reproduce (Bull et al. 1991). Similarly, the cost of host debilitation is much higher for parasites that cannot survive outside the host and depend on a live host than for parasites that are able to survive outside the host (Walther and Ewald 2004). Virulent strains of CHV-1 were shown to have a higher virus replication rate in the host and a higher virus-infection rate of asexual fungal spores (Suzuki et al. 2003; Lin et al. 2007). However, this seems to occur at a very high cost for CHV-1 since virulence is associated with attenuation of fungal growth and of production of asexual fungal spores spreading the virus within and among populations (Milgroom and Cortesi 2004). Therefore, the question why highly virulent strains of CHV-1 are prevalent in Europe has been a puzzle (Taylor 2002; Milgroom and Cortesi 2004). There seem to be additional, yet unidentified, benefits of high virulence, which counterbalance virulence costs. So far, it has been commonly assumed that the rate of virus transmission per host-to-host contact (transmissibility) was not influenced by the virus (Cortesi et al. 2001). However, more virulent viruses might be more transmissible than less virulent viruses, and thus have a higher transmission rate per host-to-host contact. This would be a major virulence benefit and may counterbalance negative effects of high virulence on asexual sporulation of the fungus. Identifying the relationship between virulence costs and virulence benefits is crucial for making predictions about virulence evolution in CHV-1 since this relationship determines the optimal degree of virulence.

Aims of this thesis

The aim of this PhD thesis was to obtain a better understanding of the characteristics that make CHV-1 an exceptional virus and that may have been responsible for its success in Europe so far. My approach was to detect and describe factors that govern the evolution of virulence in CHV-1 and to draw a clearer picture of the CHV-1 emergence in Europe. I investigated the impact of genotype-by-genotype-by-environment interactions (Chapter 2) and of transmission barriers in the host population (Chapter 3) on virulence in CHV-1. I also studied the cost-benefit relationship of virulence in CHV-1 and explored the potential for the presence of virulence benefits for virus transmission (Chapter 4). Furthermore, I analyzed nucleotide sequence variations among CHV-1 isolates sampled from a wide area in Europe to reconstruct the invasion history of CHV-1 (Chapter 5).

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Chapter 2

Temperature-dependent genotype-by-genotype interaction between a pathogenic fungus and its hyperparasitic virus

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Abstract

The outcome of host–parasite interactions may not only depend on the genotypes of the species involved but also on environmental factors. We used the fungus *Cryphonectria parasitica*, the causal agent of chestnut blight, and its hyperparasitic virus, *Cryphonectria hypovirus 1* (CHV-1), to test for genotype-by-genotype-by-environment interactions in a host–parasite system. In *C. parasitica*, infection with CHV-1 induces a hypovirulent phenotype with reduced virulence towards the chestnut tree (*Castanea* spp.) and thus controls chestnut blight in many European regions. In contrast, uninfected virulent *C. parasitica* have nearly eradicated the American chestnut in North America. We applied a full factorial design and assessed the fungal growth and sporulation of four *C. parasitica* strains, uninfected and infected with each of the four known CHV-1 subtypes, at 12°C, 18°C, 24°C and 30°C. We found a significant ($p \leq 0.0001$) genotype-by-genotype-by-environment interaction, demonstrating the potential for a selection mosaic. As a consequence, different host and parasite genotypes would be selected under different climatic conditions, affecting the coevolutionary dynamics of the host–parasite interaction and the course of chestnut blight epidemics. Genotype-by-genotype-by-environment interactions are essential to take into account when designing biological control strategies.

Introduction

Parasites and hosts attempt to manipulate each other's physiology and fitness. Ultimately, the outcome of the interaction between a host and a parasite may depend on particular combinations of genotypes (genotype-by-genotype interaction; Browder 1985; Thompson and Burdon 1992; Peever et al. 2000; Carius et al. 2001). Coevolutionary theory states that the antagonistic relationship between hosts and parasites results in an evolutionary arms race (Dawkins and Krebs 1979). Furthermore, it has been hypothesized that this arms race is influenced by environmental factors (Little 2002; Thomas and Blanford 2003; Mitchell et al. 2005; Wolinska and King 2009). One important environmental variable is temperature, which has been shown to alter infection related traits of hosts and parasites (Wolinska and King 2009). On the one hand, temperature alone can generally increase or decrease the effect of parasitism (Mitchell et al. 2005; Vale et al. 2008b). On the other hand, temperature may specifically interact with certain host genotypes, influencing their relative resistance (Browder 1985; Blanford et al. 2003), and with particular parasite genotypes, influencing their relative virulence (genotype-by-environment interaction; Laine 2008; Vale and Little 2009). This raises the question of whether temperature also alters the interaction between specific host and parasite genotypes (genotype-by-genotype-by-environment interaction). To date, the vast majority of studies has only included environmental effects on different genotypes in one of the two interacting species and has therefore not enabled statistical testing of genotype-by-genotype-by-environment interactions (Thomas and Blanford 2003; Lazzaro and Little 2009; Wolinska and King 2009). We are aware of no more than three studies in host–parasite systems that statistically analyzed this three-way interaction (Laine 2007; Tétard-Jones et al. 2007; Vale and Little 2009). A significant genotype-by-genotype-by-environment interaction was only found in one of these studies, which investigated the interaction with a biotic environmental factor (rhizobacteria; Tétard-Jones et al. 2007).

Demonstrating the existence of genotype-by-genotype-by-environment interactions may be of interest for two different reasons: first, to predict phenotypic expression and the outcome of host–parasite interactions in different environments, and second, to understand how natural selection acts on host–parasite interactions in different environments. The effect of the environment on the phenotype is described by the reaction norm, which displays the series of phenotypes expressed by a genotype across a range of environments (Gomulkiewicz and Kirkpatrick 1992). The reaction norm of the interaction between two species is called an interaction norm (Thompson 1988). Genotype-by-genotype-by-environment interactions are indicated by non-parallel slopes of interaction norms. Crossing host–parasite interaction norms would further indicate that no combination of host and parasite genotypes exists that outperforms all the others

across a range of environments and that different combinations of coevolving host and parasite genotypes are favored in different environments. Such variation in natural selection among ecosystems is hypothesized by the geographic mosaic theory of coevolution (Thompson 1999; Gomulkiewicz et al. 2000). This theory assumes the presence of a selection mosaic in which genotype-by-genotype-by-environment interactions govern the extent and the direction of natural selection on interacting species. Despite growing evidence for the presence of geographic selection mosaics (Benkman 1999; Zangerl and Berenbaum 2003; Toju and Sota 2006; Laine 2009), testing for their existence is not trivial (Gomulkiewicz et al. 2007). Most empirical studies have only assessed whether selection on coevolving species differs in different environments and not whether differences are due to variation in how selection acts on the same genotype-by-genotype interactions in different environments (Hoeksema et al. 2009). A first step in this direction would be to demonstrate the potential for a selection mosaic by proving the existence of genotype-by-genotype-by-environment interactions (Gomulkiewicz et al. 2007; Hoeksema et al. 2009).

The pathogenic fungus *Cryphonectria parasitica*, the causal agent of chestnut blight, and its hyperparasitic virus *Cryphonectria hypovirus* (CHV) constitute a model system for studying fungus–virus interactions. This system provides a textbook example of a viral disease that significantly reduces fungal virulence, a phenomenon called hypovirulence (Van Alfen et al. 1975; Taylor et al. 1998; Nuss 2005). Among the four CHV species described, most attention has been given to CHV-1 due to its role in biological control of chestnut blight and its high prevalence in Europe (Hillman and Suzuki 2004). Within CHV-1, four genetically distinguished subtypes are identified: Italian subtype I, German/Spanish subtype D and French subtypes F1 and F2 (Gobbin et al. 2003).

The goals of this study were to elucidate the influence of temperature on the host–parasite interaction between *C. parasitica* and CHV-1 and to test for a genotype-by-genotype-by-environment interaction, thereby exploring the potential for a temperature-dependent selection mosaic. In a full factorial design, we used four *C. parasitica* strains as virus-free controls and in combination with each of the four CHV-1 subtypes and assessed fungal growth and sporulation at four temperatures.

Materials and methods

The study system

C. parasitica is a tree pathogen originating from East Asia that causes lethal bark cankers on susceptible *Castanea* spp. It was introduced to both North America and Europe during the past century. In a devastating epidemic, it has nearly eradicated American chestnut (*C. dentata*) in North America (Anagnostakis 1982). In contrast, chestnut blight incidence is high on European chestnut (*C. sativa*) in Europe but maintained at low severity in most regions (Heiniger and Rigling 1994) due the infection of *C. parasitica* with CHV-1, an unencapsidated double-stranded RNA virus of the genus *Hypovirus* (Choi and Nuss 1992). CHV-1 significantly decreases canker growth, strongly attenuates asexual sporulation and almost completely inhibits sexual reproduction of its fungal host (Elliston 1985; Zhang et al. 1998; Peever et al. 2000). It is dispersed by asexual fungal spores (Prospero et al. 2006), and its transmission from one fungal individual to another by hyphal fusion (anastomosis) theoretically allows the spread of hypovirulence within the fungal population (Milgroom and Cortesi 2004). Natural dissemination and biological control efforts have led to a high prevalence of hypovirulence in many areas in Europe (Heiniger and Rigling 1994). The failure of hypovirulence in North America, however, may have resulted from environmental differences, higher blight susceptibility of the American chestnut, differences in the fungal population structure and/or differences in the virulence of the hypoviruses (MacDonald and Fulbright 1991; Dawe and Nuss 2001; Milgroom and Cortesi 2004).

The lack of an extracellular phase and the resulting complete dependence of CHV-1 on *C. parasitica* suggest a significant genotype-by-genotype interaction (Peever et al. 2000) and indicate reciprocal selection on fitness traits. Genetic variation in natural populations of *C. parasitica* (Liu et al. 1996; Breuillin et al. 2006) and CHV-1 (Gobbin et al. 2003) further provides the basis for natural selection. Additionally, both chestnuts (Anagnostakis 1987; Conedera et al. 2004) and *C. parasitica* (Roane et al. 1986) grow under a wide range of temperatures, and an effect of environmental factors, such as temperature (Anagnostakis and Aylor 1984) or light intensity (Hillman et al. 1990), on the expression of hypovirulence has been suggested.

Host and parasite isolates

We used four fungal strains in combination with each of the four CHV-1 subtypes and with no virus (in total 20 combinations). To create these fungus–virus combinations, virus-infected *C. parasitica* isolates obtained from four different geographic regions in

Europe and stored in glycerol at −80°C were used. They harboured four different subtypes of CHV-1 (Gobbin et al. 2003). Isolate M1372, obtained from Oberkirch (Germany) in 1992, harbored CHV-1 subtype D (Gobbin et al. 2003) and was given the name *baw* to identify the fungal strain. Isolate M4357, obtained from Dordogne (France) in 2003, harbored CHV-1 subtype F1 and was given the name *dor*. Isolate M2021 obtained from Var (France) in 1999 harbored CHV-1 subtype F2 (Robin et al. 2010) and was given the name *var*. Finally, isolate M4042 was obtained from Ticino (Switzerland) in 2004, harbored CHV-1 subtype I and was named *tic*. From each virus-infected isolate we obtained a virus-free culture through single conidial isolation (Prospero et al. 2006). Each hypoviral subtype was then transmitted from the infected fungal donor isolate to each of the virus-free recipient strains by hyphal anastomosis (Rigling et al. 1989; Peever et al. 2000; Robin et al. 2010). A small piece of mycelium was taken from the edge of the infected recipient culture to initiate the stock cultures for the experiment. The history of sub-cultivation was identical for all stock cultures. Each culture was checked for presence or absence of CHV-1 by extraction of double-stranded RNA, and the identity of the CHV-1 subtypes was verified by sequence analysis as described in Gobbin et al. (2003).

Experimental setup

We took three phenotypic measures in two experiments with identical design. Fungal growth and sporulation were assessed on potato dextrose agar (PDA, Difco Laboratories, Detroit) at the same colonies and fungal growth was also measured on dormant chestnut stems. In both experiments, we exposed six replicates of each of the 20 fungus–virus combinations to four different temperatures (12°C, 18°C, 24°C and 30°C) which fall within the thermal range of chestnut growing regions. Replicates were assigned to one of six blocks and randomized within blocks. The four climate chambers used in the experiment were of identical type and were ventilated with outside air through a common air duct. The temperatures were kept constant at 12°C, 18°C, 24°C or 30°C, and relative humidity was set to 70% in all chambers (JUMO DICON SM Universeller Kompaktregler, M. K. Juchheim, Germany). The actual temperature and relative humidity (rH) were recorded at 5-minute intervals with data loggers (DL-120TH Humidity/Temperature, Voltcraft, Germany) during the course of the experiment and the mean values (± SD) were: 12.1°C ± 0.2°C at 72.2% rH ± 1.5% rH, 18.0°C ± 0.4°C at 72.7% rH ± 3.1% rH, 24.0°C ± 0.2°C at 71.4% rH ± 2.5% rH, and 30.0°C ± 0.3°C at 70.3% rH ± 3.3% rH.

Growth and sporulation on PDA

For each fungal colony in the experiment, we used an individual sterile 9-cm Petri dish (84 mm inner diameter) containing 25 ml of PDA. These PDA plates had been prepared from the same lot 10 days before inoculation and kept at 4°C. On the starting day of the

experiment, we inoculated the PDA plates by placing a mycelial plug (6 mm diameter) taken from the growing edge of 5-days-old precultures into the centre of the plate. The plates were wrapped with Parafilm and arranged in adjacent blocks on a shelf in each chamber. Each shelf was illuminated by twelve new fluorescent tubes (Philips Master PL-L-36W/840/4P-ICT, Koninklijke Philips Electronics, Netherlands) set for a 14-hour photoperiod. Light intensity (mean ± SD) received by the fungal colonies was 3330 lx ± 160 lx (Illuminance Meter, Minolta, Japan). At 30°C, all cultures belonging to block 1 displayed severe growth deficiencies and were therefore excluded from analysis, allowing the use of five rather than six replicates of each isolate.

During the first 10 days of the experiment, we assessed radial growth on PDA every 24 hours. Two cardinal diameters of each colony through two orthogonal axes previously drawn on the bottom of each plate were measured with a ruler, using a millimeter scale. As the shape of the colonies was not a perfect circle, we calculated the geometric mean diameter of an ellipse. Radial growth of *C. parasitica* cultures on PDA has been described as undergoing a phase of linear growth (Anagnostakis and Aylor, 1984) and we determined this phase of linear growth for each temperature separately. Linear regression implemented in Microsoft Excel 2007 was performed on the geometric mean diameter calculations from the first 10 days of the experiment. The criterion $R^2 > 0.980$ of the linear regression fit was applied to define the time period during which all cultures at a given temperature were growing linearly. The increment of the fitted regression line was taken as the growth rate during the linear growth phase and was used for all further analyses of growth on PDA. We chose to assess growth rate, because growth rate includes data from an extended period of time, which is biologically more informative and meaningful than fungal colony size at a single time point.

After 31 days of incubation, we assessed sporulation by liberating conidia (asexual spores) with a glass rod in 10 ml of 0.15% Tween 80 (Sigma-Aldrich, USA) according to Hillman et al. (1990). Serial ten-fold dilutions were made from each spore suspension, and the number of conidia was quantified by direct counting under a light microscope (DMIL, Leica, Germany) with 400x magnification, using a hemocytometer (Thoma, 0.1mm, 0.0025mm^2). The measured concentration in conidia/ml was multiplied by the volume of the suspension recovered from the fungal colony.

Growth on chestnut stems

Six plastic containers (57 cm x 37 cm x 13 cm) each holding five dormant chestnut stems (50 cm length, 5–10 cm diameter) were used as blocks in each of the above mentioned chambers. Twenty healthy stems were cut from each of six individual *C. sativa* sprout clusters in the Ticino (Switzerland) at the beginning of November 2009, a

few days before the start of the experiment. Sprout clusters growing up from a single stump are clones of the same tree. Each sprout cluster was assigned to a block, and five stems of the same sprout cluster were put together in one container. Both ends of the stems were sealed with paraffin. Along the axis of the stem, four circular wounds (6 mm diameter) were made with a cork borer to the depth of the cambium. The wounds were arranged 12 cm apart from each other and 7 cm from the two ends, thus assuring that the fungal cultures would not influence each other. Within each block at each temperature, the 20 fungus–virus combinations were assigned to wounds at random, and we filled each wound with two mycelial mats (6 mm diameter) obtained from the growing edge of the 5-day-old precultures. The holes were then covered with transparent adhesive tape to prevent desiccation. Within the containers, the stems were placed horizontally on plastic grids 5 cm above the bottom of the container. The containers were filled with water to a depth of 2 cm and covered with a nontransparent lid. At 12°C, one stem had to be removed from the experiment due to contamination with another fungus, and at 24°C, one inoculation was missing, resulting in a total of five missing values in this experiment. After 18 days of incubation, we determined the lesion diameter on the chestnut stems, using a millimeter scale. Two diameters of each lesion were measured, one along the longitudinal and a second along the lateral axis of the stem. As the shape of the lesions resembled an ellipse, we calculated the geometric mean diameter and used it as a phenotypic value for growth on chestnut stems in all further analyses.

Data analysis

We analysed the three variables growth on PDA, sporulation on PDA and growth on chestnut stems separately, using a general linear model (GLM) in SPSS 17.0 (SPSS, USA) with the fixed factors temperature, fungus and virus and their interactions. Block was included as a random factor. In the GLM for the experiment on PDA, block was nested within temperature. In the experiment on dormant chestnut stems, stems originating from the same sprout cluster were assigned to a block. As all replicate measurements at a particular temperature were obtained within the same chamber, we cannot exclude the possibility that uncontrolled chamber effects confounded the effects we attributed to temperature. However, any potential effect of the chamber was minimized by ensuring identical growth conditions (apart from temperature). Residuals of the GLM were normally distributed and displayed constant error variances for growth on PDA and growth on chestnut stems but not so for sporulation. Therefore, we log-transformed the sporulation data, which stabilized error variances.

We also applied GLMs on the data within each temperature. The fixed factors of this reduced linear model were fungus and virus, and block was a random factor. Tukey’s test

was then implemented to detect significant (at $\alpha < 0.01$) differences among fungus–virus combinations at the same temperature.

We calculated Pearson's correlation coefficients to test for a linear relationship between growth and sporulation on PDA, as well as between growth on PDA and growth on chestnut stems. In the latter case, the mean values for each isolate were used.

To study the effect of virus infection on the fungal host, we calculated the difference between the phenotypic mean values of the virus-infected strain and the corresponding uninfected strain for each fungus–virus combination at all temperatures. The resulting value was given as a proportion of the phenotype value of the uninfected strain and termed the virus effect. The output of Tukey's test implemented in the within-temperature GLMs was used to determine whether the virus effect was significant (at $\alpha < 0.01$). GLMs and Tukey's tests were also performed on virus effects to test for significant (at $\alpha < 0.01$) differences in the effectiveness of virus infection among temperatures and among viral subtypes as well as between home and away host–parasite combinations. Residual analysis revealed no violation of GLM assumptions.

Table 1. General linear models on growth and sporulation of *Cryphonectria parasitica* strains, infected with *Cryphonectria hypovirus 1* subtypes or uninfected, at different temperatures.

		Growth rate on agar medium			Sporulation on agar medium				Growth on chestnut stems		
Source	df	MS	F	P	MS	F	P	df	MS	F	P
T	3	2336.088	487.090	≤0.0001***	111.903	517.308	≤0.0001***	3	6747.715	237.913	≤0.0001***
F	3	22.703	76.814	≤0.0001***	0.489	5.764	0.002**	3	195.869	6.409	0.005**
V	4	360.291	1912.597	≤0.0001***	10.756	107.746	≤0.0001***	4	6427.216	344.347	≤0.0001***
TxF	9	9.214	31.175	≤0.0001***	1.508	17.767	≤0.0001***	9	80.334	3.249	≤0.004**
TxV	12	48.244	256.102	≤0.0001***	3.266	32.714	≤0.0001***	12	404.638	16.689	≤0.0001***
FxV	12	6.169	30.347	≤0.0001***	0.861	11.723	≤0.0001***	12	29.905	1.271	0.259
TxFxV	36	1.868	9.188	≤0.0001***	0.884	12.037	≤0.0001***	36	20.233	0.923	0.599
Error	228	0.203			0.073			175	21.928		

Note: The effects of the fixed factors temperature (T), fungal strain (F), virus subtype or no virus (V), and their interactions were tested on growth on agar medium (growth rate in colony diameter in mm/d during the phase of linear growth), sporulation on agar medium (log number of conidia produced after 31 days of incubation) and growth on chestnut stems (lesion diameter after 18 days of incubation). The blocking factor was included in the model as a random term, and the associated MS values are reported in Table S1. The significance levels applied were: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results

Identification of significant effects and factor interactions

Growth and sporulation on PDA were significantly affected by all main factors (fungus, virus and temperature) and all interactions (Table 1). Growth on chestnut stems was also significantly affected by all main factors and by the virus-by-temperature interaction. When the data were restricted to the virus-infected cultures, the respective GLM terms remained significant for the same factors (results not shown). Figure 1 shows that the fungus-by-virus-by-temperature interaction was not only significant, as indicated by non-parallel interaction norms, but that the fungus–virus interaction norms even crossed. Crossing interaction norms included different fungal strains infected with the same virus subtype. The majority of crossings occurred between 24°C and 30°C, and were most reliably observed for growth rate on PDA (Fig. 1A), the phenotypic variable that could be most accurately measured (mean CV: for growth rate on PDA = 0.06, for sporulation on PDA = 0.51; for lesion diameter on chestnut stems = 0.21). Ranking of the fungus–virus combinations according to their phenotypic values revealed that some fungus–virus combinations switched their ranks substantially among temperatures, reaching high ranks at some temperatures and low ranks at others.

Correlation of phenotypes

Growth on PDA and growth on chestnut stems displayed the same trend across temperatures and were correlated at $r = 0.673$ ($p \leq 0.01$). The major inconsistency between the growth measurements on the two types of media was found in the performance of the uninfected cultures in relation to the virus-infected cultures. Exclusion of the virus-free cultures from the data set increased the correlation ($r = 0.751$, $p \leq 0.01$). Growth of the uninfected strains on PDA was intermediate when compared to the virus-infected strains, whereas the uninfected strains generally outperformed the virus-infected strains when grown on chestnut stems (Fig. 1A; Fig. 1C). The ranking of each viral subtype in relation to the growth of their host averaged across all temperatures and fungal strains was I > D > F2 > F1 on both PDA and chestnut stems, with subtype I allowing the highest and subtype F1 the lowest growth. Growth on PDA and sporulation on PDA (log-transformed data) were highly correlated ($r = 0.738$, $p \leq 0.01$). Both variables displayed a trend of higher values with increasing temperatures up to 24°C (Fig. 1A; Fig. 1B).

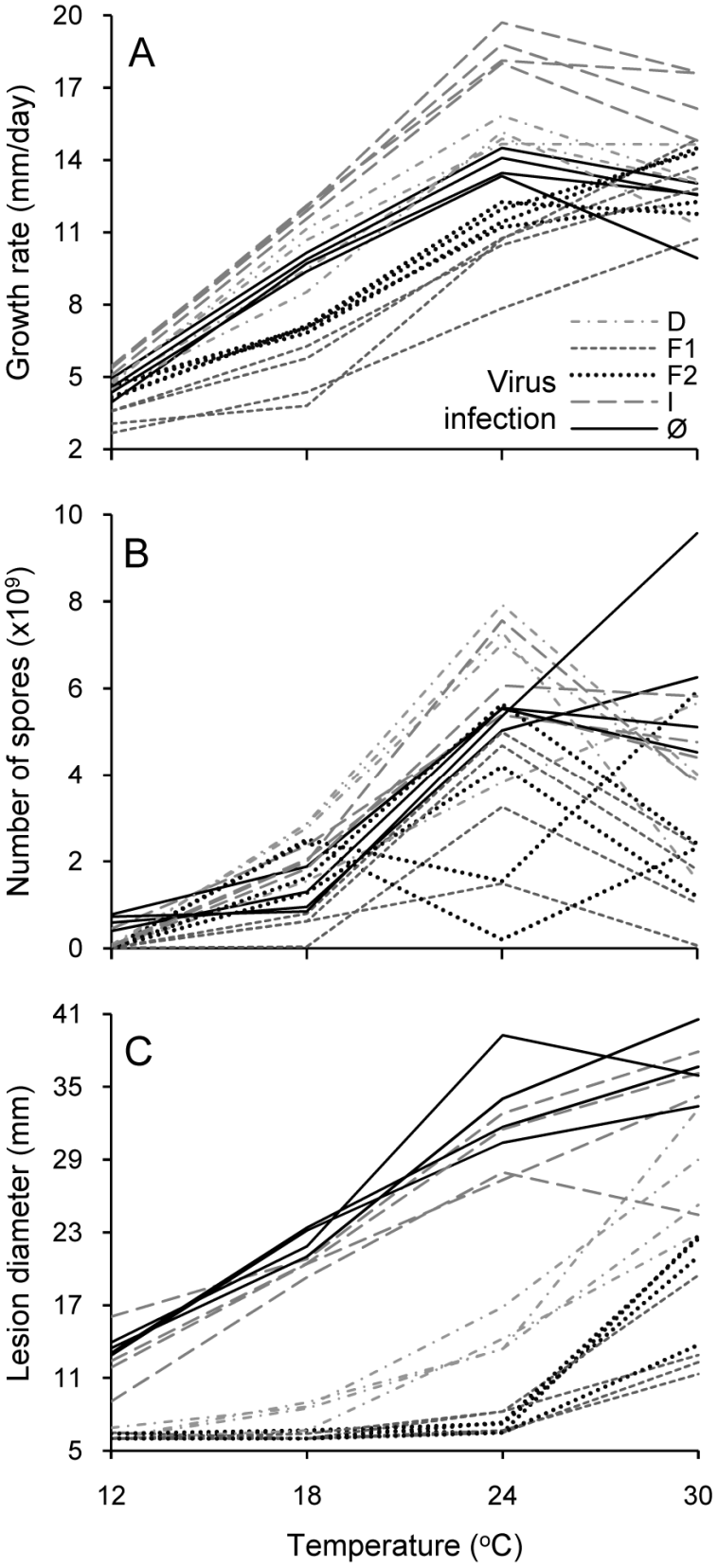

Fig. 1. Interaction norms for the growth rate on agar medium during the phase of linear growth (A), the number of spores produced on agar medium after 31 days of incubation (B) and growth (lesion diameter) on dormant chestnut stems after 18 days of incubation (C). Lines represent the mean performance (n = 6) of each combination between four *Cryphonectria parasitica* strains and the four *Cryphonectria hypovirus 1* subtypes. Different line styles refer to different subtypes (D, F1, F2 and I) and uninfected control strains (Ø).

Effect of virus infection

The effect of each virus on each strain was expressed by the relative difference in performance between the virus-infected strain and the corresponding uninfected strain at each temperature. In Figure 2, the effect of the four virus subtypes on the host phenotype averaged across all fungal strains are displayed for each temperature. The effect of virus infection on all three measured traits differed significantly (at $\alpha < 0.01$) among temperatures and viral subtypes. When focusing on each fungus–virus combination at each temperature separately, both qualitative and quantitative differences were evident (Tables S2,S3 and S4). The growth rate on PDA of nearly all strains at all temperatures was significantly (at $\alpha < 0.01$) increased after infection with subtype I. Subtype D did not significantly (at $\alpha < 0.01$) alter the growth rate of most strains at all temperatures. The subtypes F1 and F2 significantly (at $\alpha < 0.01$) reduced growth of most strains at 12°C, 18°C and 24°C but at 30°C, they significantly increased growth in four cases, significantly reduced growth in one case and exhibited no significant effect in three cases. The sporulation of most fungal strains on PDA was significantly (at $\alpha < 0.01$) reduced by all viral subtypes at 12°C when compared to the uninfected strains. At 18°C, sporulation was generally increased after virus infection but this effect was not significant (at $\alpha < 0.01$). At 24°C and 30°C, only a few significant (at $\alpha < 0.01$) effects were observed, and most of those resulted from an infection with the subtypes F1 or F2, reducing sporulation of the host. Growth on chestnut stems of all fungal strains was not significantly (at $\alpha < 0.01$) affected by infection with subtype I at any temperature. Subtypes D, F1 and F2 always significantly (at $\alpha < 0.01$) reduced lesion diameter in combination with all fungal strains at 12°C, 18°C and 24°C. In contrast, at 30°C, only subtype F1 reduced the growth of all strains significantly (at $\alpha < 0.01$), while subtype F2 reduced growth of only two strains significantly (at $\alpha < 0.01$) and subtype D did not exhibit any significant (at $\alpha < 0.01$) effect on any of the strains.

With our fully reciprocal set of fungus–virus combinations each virus was infecting its home and three away hosts, allowing to test for local adaptation. The effect of virus infection did not differ significantly (at $\alpha < 0.01$) between home and away hosts for all three measured traits with a single exception of subtype F1, which reduced the growth rate on PDA of its home host more than of the away hosts (results not shown).

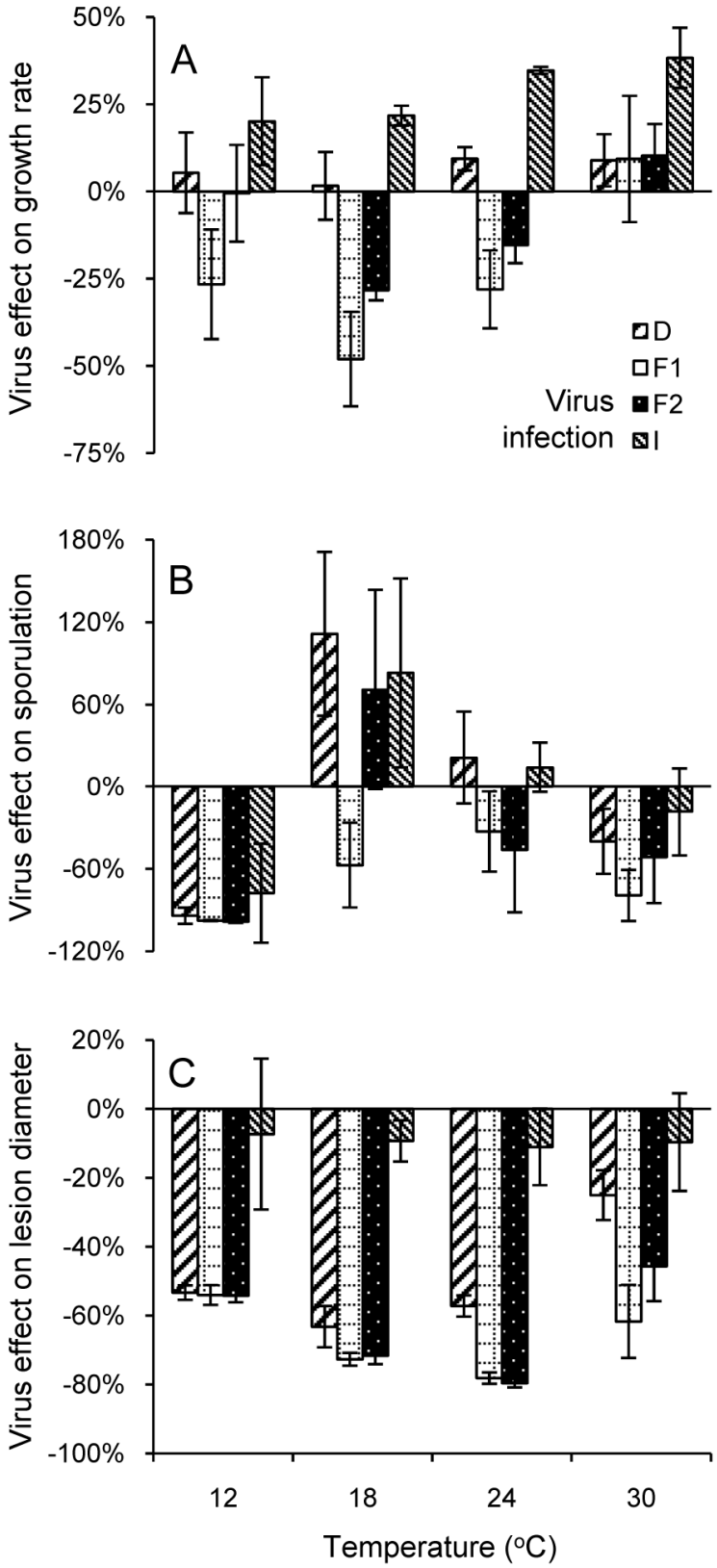

Fig. 2. The effect of virus infection on growth rate in colony diameter on agar medium during the phase of linear growth (A), sporulation on agar medium after 31 days of incubation (B) and growth (lesion diameter) on chestnut stems after 18 days of incubation (C). The virus effect is the difference in performance between the virus-infected and the corresponding uninfected control as a proportion (%) of the performance of the uninfected control. For each *Cryphonectria hypovirus 1* subtype (D, F1, F2 and I), the average effect across four strains of *Cryphonectria parasitica* at each temperature is displayed. Error bars represent standard deviations.

Discussion

In this study, we found a highly significant genotype-by-genotype-by-environment interaction between fungal strains, hypovirus subtypes and temperature in the chestnut blight pathosystem. Furthermore, we observed crossing interaction norms (i.e., rank switches of genotype-by-genotype combinations across temperatures), suggesting the potential for a selection mosaic in the studied host–parasite system. To our knowledge, this is the first report of a genotype-by-genotype interaction in a host–parasite system that is modified by the abiotic environment. These results provide evidence that temperature potentially impacts the coevolutionary trajectory of host–parasite interactions. At each of the four experimental temperatures, a significant genotype-by-genotype interaction between the host and the parasite was found on agar medium, affecting the phenotypic expression of the infection. Therefore, the ability of a certain hypovirus subtype to manipulate the physiology of a particular fungal strain seems to be temperature-dependent. Interestingly, most crossing interaction norms were observed between 24°C and 30°C, while interaction norms at 12°C to 24°C were non-parallel but mostly non-crossing. This indicated that most qualitative differences in the outcome of the host–parasite interaction in this pathosystem can be expected at increased temperatures.

To evaluate the relevance of the results obtained on agar medium in a more natural system we applied the same full factorial design in an experiment on dormant chestnut stems. As noticed in previous experiments (Chen et al. 2000; Robin et al. 2010), variation among replicates was substantially higher on dormant chestnut stems than on agar medium, where growth conditions can be better standardized. A high level of standardization is required for the resolution of factorial interactions. Variation in conditions can obscure existing effects, most likely explaining why the genotype-by-genotype-by-environment interaction was not significant with the given sample size in our experiment on chestnut stems. On agar medium, the virus-free cultures performed worse than several virus-infected cultures while they outperformed (almost all) virus-infected cultures when grown on chestnut stems, in accordance with other studies (Chen and Nuss 1999; Chen et al. 2000; Robin et al. 2010). However, as our main focus was on the host–parasite interaction, we were mostly interested in the virus-infected cultures. In our experiment, the performance of the virus-infected cultures on the two types of media was correlated; in particular, the ranking among the CHV-1 subtypes remained the same. We conclude that the pattern observed on dormant chestnut stems supports the biological relevance of the genotype-by-genotype-by-environment interaction obtained on agar medium. Previous research further showed that growth measures on agar medium explained the virulence of *C. parasitica* in living chestnut sprouts (Dunn and

Boland 1993) and the fitness and long-term persistence of CHV-1 in the field (Robin et al. 2010). The fitness measures we assessed are key components of chestnut blight epidemics. The damage that *C. parasitica* causes to the tree is strongly related to the growth characteristics of the fungus (Bazzigher 1981; MacDonald and Fulbright 1991), while the degree of sporulation defines the spread of the disease as well as the potential for hypovirus dissemination.

In natural populations, an additional level of complexity is introduced by the third species in the tritrophic interaction of the chestnut blight pathosystem, namely the chestnut tree. Several surveys in natural populations of European and American chestnut have revealed a general susceptibility to the disease with little variation (Bazzigher 1981; Roane et al. 1986). The absence of significant resistance within populations can be explained by the lack of coevolution with the pathogen (Anagnostakis 1987), as *C. parasitica* is an introduced pathogen to both Europe and North America. The impact of hypovirulence on disease severity generally outweighs the impact of partial tree host resistance (Van Alfen et al. 1975; Fulbright 1984; Roane et al. 1986), and the tree genotype might therefore have little impact on the fungus–virus interaction. This could be different in resistant Asian chestnut populations, where the native chestnut species (*Castanea crenata* and *C. mollissima*) have coevolved with *C. parasitica* (Anagnostakis 1987). In resistant trees, growth and establishment of *C. parasitica* are highly constricted (Graves 1950), and a stronger selection could be expected against phenotypes of fungus–virus interactions with little growth and sporulation. Furthermore, the degree of expressed resistance might depend on the interaction between environmental factors and tree genotype (Browder 1985; Carson and Carson 1989). In resistant chestnut populations, the influence of genetic variation in resistance might therefore be complex and could strengthen or weaken the effect of the fungus-by-virus-by-temperature interaction.

With this study, we met the need to provide statistical confirmation of genotype-by-genotype-by-environment interactions and to explore the potential for selection mosaics (Thomas and Blanford 2003; Gomulkiewicz et al. 2007; Piculell et al. 2008; Vale et al. 2008a; Lazzaro and Little 2009; Wolinska and King 2009). In line with the proposed stepwise approach for the dissection of the coevolutionary dynamics of host–parasite interactions (Gomulkiewicz et al. 2007; Piculell et al. 2008), our goal was not to demonstrate an actual selection mosaic in natural populations but to ask if a selection mosaic could serve as a starting point for divergent selection in the particular host–parasite interaction under study. Our results suggest that different host and parasite genotypes could indeed be favored under different temperatures. The climatic conditions encountered in the chestnut growing regions are very diverse and include Mediterranean, oceanic and continental climates. If the same *C. parasitica* and CHV-1 genotypes are introduced to two regions that differ in temperature, the thermal specificity of the

fungus–virus interaction would lead to different coevolutionary trajectories. Ultimately, genetically different local populations of *C. parasitica* and CHV-1 would evolve, thus shaping the course and expression of local chestnut blight epidemics.

Predicting the effect of environmental factors on phenotype expression is also important for biological control. In regions where chestnut blight is still active, biological control treatments with CHV-1 could provide a mean for containing the epidemic. However, the results of this study imply that the outcome of hypovirus applications on different fungal populations and under a different climate will be unpredictable given the fungus-by-virus-by-temperature interaction. The suitability of a certain hypovirus subtype for biological control depends on both the temperature and the fungal host genotype. Therefore, different management strategies need to be designed for different regions and the most suitable biological control agent has to be evaluated for each case individually.

Fig. 3. European chestnut forest. Some trees were killed by virulent *Cryphonectria parasitica* strains, but most trees were healthy. They were infected with virus-infected *C. parasitica* strains. Photograph by Sarah Bryner.

In most European regions, chestnut blight does not currently pose a mortal threat to chestnut forests due to the high prevalence of hypovirulence (Fig. 3). However, the disease has not been eradicated and is still widespread, making it possible that a temperature increase could disturb the subtle host–parasite interaction between *C. parasitica* and CHV-1. Climate change is expected to lead to more frequent temperature

extremes during summer (Easterling et al. 2000). The significant fungus-by-virus-by-temperature interaction indicates that any given fungus–virus interaction could change its outcome when temperatures increase and that an extreme summer heat event could turn a formerly hypovirulent fungus more virulent.

In evolutionary terms, chestnut blight is still a young disease in Europe, presumably explaining why we did not find evidence for local adaptation in our study. Little is known about the evolutionary trajectory of the fungus–virus interaction, which makes predictions for the future difficult. Such knowledge, however, would be essential to design sustainable management strategies. Therefore, further research should be directed at investigating the fungus–virus coevolution in various environments and over an extended period of time. Our study is a first step to understand how reciprocal selection acts in this pathosystem, and it highlights the importance of genotype-by-genotype-by-environment interactions for coevolution.

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Supporting Information

Table S1. Mean square values associated with the random blocking factor resulting from the GLMs.

		Growth rate on agar medium	Sporulation on agar medium			Growth on chestnut stems
Source	df	MS	MS	Source	df	MS
B(T)	19	4.796	0.216	B	5	136.723
B(T)xF	57	0.296	0.085	BxF	15	30.598
B(T)xV	76	0.188	0.100	BxV	20	18.655
				BxT	15	28.389
				BxFxV	60	23.537
				BxTxF	45	24.748
				BxTxV	60	24.261

Note: This table complements the general linear model (GLM) results reported in Table 1.

Table S2. Growth rate in colony diameter on agar medium during the phase of linear growth (mm/day).

Fungus	Virus	Temperature (°C)							
		12		18		24		30	
baw	D	4.7 (0.1)	efghi	11.1 (0.3)	ghi	15.8 (0.2)	i	13.2 (1.7)	efgh
	F1	3.1 (0.3)	ab	3.8 (1.2)	a	10.7 (0.8)	bc	14.9 (1.4)	ij
	F2	4.2 (0.2)	cdef	7.0 (0.5)	c	12.0 (0.3)	cde	14.3 (1.3)	ghi
	I	5.3 (0.2)	ij	12.0 (0.5)	i	19.7 (0.6)	k	17.7 (0.8)	k
	Ø	4.9 (0.1)	ghij	10.2 (0.5)	efg	14.5 (0.5)	ghi	13.0 (1.1)	defg
dor	D	4.6 (0.1)	efgh	9.5 (0.4)	de	14.9 (0.4)	hi	13.1 (1.2)	defg
	F1	2.7 (0.2)	a	4.4 (0.5)	a	7.8 (0.9)	a	10.7 (0.4)	ab
	F2	4.7 (0.1)	efgh	7.0 (0.8)	c	11.2 (0.1)	bcd	12.3 (1.1)	cde
	I	5.4 (0.2)	j	11.9 (0.7)	i	18.8 (0.3)	jk	16.1 (0.8)	j
	Ø	4.6 (0.5)	defgh	9.9 (0.5)	ef	14.1 (0.4)	gh	12.5 (0.7)	cdef
tic	D	4.8 (0.2)	fghij	10.7 (0.6)	fgh	14.7 (0.7)	ghi	14.7 (1.0)	i
	F1	3.6 (0.1)	bc	5.8 (0.5)	b	10.8 (0.8)	bc	13.7 (0.3)	fghi
	F2	4.6 (0.2)	efgh	6.8 (0.3)	bc	11.4 (0.7)	bcd	14.5 (1.1)	hi
	I	5.4 (0.1)	j	12.1 (0.6)	i	18.1 (0.1)	j	17.6 (1.0)	k
	Ø	3.9 (0.8)	cd	9.7 (0.4)	ef	13.5 (0.4)	fg	12.6 (0.3)	cdef
var	D	4.5 (0.3)	defgh	8.5 (0.4)	d	15.2 (0.5)	hi	11.3 (1.2)	bc
	F1	3.6 (0.3)	bc	6.3 (0.9)	bc	10.5 (0.5)	b	12.8 (1.3)	def
	F2	4.1 (0.1)	cde	7.1 (0.4)	c	12.3 (0.4)	def	11.8 (1.4)	bcd
	I	5.1 (0.2)	hij	11.6 (0.6)	hi	18.0 (0.5)	j	14.8 (1.1)	ij
	Ø	4.4 (0.2)	defg	9.4 (0.3)	de	13.3 (0.7)	efg	9.9 (1.5)	a

Note: The means and the standard deviations in parentheses of four *Cryphonectria parasitica* strains (baw, dor, tic and var), infected with each of the four *Cryphonectria hypovirus 1* subtypes (D, F1, F2 and I) and uninfected (Ø), at each of four temperatures are displayed (n = 6). Mean values within the same column followed by the same letter are not significantly different according to the Tukey's test at $\alpha < 0.01$.

Table S3. Sporulation on agar medium after 31 days of incubation (number of conidia x10^6).

Fungus	Virus	Temperature (°C)											
		12			18			24			30		
baw	D	80	(30)	de	2761	(722)	e	7005	(1873)	d	3866	(1022)	cdef
	F1	14	(14)	bcd	36	(21)	a	3256	(2425)	bcd	1039	(205)	b
	F2	9	(3)	bc	2502	(745)	e	1546	(2581)	ab	5931	(3672)	def
	I	450	(305)	ef	2360	(1468)	de	5386	(1654)	d	4764	(2177)	cdef
	Ø	591	(482)	ef	950	(218)	bcde	5029	(1678)	d	6252	(1911)	ef
dor	D	5	(3)	ab	2873	(1209)	e	7926	(2245)	d	4013	(1472)	cdef
	F1	22	(819)	bcd	622	(325)	bc	1490	(1588)	abc	65	(56)	a
	F2	25	(14)	bcd	2444	(511)	e	202	(91)	a	2309	(786)	bcde
	I	64	(79)	cd	2044	(694)	cde	5532	(1042)	d	4409	(2635)	cdef
	Ø	777	(498)	f	1876	(299)	cde	5530	(1564)	d	4529	(1677)	cdef
tic	D	31	(9)	cd	2857	(667)	e	7296	(1211)	d	1573	(731)	bc
	F1	8	(7)	abc	796	(465)	bcd	4992	(1666)	d	2356	(1079)	bcde
	F2	6	(6)	ab	1276	(8379)	bcde	4202	(1950)	cd	1185	(578)	b
	I	2	(1)	a	1829	(1102)	bcde	6066	(3158)	d	5816	(2823)	def
	Ø	398	(181)	ef	1294	(965)	bcde	5549	(1637)	d	5110	(1632)	def
var	D	15	(7)	bcd	1545	(759)	bcde	3823	(1766)	bcd	5656	(977)	ef
	F1	20	(19)	bcd	616	(440)	b	4682	(1917)	cd	1806	(611)	bcd
	F2	6	(3)	abc	1626	(1109)	bcde	5638	(3326)	cd	2401	(1683)	bcde
	I	34	(35)	bcd	1988	(756)	cde	7560	(2813)	d	3803	(2591)	bcdef
	Ø	725	(356)	f	849	(382)	bcde	5361	(888)	d	9572	(3255)	f

Note: The means and the standard deviations in parentheses of four *Cryphonectria parasitica* strains (baw, dor, tic and var), infected with each of the four *Cryphonectria hypovirus 1* subtypes (D, F1, F2 and I) and uninfected (Ø), at each of four temperatures are displayed (n = 6). Mean values within the same column followed by the same letter are not significantly different according to the Tukey's test at $\alpha < 0.01$ on the log-transformed sporulation data.

Table S4. Growth (lesion diameter) on chestnut stems after 18 days of incubation (mm).

Fungus	Virus	Temperature (°C)								
		12			18			24			30		
baw	D	6.0	(0.0)	a	8.5	(2.8)	a	13.4	(5.2)	ab	25.3	(5.0)	abcd
	F1	6.5	(1.2)	a	6.0	(0.0)	a	6.5	(1.2)	a	12.3	(2.8)	a
	F2	6.0	(0.0)	a	6.7	(1.6)	a	6.5	(1.2)	a	22.7	(5.0)	abc
	I	16.1	(4.1)	c	20.8	(3.7)	b	32.8	(6.7)	e	37.9	(6.0)	de
	Ø	13.0	(3.6)	bc	23.4	(6.8)	b	31.7	(6.2)	e	36.6	(13.3)	cde
dor	D	6.0	(0.0)	a	9.0	(3.4)	a	13.4	(2.1)	ab	33.2	(10.6)	bcde
	F1	6.0	(0.0)	a	6.0	(0.0)	a	8.2	(2.7)	a	12.9	(4.5)	a
	F2	6.4	(1.0)	a	6.7	(1.6)	a	7.2	(3.0)	a	21.0	(4.2)	ab
	I	11.9	(4.1)	bc	20.4	(2.9)	b	27.3	(12.4)	bcde	34.2	(4.6)	bcde
	Ø	13.5	(2.1)	bc	21.0	(9.7)	b	34.0	(7.2)	e	40.6	(8.4)	e
tic	D	6.0	(0.0)	a	6.7	(1.6)	a	14.2	(8.0)	abc	22.9	(1.0)	abc
	F1	6.0	(0.0)	a	6.0	(0.0)	a	6.7	(1.6)	a	11.3	(5.3)	a
	F2	6.0	(0.0)	a	6.0	(0.0)	a	6.5	(1.2)	a	13.7	(1.6)	a
	I	9.1	(3.8)	ab	19.3	(8.3)	b	27.9	(8.2)	cde	24.5	(7.2)	abcd
	Ø	12.8	(3.0)	bc	23.2	(6.9)	b	30.4	(6.9)	de	33.4	(3.1)	bcde
var	D	6.9	(2.0)	a	8.6	(3.6)	a	16.8	(11.4)	abcd	29.0	(6.6)	bcde
	F1	6.0	(0.0)	a	6.4	(1.0)	a	8.2	(1.9)	a	19.5	(4.7)	ab
	F2	6.0	(0.0)	a	6.0	(0.0)	a	7.3	(2.1)	a	22.5	(8.2)	abc
	I	12.4	(2.6)	bc	20.5	(4.3)	b	31.5	(6.0)	e	36.1	(2.9)	cde
	Ø	13.9	(2.9)	c	21.8	(4.7)	b	39.2	(5.5)	e	35.9	(2.3)	cde

Note: The means and the standard deviations in parentheses of four *Cryphonectria parasitica* strains (baw, dor, tic and var), infected with each of the four *Cryphonectria hypovirus 1* subtypes (D, F1, F2 and I) and uninfected (Ø), at each of four temperatures are displayed (n = 6). Mean values within the same column followed by the same letter are not significantly different according to the Tukey's test at $\alpha < 0.01$.

Chapter 3

Hypovirus virulence and vegetative incompatibility in populations of the chestnut blight fungus

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Abstract

Cryphonectria hypovirus 1 hyperparasitizes the chestnut blight fungus *Cryphonectria parasitica* and acts as a biocontrol agent of this serious tree disease. The virus is transmitted cytoplasmatically between fungal individuals. However, highly virulent viruses strongly debilitate their host and, thus, reduce their own transmission probability. Furthermore, vegetative incompatibility between fungi is an important transmission barrier. Virulent viruses are therefore expected to be strongly selected against in fungal populations with high levels of vegetative incompatibility, eventually leading to the erosion of biocontrol. To test this prediction, we assessed the virulence of the virus in four *C. parasitica* populations with high and in four populations with low diversity of vegetative compatibility types. We expected the degree of virus virulence to be lower in fungal populations with high levels of vegetative incompatibility. However, our results did not reveal such a trend. No significant differences in virus virulence between populations with low vs. high diversity of vegetative compatibility types were observed. There was no evidence for an erosion of disease control due to the presence of these transmission barriers. Thus, the findings of this study are promising for the sustainability of *Cryphonectria hypovirus 1* as a biocontrol agent for chestnut blight in Europe.

Introduction

Biological control of pests and diseases makes use of host-parasite interactions that result in the debilitation of the host. The degree of virulence (i.e. damage to the host) that a biocontrol agent exhibits on the pest or disease organism is pivotal to the level of control achieved. In extensively managed ecosystems, such as forests and pastures, the long-term establishment of biocontrol agents is attempted (Payne et al. 1988; Heiniger and Rigling 1994). In these systems, biocontrol agents need to be virulent enough to achieve sufficient disease control but as well should be able to infect many hosts (Hufbauer and Roderick 2005). They are expected to spread independently and persist in the ecosystem, exerting continuous control. The evolution of virulence in biocontrol agents is therefore crucial to the sustainability and effectiveness of the biocontrol, however, little is known about it (Hufbauer and Roderick 2005).

Current theory suggests that the evolution of virulence underlies a trade-off between virulence costs and virulence benefits (Anderson and May 1982; Bull 1994; Frank 1996). The fitness of a parasite is the result of its multiplication within the host and its transmission from infected to non-infected hosts. Highly virulent parasites multiply rapidly and exploit their host efficiently, however, at the cost of increased host mortality or debilitation, reducing the chance for the parasite to be transmitted. Several models were developed to describe the evolution of virulence in host-parasite interactions (Levin and Pimentel 1981; Frank 1992; Antia et al. 1994; Day 2001; Koella and Restif 2001; Day 2002) and generally suggest: The optimal degree of virulence, maximizing the parasite's fitness, depends on the particular cost-benefit relationship of the host-parasite interaction. Some parasites rely on relatively normal host function and, hence, lower virulence for transmission, while other parasites may inflict more damage to the host without inhibiting transmission (Ewald 1995). Vertically (to offspring) transmitted parasites, for example, depend on reproduction of the infected host. Their cost of host debilitation is much higher than for horizontally (to other hosts) transmitted parasites that do not require their host to reproduce (Bull et al. 1991). Similarly, the cost of host debilitation is much higher for parasites that cannot survive outside the host and depend on a live host than for parasites that are able to survive outside the host (Walther and Ewald 2004).

Cost-benefit relationships are important to take into account when evaluating the sustainability of a biocontrol system (Hufbauer and Roderick 2005). According to the theory described above biocontrol agents with high costs of virulence for transmission would evolve towards avirulence, since virulent strains would not be able to spread and

persist within the population. A loss of virulence in the biocontrol agent, however, results in the erosion of disease control.

The *Cryphonectria*-hypovirus pathosystem is a famous and (so far) successful biocontrol system (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). The fungus *Cryphonectria parasitica* causes lethal bark cankers on chestnut trees (*Castanea spp.*) and is responsible for the serious tree disease called chestnut blight. It was introduced from Asia to both North America and Europe early in the 20th Century. In North America, it destroyed the native chestnut forests (Milgroom and Cortesi 2004). In Europe, however, a few years after the first disease records, superficial cankers were observed, which did not result in dieback of the infected trees (Heiniger and Rigling 1994). These so called healing cankers were found to be the result of the emergence of a viral disease of *C. parasitica* (Choi and Nuss 1992). The virus *Cryphonectria hypovirus 1* (CHV-1) hyperparasitizes *C. parasitica* and reduces its pathogenicity. Infection with CHV-1 is persistent and does not kill *C. parasitica*, however, it inhibits sexual reproduction and strongly attenuates fungal growth and asexual sporulation of the fungus (Peever et al. 2000). The reduction of fungal growth and sporulation is commonly regarded the major virulence determinant in CHV-1 (Peever et al. 2000; Milgroom and Cortesi 2004; Robin et al. 2010). Following its emergence, the virus spread spontaneously throughout the *C. parasitica* populations in Europe. Natural dissemination and active biocontrol efforts have led to a high prevalence of virus infection, successfully controlling the chestnut blight disease in most regions (Heiniger and Rigling 1994).

The vast majority of fungal viruses infects the host without any apparent symptoms (Nuss 2005) as expected under the theoretical considerations described above. Fungal viruses are obligate parasites that infect their hosts persistently. They lack an extracellular stage outside the host and therefore completely depend on their host for survival and fitness (Nuss 2005). CHV-1 is a rare exception among fungal viruses, causing comparatively severe damage to its host. This is particularly intriguing since CHV-1 also depends on vertical transmission for spread. CHV-1 is transferred into the asexual spores of its fungal host, dispersed in these spores and then transmitted from the outgrowing spores to virus-free fungal individuals upon hyphal fusion (Hoegger et al. 2003). The cost of virulence should be very high for CHV-1 since virulence is associated with reduced fungal growth and reduced production of asexual spores. Virulence therefore compromises the spread of the virus directly and CHV-1 had been predicted to evolve towards lower virulence over time (Milgroom 1999; Day 2001).

Furthermore, in the *Cryphonectria*-hypovirus pathosystem an additional factor is expected to promote the evolution of low virulence. Virus transmission in *C. parasitica* is restricted by vegetative incompatibility between fungal individuals (Liu and Milgroom

1996). Fusion between cells of incompatible individuals results in cell death, hampering the transmission of viruses and other cytoplasmic elements. Between compatible individuals of *C. parasitica* (i.e. identical vegetative compatibility (vc) types), CHV-1 is transmitted in virtually 100% of host-to-host contacts. Among different vc types transmission can also occur, but at much reduced rates (Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). In *C. parasitica* populations with high vc type diversity, the transmission probability of CHV-1 is expected to be greatly limited (Cortesi et al. 2001; Milgroom and Cortesi 2004). Under the presence of such transmission barriers, virulent viruses, which reduce their own transmission probability in excessively debilitating their host, are expected to be strongly selected against (Milgroom 1995). It was assumed that the failure of biological control with CHV-1 in North America – but not in Europe – was (at least in part) due to the much higher vc type diversity in North America (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). However, the vc type diversity could also increase in European populations as a result of new introductions or sexual reproduction between divergent genotypes of *C. parasitica* (Jezic et al. 2012; Prospero and Rigling 2012). Evolution of avirulence in CHV-1 is therefore of great concern. To date, populations of *C. parasitica* with different degrees of vc type diversity exist in Europe. Over a wide area, they are infected with the same subtype of CHV-1 (Gobbin et al. 2003). This provides the opportunity to study the influence of the vc type diversity on the virulence of CHV-1 within the same hypovirus subtype and in several geographically separated natural populations of *C. parasitica*.

In this study, our aim was to make predictions about the evolution of virulence in the biocontrol agent CHV-1 resulting from the diversity of the fungal host population. We hypothesized that the cost of high virulence for transmission of CHV-1 was higher in populations with transmission barriers than in populations without. We assumed that the degree of virulence was lower in host populations with high vc type diversity (transmission barriers present) than in populations with low vc type diversity (no transmission barriers). To test this hypothesis, we sampled four *C. parasitica* populations with high vc type diversity and four populations with low vc type diversity and assessed the virulence of the virus in each population.

Materials and methods

Sampling of chestnut blight cankers

We sampled a total of eight geographically separated *C. parasitica* populations in Europe (Fig. 1) where CHV-1 had established naturally and where the Italian subtype of CHV-1 (Gobbin et al. 2003) was the only subtype present. All populations were sampled within an area <1 ha. Four populations were obtained from regions with known low *C. parasitica* vc type diversity (i.e. only one dominant vc type) and four from regions with known high vc type diversity (Robin and Heiniger 2001; Milgroom et al. 2008). All sampling sites were coppice forests with 10 – 20 years old chestnut sprouts and a high incidence of chestnut blight. No *C. parasitica* and CHV-1 isolates had ever been released in these areas. Bark samples were taken with a cork borer (5 mm diameter) from chestnut blight cankers at intervals of at least 5 m between trees. Only one canker per tree was sampled and the cork borer was sterilized with 70% ethanol and flaming between cankers.

Isolation of *C. parasitica*, determination of virus-infection and assessment of vc type diversity

We obtained a total of 634 pure cultures of *C. parasitica* (Table 1) by isolation from the bark samples on water agar followed by culturing on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI; Robin et al. 2010). Since culture morphology is an indicator of CHV-1 infection (Choi and Nuss 1992), *C. parasitica* isolates were determined to be virus-infected if they displayed the white culture morphology and to be virus-free if they displayed the orange morphology (Peever et al. 2000; Robin et al. 2010). The presence and identity of CHV-1 subtype I was verified by extraction of the viral double-stranded (ds) RNA and subsequent sequence analysis (Gobbin et al. 2003).

The vc type of each *C. parasitica* isolate was determined by pairing with vc type tester strains (Cortesi and Milgroom 1998). To estimate the diversity of vc types in each population the Shannon-Wiener's index was calculated based on the vc type frequency observed (H'_{obs}) and based on the vc type frequency expected (H'_{44}) for the smallest sample size being analyzed (n=44). The expected diversity was determined by rarefaction analysis implemented by the vegan package in the software R 2.6.2 (R-Development-Core-Team 2008). To estimate the potential of hypovirus transmission in each population we used the logistic regression model described in Cortesi et al. (2001), which calculates the mean probability of virus transmission based on the frequency distribution of the vc types observed in the population.

Preparation of experimental isolates

To assess the viral virulence we conducted an inoculation experiment. A subsample of 16 virus-infected isolates was selected from each population. To incorporate the vc type diversity within populations a stratified procedure was applied. Isolates with vc types that were heteroallelic at the vegetative incompatibility locus *vic4* but homoallelic at all other vegetative incompatibility loci, were assigned to a pooled vc type. It has been previously described that locus *vic4* did not restrict virus transmission (Cortesi et al. 2001). One isolate from each pooled vc type was selected at random. The additional samples required to reach a sample size of 16 were then randomly selected from the entire population. Randomization was performed in R. The number of isolates selected from each vc type is shown in Table S1.

From each of the 16 isolates per population we produced a virus-free culture through isolations of single conidia, of which some had escaped virus-infection. We spread conidial suspensions on PDA and incubated them at 24°C for 3 days in the dark followed by 7 days under low light conditions. Virus-free pycnidia were identified by their orange colour and the exudation of conidia (Choi and Nuss 1992). This conidial mass was transferred to PDA with a sterile dissection needle and incubated at 24°C for 7 days in the dark followed by 2 days under low light conditions. Pure cultures that had been cured successfully from the virus were identified by their orange culture morphology.

Assessment of viral virulence

Virulence was measured as the reduction of fungal growth due to virus infection, which was previously shown to be correlated with the reduction in sporulation (Peever et al. 2000; Bryner and Rigling 2011). These effects are common virulence measures of fungal viruses (Nuss 2005; Pearson et al. 2009) and they are relevant for biocontrol of chestnut blight (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). Findings of significant genotype-by-genotype interactions between CHV-1 and *C. parasitica* (Peever et al. 2000; Bryner and Rigling 2011), prohibited the use of a common genetic background in our study. Instead, we assessed the virulence of each virus in its natural host by comparing the growth of the virus-infected fungal culture with the growth of the virus-free clone (Bryner and Rigling 2011). For each population, we inoculated two cultures of the 16 virus-infected and the 16 virus-free fungi (in total 512 cultures) on dormant chestnut stems. Healthy *C. sativa* stems (50 cm length, 5–10 cm diameter) were cut in the Ticino (Switzerland) in February 2011, a few days before the start of the experiment. Both ends of the stems were sealed with paraffin. Along the axis of each stem, four circular wounds were made and filled with the mycelia mats as described in (Bryner and Rigling 2011). The virus-free and the virus-infected cultures of the same fungal strain were always put

into neighboring wounds on the same stem. The two cultures of each virus-infected virus-free pair of the same fungal strain were randomly assigned to stems and to positions on stems. The stems were incubated in opaque plastic containers inside a climate chamber (Bryner and Rigling 2011). The temperature was kept constant at 20°C, and relative humidity was set to 70% (JUMO DICON SM Universeller Kompaktregler, M. K. Juchheim, Fulda, Germany). After 22 days of incubation, we determined the geometric mean diameter of each lesion to assess the fungal growth on chestnut stems (Bryner and Rigling 2011).

Data analysis

To quantify the effect of each virus on its natural host we calculated the growth difference between the virus-infected and the virus-free clone incubated on the same chestnut stem. The difference was given in proportion to the growth of the virus-free culture and termed the virus effect (Bryner and Rigling 2011). The virus effect indicated to which percentage the growth of the fungus was changed due to infection with the virus. It was our measure for virus virulence towards its natural host. For each virus we made two assessments to obtain a higher precision of the virulence measurement and calculated their mean. We analyzed the virus effect using a general linear model (GLM) in SPSS 19.0 (SPSS, Somers, NY) with the fixed factors population type "Type" (i.e. low vs. high vc type diversity) and population nested within population type "Pop(Type)". We also applied a GLM on the standard deviation of each population to determine if "Type" had an effect on the within-population variation. Furthermore, we analysed the effect of the frequency of infected vc types on the virus effect. A GLM with the fixed factors frequency of the vc type "Freq" (i.e. rare vs. common vc types) and population "Pop" was used. A vc type was considered rare when the pooled vc type (see above) occurred at a frequency of less than 7% within the population; otherwise it was considered common. The residuals of all GLMs were normally distributed and displayed constant error variances.

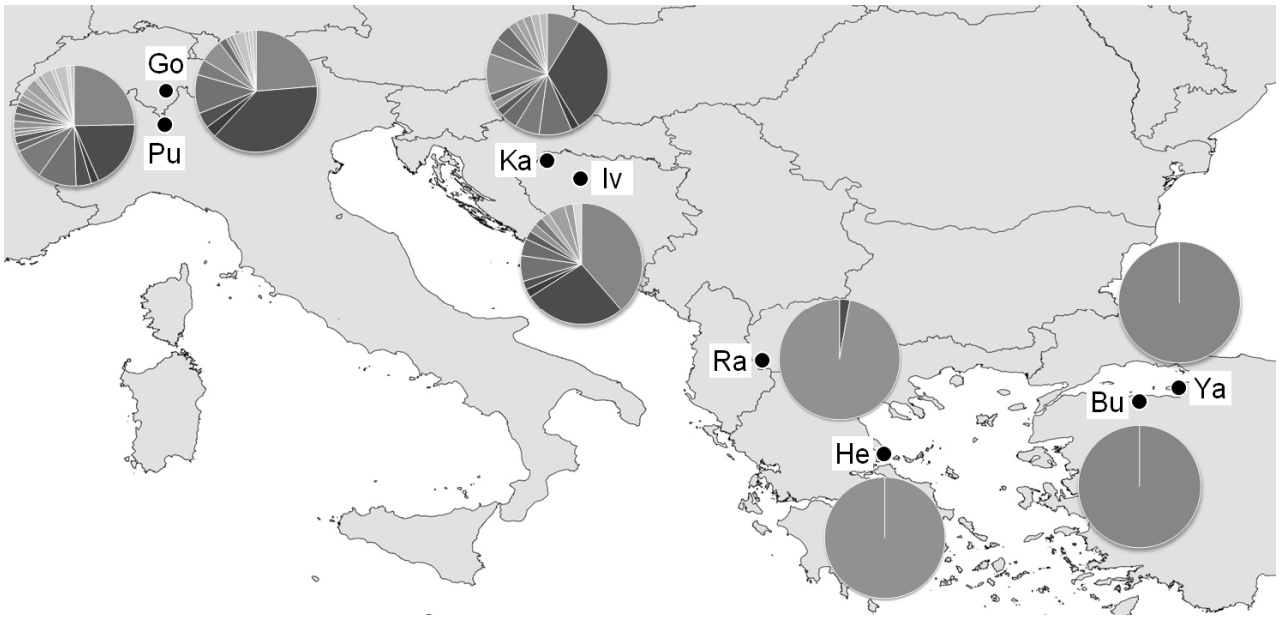

Fig. 1. Map of Europe showing the eight sampling sites. In 2010, Go and Pu were sampled in Switzerland, He in Greece, Ra in Macedonia, and Bu and Ya in Turkey. Ka and Iv were sampled in Bosnia in 2008. Pie graphs display the diversity of vegetative compatibility types that we observed in each population of *Cryphonectria parasitica*.

Table 1. Diversity of vegetative compatibility types and potential for virus transmission in the eight *Cryphonectria parasitica* populations used in this study.

Population	N	Virus-infected	Vc types	H'_{obs}	H'_{44}	Potential of virus transmission
Go	97	50 (52%)	14	1.91	1.63	0.45
Pu	97	42 (43%)	24	2.57	2.54	0.40
Ka	46	39 (85%)	17	2.37	2.26	0.35
Iv	44	31 (70%)	13	1.87	1.87	0.48
He	93	41 (44%)	1	0.00	0.00	1.00
Ra	72	55 (76%)	2	0.13	0.11	0.93
Bu	96	33 (34%)	1	0.00	0.00	1.00
Ya	89	18 (20%)	1	0.00	0.00	1.00

Note: Populations were sampled within an area <1 ha each in Switzerland (Go and Pu), Bosnia (Ka and Iv), Greece (He), Macedonia (Ra) and in Turkey (Bu and Ya).N is the total number of collected isolates, followed by the number of virus-infected isolates (proportion of total in parentheses). Furthermore, the number of different vegetative compatibility (vc) types is given. The Shannon-Wiener's index is expressed in terms of the observed vc types (H'_{obs}) and in terms of the vc type distributions expected (H'_{44}) by rarefaction analysis for the smallest sample (n=44). The estimated mean potential of virus transmission within the population was calculated based on the observed vc type frequency distribution (Cortesi et al. 2001).

Results

Vc type diversity

Table 1 displays for each population the total number of isolates sampled, the total number of vc types detected, the Shannon-Wiener's index for vc type diversity and the estimated potential of virus transmission. In the populations from Switzerland and Bosnia (high-diversity populations), the vc type diversity was substantially higher than in the populations from Greece, Macedonia and Turkey (low-diversity populations), which also became manifest in the estimated potential of virus transmission. Between 13 and 24 different vc types per population were detected in the high-diversity populations (Go, Pu, Ka and Iv). In three of the four low-diversity populations only one vc type (EU-1 in Ya and Bu, and EU-12 in He) was found. The population from Macedonia (Ra) was also dominated by one vc type (EU-12), but two of the 72 sampled isolates were of a second vc type (EU-2).

The estimated potential of virus transmission (i.e. the mean probability of virus transmission across the population) ranged from 0.35 to 0.48 in the high-diversity populations and from 0.93 to 1.00 in the low-diversity populations.

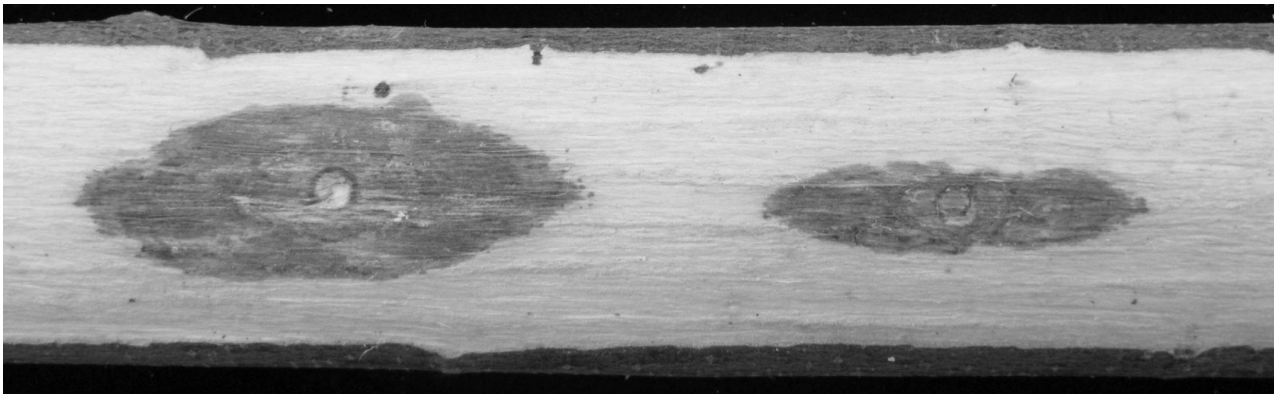

Fig. 2. Chestnut blight lesions produced on a chestnut stem by clones of the fungus *Cryphonectria parasitica,* virus-free (left) or infected with *Cryphonectria hypovirus 1* (right). The bark was peeled off for better visibility of the lesions. Photograph by Sarah Bryner.

Viral virulence in fungal populations with low and high vc type diversity

In all populations, the virus-free isolates produced on average larger lesions than the virus-infected isolates on dormant chestnut stems (Fig. 2; Table S2). Since the effect of each virus on fungal growth was expressed by the relative difference in lesion size

between the virus-infected strain and the corresponding virus-free strain, negative virus effects were observed in most cases. Figure 3 depicts the population's mean virus effects in ascending order. It shows that the populations of low and high vc type diversity were intermixed. Both the strongest (highest virulence) and the lowest mean virus effect (lowest virulence) were observed in high-diversity populations.

The GLM on the full data set revealed that the virus effect on fungal growth was not significantly ($p > 0.05$) affected by the vc type diversity of the fungal host population ("Type", Table 2A). The population had a significant ($p = 0.027$) effect ("Pop(Type)", Table 2A). This result was confirmed when, as a control, the same analysis was performed on additional measurements done 18 and 26 days after inoculation (results not shown). Furthermore, the population type had no significant ($p > 0.05$) effect on the within-population variation, which was high in all eight populations (results not shown).

The GLM restricted to the high-diversity populations revealed that whether the virus was infecting a rare or a common vc type did not significantly ($p > 0.05$) affect the virus effect ("Freq", Table 2B). The population had again a significant ($p = 0.029$) effect ("Pop", Table 2B).

Table 2. General linear models on the effect of virus infection on the growth of *Cryphonectria parasitica*.

A: Effect of population type on virus effect					B: Effect of vc type frequency on virus effect				
Source	df	MS	F	P	Source	df	MS	F	P
Type	1	0.037	0.841	0.361	Freq	1	0.034	0.810	0.372
Pop(Type)	6	0.110	2.483	0.027	Pop	3	0.137	3.222	0.029
Error	120	0.044			Error	59	0.043		

Note: The effects of the fixed factors population type ("Type"; low vs. high vc type diversity) and population nested within population type ("Pop(Type)") were tested on the effect of virus infection on the growth of *C. parasitica* on dormant chestnut stems (lesion diameter after 22 days of incubation) using a total number of eight populations: four of low vegetative compatibility (vc) type diversity and four of high vc type diversity. Using the four populations with high vc type diversity only, the effect of the fixed factors vc type frequency ("Freq"; common vs. rare) and population ("Pop") were tested.

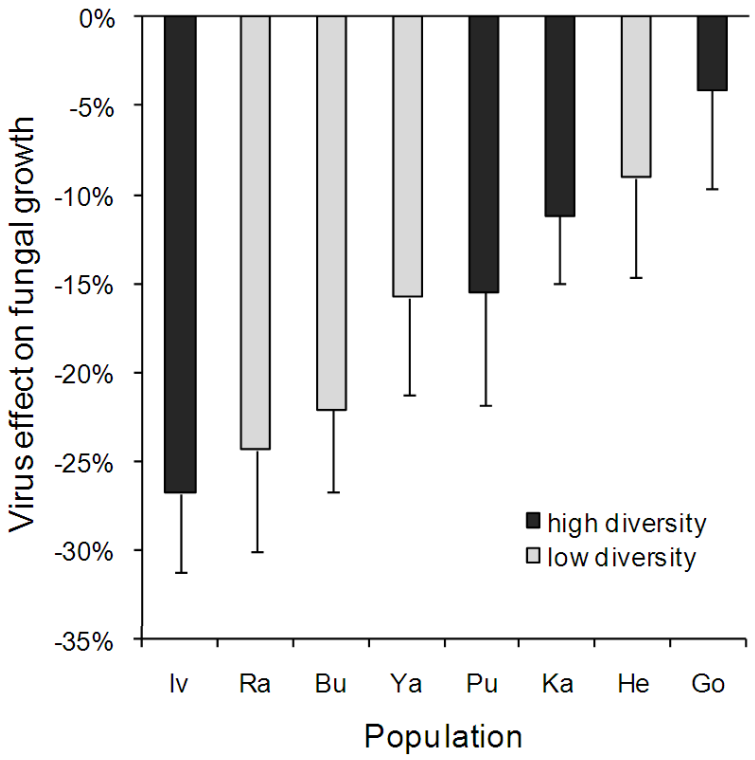

Fig. 3. Effect of virus infection on the growth of *Cryphonectria parasitica* on chestnut stems after 22 days of incubation. The virus effect is the difference in growth (lesion diameter) between the virus-infected and the virus-free clone as a proportion (%) of the virus-free clone. For each population (Go, Pu, Ka, Iv, He, Ra, Bu, Ya), the mean virus effect (n=16) is displayed. Error bars represent standard errors. The pattern of the bars refers to the diversity of vegetative compatibility types in the *C. parasitica* populations.

Discussion

The results of our study did not support the hypothesis that a lower transmission probability promotes the evolution of reduced parasite virulence. We estimated the transmission probability of the hyperparasitic virus CHV-1 from the vc type diversity within the fungal host population. The virus transmission probability was considerably restricted in the populations with high vc type diversity (H'_{obs} = 1.87–2.57), whereas it was unrestricted in the populations with low diversity (H'_{obs} = 0.00–0.13). The vc type diversities that we observed had been at the same levels for more than 30 years in all populations (Robin and Heiniger 2001). This indicated that there was no or only very little migration between high and low diversity populations (Milgroom et al. 2008), providing opportunity for coevolution over an extended period of time. However, the presence or absence of these transmission barriers neither seemed to have an effect on the average virulence of virus populations nor on the variation of virulence within populations. Furthermore, the highest virulence of CHV-1 was observed in a population with high vc type diversity. We further examined the virulence of viruses infecting rare versus common vc types. Rare vc types were frequently virus-infected and the virulence of those viruses was not lower than the virulence of viruses infecting common vc types. All these findings suggested that transmission barriers between different vc types did not exhibit a discernable selective pressure on viral virulence.

In all eight populations of CHV-1, we found high variation in virulence, which would have provided the raw material for evolution. The apparent lack of selection may indicate that the selective advantage or disadvantage of viruses with low vs. high virulence was not significant. *C. parasitica* and CHV-1 were both introduced to Europe during the past century, the first official records dating back to 1938 for *C. parasitica* and 1951 for healing cankers that were identified in 1964 to be associated with the dsRNA of CHV-1 (Heiniger and Rigling 1994). In evolutionary terms, a coevolution of 60 – 70 years is not long. However, parasites – and RNA viruses in particular – are known to evolve very rapidly (Moya et al. 2004; Walther and Ewald 2004). Furthermore, hypoviruses follow more closely the properties of single-stranded (ss) RNA viruses than true dsRNA viruses. It is generally thought that the dsRNA is most likely an artefact of the replication cycle during which the nascent and template RNA strands anneal and copurify (Fahima et al. 1993).

CHV-1 is therefore fundamentally an ssRNA virus, which evolves even faster than dsRNA viruses (Hillman 2001). A classic example of rapidly evolved virulence comes from the biological control of introduced rabbits in Australia with myxoma virus (Fenner 1983). Within only one year of coevolution, the mortality rate of infected rabbits decreased from

100% to 90%.Overall, it is therefore not apparent why virulence in CHV-1 would not evolve after half a century of coevolution – unless the selective pressure was not strong enough.

In our study, all vc types were equally virus-infected, which could also indicate that the vc type barrier was not as restrictive as previously assumed. The prevalence of CHV-1 in populations with high vc type diversity was not lower than in populations with low vc type diversity. The restriction on transmission imposed by the vc type diversity may therefore be questioned despite its wide acceptance in the *Cryphonectria*-hypovirus literature (Milgroom and Cortesi 2004). A population genetics study by Carbone et al. (2004) also indicated that migration of CHV-1 among vc types occurs within (but insignificantly among) natural populations. These results suggested that, in natural populations, the virus transmission probability might be higher than expected from observations in short-term laboratory experiments (Cortesi et al. 2001; Papazova-Anakieva et al. 2008) or from modelling (Liu et al. 2000). Thus, the relevance of the vc type barrier for virus transmission in nature has to be better quantified. In addition, the high vc type diversities in Europe might not be high enough to exert a selective pressure on virulence. Vc type diversities considered high in Europe are only moderate when compared to vc type diversities in North America and especially in Asia, the centre of origin of *C. parasitica* (Liu et al. 2007). Theoretical studies indicated that viruses might not be able to invade *C. parasitica* populations if their vc type diversity exceeded a certain threshold (Liu et al. 2000). This might have been the case in North America and would explain why biocontrol efforts have not been successful there (Milgroom and Cortesi 2004).

Furthermore, modelling of the relationship between vc type diversity, CHV-1 virulence and CHV-1 prevalence indicated a certain stability of the system. The model of Brusini et al. (2011) suggests that the system is robust against change at moderate levels of both the vc type diversity and the CHV-1 virulence. A significant feedback effect on the other model component is only expected when either the vc type diversity or the virulence increases drastically. The virulence of CHV-1 subtype I, the subtype that we have investigated in this study, is moderate compared to other (less prevalent) subtypes present in Europe (Gobbin et al. 2003; Robin et al. 2010; Bryner and Rigling 2011). The results of our empirical study (i.e. no feedback effects) therefore correspond with the model predictions.

In this study, we investigated the impact of transmission barriers provided by the host population on parasite virulence. The impact of the host population on parasite virulence is of particular importance in biocontrol systems, where the release of a biocontrol agent specifically aims at affecting the host population (Hufbauer and Roderick 2005). If certain characteristics of host populations would promote the evolution of avirulence in the

biocontrol agent, biocontrol would become ineffective in those populations. In order to design sustainable disease management strategies, it is therefore crucial to understand the interactions and interrelations of the host and parasite populations in each biocontrol system. It has been shown previously that the probability for parasite transmission can differ among host populations and that this difference may affect the evolution of parasite virulence. Herre (1993) found that high virulence evolved in parasitic nematodes when the fig wasp populations provided increased transmission probabilities (multiple foundress broods) and low virulence evolved when they provided limited transmission probabilities (single foundress broods). Our findings, however, did not indicate an association of transmission probability and virulence in CHV-1 and, thus, have positive implications for the biocontrol system. CHV-1 is playing an indispensable role for the biological control of chestnut blight in Europe, where it has been persisting within fungal host populations. The incidence of chestnut blight is very high but infection of *C. parasitica* with CHV-1 is preventing severe damage, including dieback of the trees (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). The stability of this natural biological control system has been a major concern and a loss of CHV-1 virulence due to a potential increase in vc type diversity has been suspected. New vc types may be introduced or could emerge through sexual reproduction of *C. parasitica*. In our study, fortunately for the biological control, no trend for reduced virulence of CHV-1 at high vc type diversities became apparent. This suggests that the biocontrol of *C. parasitica* with CHV-1 in Europe is not eroding due to (moderate) increases in vc type diversity. More generally, the results of this study indicate that biocontrol agents may be very effective in controlling a disease in a sustainable and self-perpetuating way over a long period of time – once they have been well established within a system. In the case of fungal pathogens, the vc type diversity of the pathogen population may be of less importance than previously expected, provided that the diversity does not pass a threshold (Liu et al. 2000) above which invasion of the biocontrol agent is inhibited completely.

In conclusion, the vc type diversity of *C. parasitica* populations did not have a significant effect on the virulence of CHV-1. Furthermore, virulent viruses were able to persist in populations with high vc type diversity, showing great promise for the sustainability of the biological control system. There was no sign for the evolution of lower virulence in the biocontrol agent due to differences in parasite transmission probability provided by the host population. These findings are promising for the sustainability and long-term effectiveness of the biocontrol of chestnut blight in Europe and they are an important contribution to research on the evolution of biocontrol systems.

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Supporting information

Table S1. Vegetative compatibility (vc) types observed in the eight populations of *Cryphonectria parasitica*.

Vc type	Go	Pu	Ka	Iv	He	Ra	Bu	Ya
EU-1	23 (10;3)	24 (11;3)	4 (4;2)	17 (13;4)			96 (33;16)	89 (18;16)
EU-2	37 (23;6)	18 (10;4)	15 (13;4)	12 (7;4)		2 (2;1)		
EU-3	3 (3;1)	2 (1;1)	1 (0;0)	1 (0;0)				
EU-4	4 (2;1)	4 (2;1)		1 (1;1)				
EU-5	10 (6;1)	10 (3;1)	4 (4;1)	3 (1;1)				
EU-6	4 (1;1)	8 (5;0)	3 (3;0)					
EU-7		2 (0;0)	2 (1;0)	2 (2;1)				
EU-8		2 (0;0)	1 (1;1)	1 (1;1)				
EU-9		1 (0;0)	1 (1;0)					
EU-10			1 (1;1)					
EU-11		1 (1;0)						
EU-12	6 (2;1)	2 (1;1)	5 (4;1)	1 (1;1)	93 (41;16)	70 (53;15)		
EU-13		2 (1;0)	2 (2;1)	1 (0;0)				
EU-14		2 (1;1)						
EU-16				1 (1;1)				
EU-17	2 (0;0)	1 (0;0)	2 (1;1)					
EU-18	1 (0;0)	2 (1;1)	1 (1;1)					
EU-19			1 (1;1)					
EU-21		2 (0;0)						
EU-22	1 (0;0)	3 (0;0)	1 (1;1)	2 (2;0)				
EU-23	3 (1;1)	1 (0;0)						
EU-25		1 (0;0)		1 (1;1)				
EU-26		3 (2;1)	1 (0;0)					
EU-27	1 (1;1)		1 (1;1)					
EU-28	1 (0;0)	1 (1;1)						
EU-29	1 (1;0)	3 (1;1)						
EU-31		1 (1;0)		1 (1;1)				
EU-43		1 (0;0)						

Note: For each population (Go, Pu, Ka, Iv, He, Ra, Bu, Ya) the total number of collected isolates of each vegetative compatibility (vc) type is displayed. In parentheses, the number of virus-infected isolates followed by the number of isolates selected for the experiment is given.

Table S2. Growth (mm) of *Cryphonectria parasitica* on dormant chestnut stems after 22d of incubation.

Population	Virus-free *C. parasitica*		Virus-infected *C. parasitica*	
Go	26.65	(5.24)	24.85	(5.67)
Pu	26.77	(3.65)	22.10	(5.14)
Ka	27.28	(3.41)	24.12	(6.04)
Iv	28.35	(6.08)	20.13	(5.23)
He	25.24	(2.64)	22.01	(4.92)
Ra	26.57	(4.46)	19.69	(5.84)
Bu	24.51	(4.21)	18.80	(4.71)
Ya	25.52	(3.42)	21.30	(5.67)

Note: The means and the standard deviations in parentheses for the growth of the 16 virus-free isolates and the 16 virus-infected clones of *C. parasitica* in each population are displayed.

Chapter 4

Virulence not only costs but also benefits the transmission of a fungal virus

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Abstract

Current theory suggests that cost-benefit relationships govern the evolution of parasite virulence. The cost of virulence is expected to be high for fungal viruses, which are obligate parasites and completely dependent on their hosts. The majority of fungal viruses infect their hosts without any apparent symptoms. *Cryphonectria hypovirus 1* (CHV-1), in contrast, is virulent and debilitates its host, *Cryphonectria parasitica*. However, the virulence of CHV-1 is associated with high costs for virus transmission, such as an attenuated fungal growth and reduced production of the fungal spores spreading the virus. In this study, we tested the hypothesis that virulence may not only have costs but also benefits for transmitting CHV-1 across vegetative incompatibility barriers between fungi. We investigated viruses with low, medium and high virulence, and determined their transmission rate per host-to-host contact (transmissibility). The average transmission rate across all combinations tested was 53% for the most virulent virus, 37% for the virus with intermediate virulence and 20% for the virus with lowest virulence. These results showed that increased virulence was strongly correlated with increased transmissibility, potentially counterbalancing virulence costs. This association of virulence and transmissibility may explain why CHV-1 spread widely and evolved higher virulence than most other fungal viruses.

Introduction

Why do parasites harm their hosts when – at the same time – they depend on hosts for survival and fitness? Current theory suggests that the evolution of virulence (i.e. harm to the host) depends on a cost-benefit relationship. The benefits of host exploitation on parasite multiplication within hosts are traded off against the cost of this exploitation for parasite transmission between hosts (Anderson and May 1982; Bull 1994; Frank 1996). Highly virulent parasites multiply rapidly and exploit their hosts efficiently, but at the cost of increased host mortality or debilitation, and thus limit the hosts' ability to transmit the parasite.

Several models have been developed to describe the evolution of virulence in host-parasite systems (Levin and Pimentel 1981; Frank 1992; Antia et al. 1994; Day 2001; Koella and Restif 2001; Day 2002). These generally suggest that the optimal degree of virulence maximizes the parasite's fitness and, thus, depends on the particular cost-benefit relationship in the host-parasite interaction. Some parasites rely on relatively normal host functions for transmission and, hence, cannot be highly virulent, while other parasites may debilitate their host without strongly limiting transmission (Read 1994; Ewald 1995). Vertically (to offspring) transmitted parasites, for example, depend on the reproduction of their infected hosts. For them, the cost of reduced host reproduction is much higher than for horizontally (to other hosts) transmitted parasites, which do not require their host to reproduce (Bull et al. 1991). Similarly, the cost of host debilitation is much higher for parasites that cannot survive outside their hosts and depend on a live host than for parasites that are able to survive without their hosts (Walther and Ewald 2004).

Direct investigations of virulence and transmission are required to define the cost-benefit relationship within a host-parasite system. For instance, higher virulence was associated with reduced host fitness, but also with increased parasite replication, in: microsporidian parasites of the yellow fever mosquito (Agnew and Koella 1997); protozoan parasites of the monarch butterfly (De Roode et al. 2008); bacterial parasites of water fleas (Jensen et al. 2006); malaria parasites of mice (Mackinnon and Read 1999); myxoma virus of rabbits (Fenner 1983) and the human immunodeficiency virus (Fraser et al. 2007).

Fungal viruses are obligate parasites, which infect their host persistently (Nuss 2005). They generally lack an extracellular stage outside the host and therefore completely depend on their host for survival and transmission. According to the theory described above, the cost of their virulence is, thus, predicted to be high. Unfortunately, little research has been done on fungal viruses and this field is still in its infancy (Pearson et

al. 2009). The vast majority of fungal viruses characterized so far, however, infect their host without any apparent symptoms (Nuss 2005). *Cryphonectria hypovirus 1* (CHV-1) is an exception among these fungal viruses and causes marked and quantifiable symptoms in its host *Cryphonectria parasitica* (Milgroom and Cortesi 2004). This may explain why CHV-1 is one of the fungal viruses that has been best studied.

C. parasitica is the causal agent of the tree disease chestnut blight, and hyperparasitation by CHV-1 results in debilitation of the fungus. CHV-1 does not kill *C. parasitica* but inhibits its sexual reproduction, strongly attenuates its growth and asexual sporulation, and reduces the pathogenicity of *C. parasitica* towards the chestnut tree (Milgroom and Cortesi 2004). CHV-1 is therefore used for the biological control of chestnut blight (Heiniger and Rigling 1994). Several subtypes of CHV-1 exist, which greatly differ in their virulence towards *C. parasitica* (Gobbin et al. 2003; Bryner and Rigling 2011), providing variation in virulence in this pathosystem.

One way to quantify the virulence of CHV-1 is by assessing the reduction in fungal growth (Peever et al. 2000; Bryner and Rigling 2011), which is a common and biologically relevant measure of virulence in fungal viruses (Pearson et al. 2009). In CHV-1, this growth reduction is highly correlated with other virulence factors, in particular with a reduction in asexual sporulation (Chen and Nuss 1999; Peever et al. 2000; Bryner and Rigling 2011). The spread of CHV-1 occurs mainly by transmission to virus-free individuals of *C. parasitica* (Hoegger et al. 2003). CHV-1 is transferred into the asexual spores of the fungus, dispersed in these spores and then transmitted from the outgrowing spores to other fungal individuals by hyphal fusion (Milgroom and Cortesi 2004). Virus-infected spores do not, however, seem to be a major source of new chestnut blight infections (Hoegger et al. 2003). The virus, thus, depends on the combination of vertical (into spores) and subsequent horizontal (from outgrowing spores to new hosts) transmission. Virus-infected individuals of *C. parasitica* are white, while virus-free individuals are orange when grown in the laboratory (Choi and Nuss 1992a). Therefore, the change from orange to white (Fig. 1) can be used to determine successful virus transmissions (Peever et al. 2000; Bryner and Rigling 2011).

Similar to the other parasites cited above, virulent strains of CHV-1 have been shown to have a higher virus replication rate in the host and a higher virus-infection rate of asexual fungal spores (Suzuki et al. 2003; Lin et al. 2007). However, this occurs at a very high cost for CHV-1 since virulence is associated with the inhibition of fungal growth and of production of the asexual fungal spores carrying the virus (Milgroom and Cortesi 2004). This, therefore, raises the question why highly virulent strains of CHV-1 should be so prevalent (Taylor 2002; Milgroom and Cortesi 2004), in particular because the spreading of CHV-1 depends on the reproduction of its host. In accordance with the

theory described above, a strong selective pressure favouring low virulence would be expected in CHV-1, especially under conditions where the transmission potential of CHV-1 is further reduced by the presence of increased transmission barriers within fungal populations (Milgroom 1995; Taylor 2002).

The transmission of CHV-1 is restricted by vegetative incompatibility between fungal host genotypes (Anagnostakis and Day 1979; Anagnostakis and Waggoner 1981; Cortesi et al. 2001; Choi et al. 2012). Vegetative incompatibility is a self/nonself recognition system in fungi that prevents transmission of viruses and other cytoplasmic elements (reviewed in Glass and Kaneko 2003). Vegetative incompatibility, thus, presents an additional transmission barrier in fungi. In *C. parasitica,* vegetative incompatibility is controlled by at least six bi-allelic unlinked vegetative incompatibility (*vic*) loci (Cortesi and Milgroom 1998), some of which have recently been characterized at the molecular level (Choi et al. 2012). Compatible individuals share the same alleles at all six *vic* loci, while incompatible individuals differ in their alleles at one or more loci. Hence, the *vic* haplotype defines the vegetative compatibility (vc) type (Cortesi and Milgroom 1998). Between individuals of *C. parasitica* that share the same vc type, CHV-1 is transmitted in virtually 100% of host-to-host contacts. Between different vc types, virus transmission sometimes occurs, but at reduced rates (Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). The presence of these vegetative incompatibility barriers in fungi is another factor that has most likely promoted the evolution of low virulence in most fungal viruses (Milgroom 1999). In a recent study of CHV-1, however, no evidence for a trend towards lower virus virulence was found in fungal populations with high levels of vegetative incompatibility (Bryner and Rigling, submitted). Virulence and prevalence of CHV-1 were equally high in *C. parasitica* populations with and without transmission barriers. This indicates that there must be certain benefits of virulence for transmission that allow CHV-1 to be virulent without compromising its effective transmission.

In the *Cryphonectria* literature, it has been commonly assumed that the rate of transmission per host-to-host contact (transmissibility) is not influenced by the virus strain (Cortesi et al. 2001). However, more virulent viruses might be more transmissible and, thus, have a higher transmission rate per host-to-host contact than less virulent viruses. This virulence benefit would counterbalance the virulence cost for transmission, i.e. the lower number of asexual fungal spores carrying the virus would be counterbalanced by the higher infectivity of these spores. We therefore suggest clearly distinguishing between two main virus-dependent factors that potentially influence virus transmission: First, the number of virus-infected asexual spores that are produced by the fungus and that carry the virus to other hosts; and second, the rate at which the virus is finally transmitted after a successful host-to-host contact. While the effect of virulence on asexual sporulation of the fungus is well known (Peever et al. 2000; Taylor 2002;

Milgroom and Cortesi 2004; Bryner and Rigling 2011), its effect on virus transmissibility has remained unexplored.

The aim of this study was to find out more about the effect of virulence on transmissibility and how, if at all, it benefits virus transmission by counterbalancing the known virulence costs in CHV-1. To test the hypothesis that increased virulence is associated with increased transmissibility, we investigated three virus strains with different virulence (determined as the reduction in fungal growth), and assessed their transmissibility among fungal hosts.

Materials and methods

Fungus and virus isolates

We assessed the transmission rate of three virus strains between different pairs of fungal donor and recipient strains. The three virus strains represented three subtypes of CHV-1 (I, D and F1 (Gobbin et al. 2003)), which are known to differ significantly in virulence (Chen and Nuss 1999; Peever et al. 2000; Robin et al. 2010; Bryner and Rigling 2011). The vc type (Cortesi and Milgroom 1998) of the *C. parasitica* isolates used as donor strains was EU-2. Virus-free recipient strains of three different vc types, all heteroallelic to vc type EU-2 at one *vic* locus, were used: EU-4 heteroallelic at *vic1*, EU-1 heteroallelic at *vic2* and EU-14 heteroallelic at *vic6*. To include some genetic background variation in the fungal host, we used two genetically different isolates originating from different populations as donor strains (isolate "baw" from Germany and isolate "var" from France (Bryner and Rigling 2011)), and two different isolates for each of the three vc types as recipient strains (isolates with suffix "A" from Italy and isolates with suffix "B" from Switzerland (Cortesi et al. 1998)). As a control, we also assessed virus transmission rates between vegetatively compatible donor and recipient strains, namely from the virus-infected donor strains "baw" and "var" two virus-free cultures of "baw" and "var".

Virus transmission assessment

The transmission rate of the three viruses was assessed using a previously described method (Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). The six donor cultures (two fungal strains infected with each of the three viruses) were paired with the six recipient cultures (two fungal strains from each of the three vc types), giving a total of 36 different combinations, plus the vegetatively compatible control pairings. For each combination, 25 replicates were tested. A 9-cm petri dish (84-mm inner diameter) was used, containing 25 ml of potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for each pairing of a virus-infected donor with a virus-free recipient culture. Plates were inoculated with two mycelial plugs (6-mm diameter) from the growing edge of 5-day-old precultures, one from the virus-infected donor strain and one from the virus-free recipient strain. The two plugs were placed approximately 4 mm apart and approximately 5 mm from the edge of the plate. The cultures were incubated at 24°C and 70% relative humidity for 7 days in the dark and subsequently for 14 days under low light conditions. After 21 days, virus transmission was assessed and double checked by a second person. In a few ambiguous cases, the success or failure of virus transmission was verified by subculturing the recipient culture. Virus transmission was considered successful if the recipient culture took on the morphology and the colour of

the donor, i.e. unpigmented, white mycelium (Fig. 1). Successful transmissions were coded as "1" and unsuccessful transmissions as "0", which resulted in a binary data set.

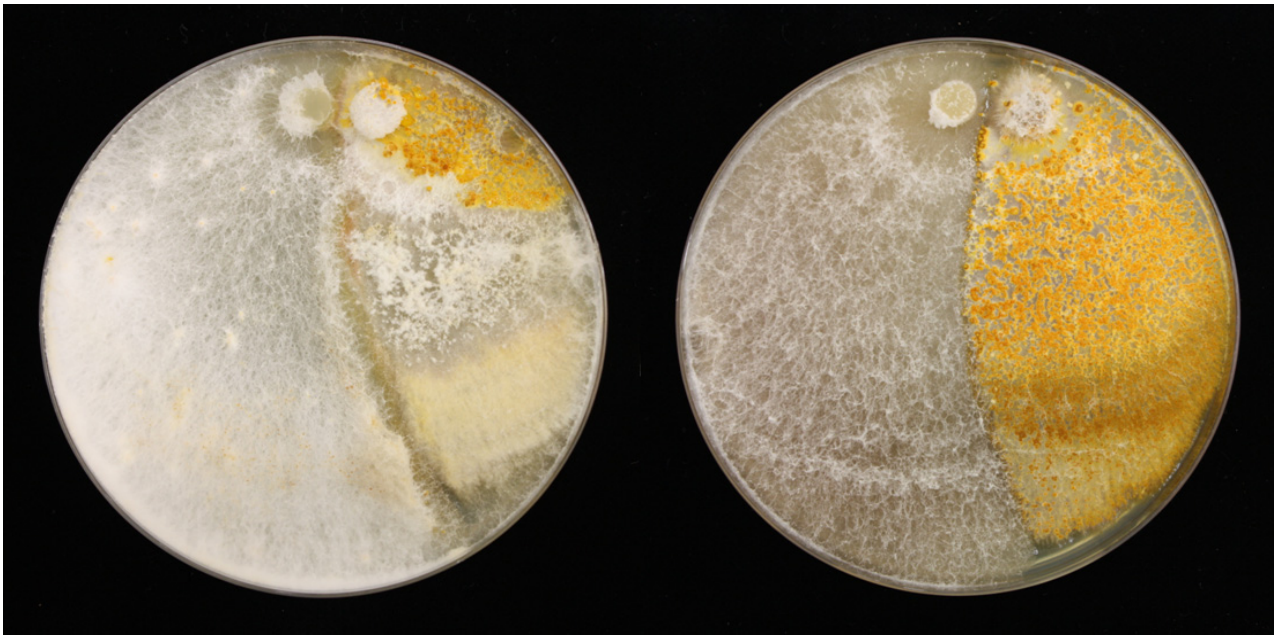

Fig. 1. Morphological determination of virus transmission between vegetatively incompatible strains of *Cryphonectria parasitica*. Successful transmission of *Cryphonectria hypovirus 1* (left): the virus-free recipient culture (orange) took on the colour of the virus-infected donor culture (white). No transmission (right): the virus-free recipient culture remained orange and thus virus-free. Photograph by Sarah Bryner.

Virus virulence assessment

To obtain a measure for the virulence of the three virus strains, we assessed their effect on the growth of the two fungal donor strains "baw" and "var" under the same experimental conditions as we assessed transmission, i.e. at 24°C and 70% humidity. The same stock cultures as in the virus transmission experiments were used. We performed two virulence experiments with identical design, one on PDA and one on dormant chestnut stems. While virulence experiments on dormant chestnut stems provide a more natural setting to obtain measures of virus virulence, experiments on PDA can be better controlled (higher standardization). The power to detect significant differences in virulence among viruses is therefore much higher in experiments on PDA (Bryner and Rigling 2011). However, on PDA, *C. parasitica* cultures infected with viruses of low virulence often grow faster than virus-free cultures (Chen and Nuss 1999; Chen et al. 2000; Robin et al. 2010; Bryner and Rigling 2011). Assessments on PDA can therefore only be used for comparisons among viruses and not to obtain absolute measures of virus virulence (Bryner and Rigling 2011).

Here, we used six replicates of each fungus-virus combination and six replicates of the virus-free cultures of both fungal strains in each experiment. Replicates were assigned to one of six blocks and randomized within blocks. The virulence of each virus strain on each fungal strain was determined by calculating the growth difference between the virus-infected and the virus-free culture of the same fungal strain. The difference (i.e. the virus effect) was given in proportion (%) to the growth of the virus-free culture, indicating the percentage to which the growth of the fungus was changed due to virus infection.

In the experiment on PDA, we used an individual sterile 9-cm Petri dish (84mm inner diameter), containing 25 ml of PDA for each fungal colony. The centre of the plates were inoculated with a mycelial plug (6 mm diameter) taken from the growing edge of 5-day-old precultures. The plates were wrapped with Parafilm, arranged in adjacent blocks on a shelf and illuminated at approximately 3330 lx (Illuminance Meter, Minolta, Japan) for a 14-hour photoperiod. We measured two cardinal diameters of each colony through two orthogonal axes previously drawn on the bottom of each plate after 2, 3 and 4 days at 24 hour intervals. As the shape of the colonies was not a perfect circle, we calculated the geometric mean diameter of an ellipse. Linear regression implemented in Microsoft Excel 2007 was performed to determine the rate of linear growth on PDA (increment of the fitted regression line, $R^2 = 0.980$).

For the experiment on dormant chestnut stems, healthy *C. sativa* stems (50 cm length, 5–10 cm diameter) were cut from six different chestnut sprout clusters in Ticino (Switzerland) after the end of the vegetation period, a few days before the start of the experiment. Stems originating from the same sprout cluster (i.e. isogenic stems) were put into the same container for the experiment. Six opaque plastic containers (57 cm x 37 cm x 13 cm) were used, each representing one of six experimental blocks. The ends of the stems were sealed with paraffin and four circular wounds were made along the axis of the stem. The wounds were arranged 12 cm apart from each other and 7 cm from the two ends. Each was filled with two mycelial mats (6 mm diameter) obtained from the growing edge of the 5-day-old precultures. After 18 days of incubation, we determined the lesion diameter on the chestnut stems, using a millimetre scale. Two diameters of each lesion were measured, one along the longitudinal and a second along the lateral axis of the stem. As the shape of the lesions resembled an ellipse, we calculated the geometric mean diameter.

Statistical analysis

To describe virus transmission, we used a logistic regression model. This is a common model for describing the presence or absence of a trait (i.e. binary data), and has

previously been applied to describe the probability of virus transmission in *C. parasitica* (Cortesi et al. 2001). We used the generalized linear mixed model procedure in R version 2.6.2 (R Development Core Team (2008), function glmer with family binomial from the lme4 package) to fit the logistic regression. The probability of virus transmission between pairs of fungal isolates was modelled as a function of the virus and the *vic* locus at which the fungal donor and recipient isolates were heteroallelic. The effects of the virus and the *vic* locus were therefore included as fixed factors. The effects of the donor and recipient, on the other hand, were defined as genetic background effects, and thus, included as random factors. Recipient was nested within heteroallelic *vic* locus. As a base model, the following logistic regression was employed:

$$\log[p_{ijkl}/(1 - p_{ijkl})] = \mu + \beta_i + \gamma_j + d_k + r_{l(j)}$$

where

p_{ijkl} is the transmission probability of virus *i* from fungal donor *k* to fungal recipient *l,* which are heteroallelic at *vic* locus *j*

μ is the intercept of the logistic regression

β_i is the effect of virus *i*, *i* = F1, D or I

γ_j is the effect of the *vic* locus *j* at which the fungal donor and recipient isolates are heteroallelic, *j* = *vic1*, *vic2* or *vic6*

d_k is the effect of the fungal donor isolate *k*, $N(0, \sigma^2_d)$

$r_{l(j)}$ is the effect of the fungal recipient isolate *l* within the heteroallelic *vic* locus *j*, $N(0, \sigma^2_r)$

The function anova implemented in R was employed to identify significant model terms. The base model (Table 1: Model 0) was compared to several simpler models with fewer factors (Table 1: Models 1-4), and to a model that also included an interaction effect between virus and *vic* locus (Table 1: Model 5). The model with the lowest Akaike's Information Criterion (AIC) value was preferred and applied to estimate the parameter values.

To analyse the virus effect (virulence), we applied a general linear model (GLM) in SPSS 19.0 (SPSS, Somers) with the fixed factors "Virus", "Fungus" and "Virus x Fungus" and with "Block" as a random factor. Tukey's test implemented in the GLM was performed to detect significant (at $\alpha \leq 0.05$) differences among viruses. Pearson's correlation coefficients were calculated in SPSS to test for a linear relationship between the virus effects measured on PDA and on dormant chestnut stem, as well as between the virus effects and virus transmissibility.

Results

Selection of the logistic regression model describing virus transmission

The lowest AIC value was reached using the base model (Model 0, Table 1), and it was therefore considered the appropriate model to use for parameter estimation. The chi-square values of the comparisons between the base model and the models that included fewer factors (Models 1-4, Table 1) revealed that these simpler models were significantly different from the base model. This indicated that all factors in the base model, i.e. virus, *vic* locus, donor and recipient, had a significant effect on the probability of virus transmission. The model that included an interaction effect between the virus and *vic* locus (Model 5, Table 1) was not significantly different from the base model. This indicates that there was no significant interaction and that the effect of the virus was independent of the *vic* locus at which the donor and recipient isolates were heteroallelic.

Table 1. Comparison of the base model (0) with alternative logistic regression models describing virus transmission.

No.	Model	AIC	Est. df	χ^2
0	$\mu + \beta_i + \gamma_j + d_k + r_{l(j)}$	420.80	7	n.a.
1	$\mu + \gamma_j + d_k + r_{l(j)}$	608.88	5	192.08****
2	$\mu + \beta_i + d_k + r_{l(j)}$	428.80	5	12.004**
3	$\mu + \beta_i + \gamma_j + r_{l(j)}$	435.99	6	17.195****
4	$\mu + \beta_i + \gamma_j + d_k$	488.00	6	69.198****
5	$\mu + \beta_i + \gamma_j + d_k + r_{l(j)} + (\beta*\gamma)_{ij}$	426.53	11	2.2684

Note: The significance levels applied were: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Table 2. Estimates of the logistic regression model for virus transmission probability.

Term	Parameter	Estimate	SE	Z
Intercept	μ	-2.7199	1.1236	-2.421*
Virus	β_D	0	n.a.	n.a.
	β_{F1}	1.9502	0.3356	5.810****
	β_I	-3.8228	0.5706	-6.700****
Vic locus	γ_{vic6}	0	n.a.	n.a.
	γ_{vic1}	7.2968	1.4838	4.918****
	γ_{vic2}	-0.5217	1.3819	-0.378
Donor	σ^2_d	1.8082	n.a.	n.a.
Recipient	σ^2_r	0.5106	n.a.	n.a.

Note: Transmission probability was estimated for the three viruses representing one of the *Cryphonectria hypovirus 1* subtypes D, F1 and I between different pairings of *Cryphonectria parasitica* strains heteroallelic at one vegetative incompatibility (*vic*) locus. The significance levels applied were: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

Table 3. Virus transmission rates.

Virus	Heteroallelic *vic* locus	Fungal donor	Fungal recipient	No. transmissions	No. trials	Transmission rate	Average transmission rate
F1	*vic1*	var	EU-4 A	25	25	100%	
		var	EU-4 B	25	25	100%	
		baw	EU-4 A	25	25	100%	
		baw	EU-4 B	25	25	100%	100%
	vic2	var	EU-1 A	14	25	56%	
		var	EU-1 B	6	25	24%	
		baw	EU-1 A	3	20	15%	
		baw	EU-1 B	0	20	0%	24%
	vic6	var	EU-14 A	6	25	24%	
		var	EU-14 B	14	20	70%	
		baw	EU-14 A	3	19	16%	
		baw	EU-14 B	4	14	29%	35%
D	*vic1*	var	EU-4 A	25	25	100%	
		var	EU-4 B	24	25	96%	
		baw	EU-4 A	25	25	100%	
		baw	EU-4 B	21	25	84%	95%
	vic2	var	EU-1 A	3	25	12%	
		var	EU-1 B	1	25	4%	
		baw	EU-1 A	1	25	4%	
		baw	EU-1 B	1	25	4%	6%
	vic6	var	EU-14 A	2	25	8%	
		var	EU-14 B	7	25	28%	
		baw	EU-14 A	0	25	0%	
		baw	EU-14 B	0	25	0%	9%
I	*vic1*	var	EU-4 A	23	25	92%	
		var	EU-4 B	6	25	24%	
		baw	EU-4 A	24	25	96%	
		baw	EU-4 B	6	25	24%	59%
	vic2	var	EU-1 A	0	25	0%	
		var	EU-1 B	0	25	0%	
		baw	EU-1 A	0	25	0%	
		baw	EU-1 B	0	25	0%	0%
	vic6	var	EU-14 A	0	25	0%	
		var	EU-14 B	0	18	0%	
		baw	EU-14 A	0	25	0%	
		baw	EU-14 B	0	21	0%	0%

Note: Transmission rates of three virus strains were calculated, each representing one of the *Cryphonectria hypovirus 1* subtypes F1, D and I, between different pairings of *Cryphonectria parasitica* strains heteroallelic at one vegetative incompatibility (*vic*) locus.

Effect of virus strain and heteroallelic *vic* locus on transmission rate

Logistic regression revealed that the probability of virus transmission significantly differed among all three viruses tested (Table 2). In the model, virus D was used as the reference category for the term virus, and its parameter value was therefore set to 0. The transmission probability for this virus was intermediate, while the transmission probability of virus F1 was significantly higher and the transmission probability of virus I significantly lower. Table 3 shows the transmission rate of each virus for each heteroallelic *vic* locus between each donor and recipient strain. Averaged over all *vic* loci and all combinations of donor and recipient strains, the mean transmission rate was 53% for virus F1, 37% for virus D and 20% for virus I (Table 3).

The probability of virus transmission also depended on the heteroallelic *vic* locus. In the model, *vic6* was used as the reference category for the term *vic* locus, and its parameter value was therefore set to 0 (Table 2). Heteroallelism at *vic2* compared to heteroallelism at *vic6* did not have a significantly different impact on transmission probability. However, transmission probability was significantly higher when *vic1* was heteroallelic. Averaged across all three viruses and all combinations of donor and recipient strains, the mean transmission rate was 85% when *vic1* was heteroallelic, 15% when *vic6* was heteroallelic and 10% when *vic2* was heteroallelic (Table 3). In the control experiment, where the same two fungal strains were used as donors and as recipients, the transmission rate was 100% for all pairings. To some extent, the probability of virus transmission was also influenced by the genetic background of the donor and recipient strains sharing the same vc type. The estimated variances attributable to specific donor and recipient strains were greater than zero (Table 2).

Table 4. General linear models on virus virulence.

		Growth rate on PDA			Lesion diameter on dormant chestnut stems		
Source	df	MS	F	P	MS	F	P
Fungus	1	0.008	2.197	0.151	0.061	0.930	0.344
Virus	2	1.067	294.292	≤0.001	1.775	27.178	≤0.001
Fungus x Virus	2	0.003	0.778	0.470	0.080	1.233	0.309
Block	5	0.005	1.491	0.228	0.005	0.839	0.353
Error	25	0.004			0.065		

Note: The effect of virus infection on fungal growth on potato dextrose agar (PDA) (growth rate in colony diameter during the phase of linear growth) and on dormant chestnut stems (lesion diameter after 18 days of incubation) were assessed. The virus effect is the difference in growth between the virus-infected and the corresponding virus-free strain of *Cryphonectria parasitica* as a proportion (%) of the virus-free strain. In the model "Virus" (n = 3), "Fungus" (n = 2) and "Fungus x Virus" (n = 6) were used as fixed factors. "Block" (n = 6) was included as a random term.

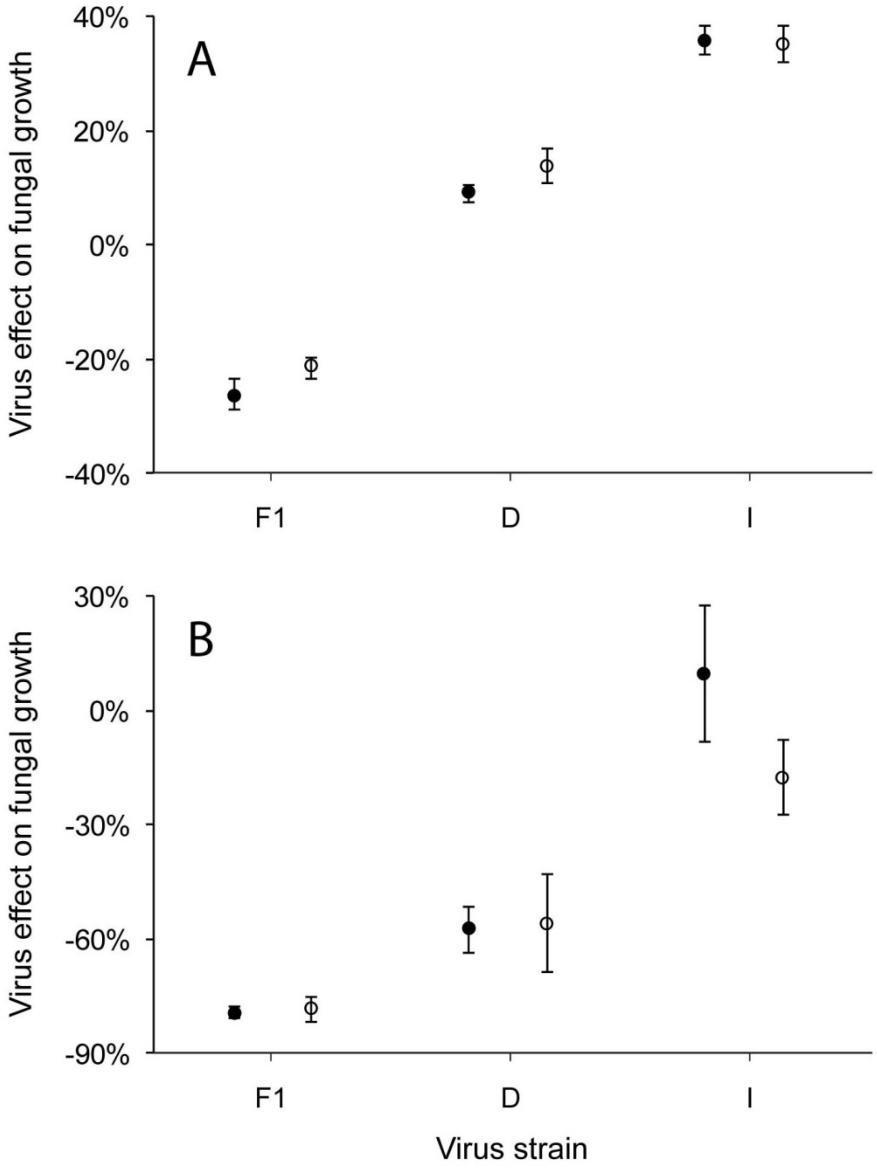

Fig. 2. Effect of three *Cryphonectria hypovirus 1* strains, F1, D and I, on the growth of the two *Cryphonectria parasitica* strains var (open circles) and baw (closed circles): (A) on potato dextrose agar (growth rate in colony diameter during the phase of linear growth) and (B) on dormant chestnut stems (lesion diameter after 18 days of incubation). The virus effect is the difference in growth between the virus-infected and the corresponding virus-free strain of *C. parasitica* as a proportion (%) of the virus-free strain. Error bars represent standard errors (n = 6).

Virus virulence

GLM results are displayed in Table 4. The factor "Virus" had a significant effect on the virus effect, but none of the other factors did. In both experiments, on PDA and on dormant chestnut stems, virus F1 had the strongest effect on fungal growth, virus I the mildest effect and virus D an intermediate effect (Fig. 2; Table S1), as expected. The virulence measurements on PDA and on dormant chestnut stems were highly correlated ($r = 0.914$, $p \leq 0.05$). Differences in virulence among all three viruses were significant in the experiment on PDA. In the experiment on dormant chestnut stems, virus I was

significantly less virulent than the other two viruses, while the difference in virulence between virus D and F1 was not significant at $\alpha \leq 0.05$.

Correlation of virus virulence and transmissibility

Virulence and transmissibility were highly correlated ($p \leq 0.05$) at $r = -0.870$ when virulence was assessed on PDA, and at $r = -0.854$ when virulence was assessed on dormant chestnut stems, as displayed in Figure 3. The sign of the correlation was negative as virulence was expressed by the reduction in fugal growth due to virus infection (negative value). The highest transmissibility was obtained with virus F1, which had the highest virulence. The lowest transmissibility was obtained with virus I, which had the lowest virulence. Finally, virus D was intermediate for both transmissibility and virulence. These differences in transmissibility were consistent across all pairings of fungal donor and recipient strains (Table 3), and thus independent of the degree of vegetative incompatibility between fungal hosts strains (Fig. 3).

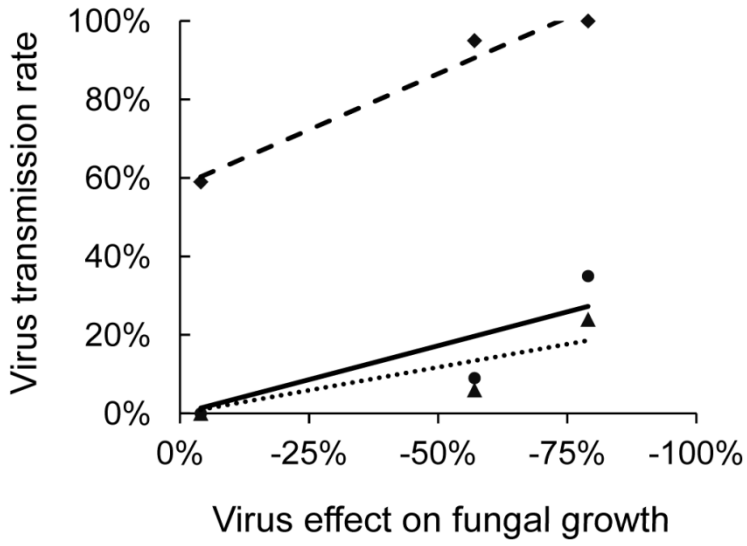

Fig. 3. The virus transmission rate (transmissibility) plotted in relation to the virus effect (virulence) on fungal growth. Here, the virus effect on fungal growth on dormant chestnut stems is shown. The assessments on dormant chestnut stems and on potato dextrose agar were highly correlated ($r = 0.914$, $p \leq 0.05$). The transmission rate is the proportion (%) of successful transmissions. The virus effect is the difference in growth between the virus-infected and the corresponding virus-free strain of *Cryphonectria parasitica* as a proportion (%) of the virus-free strain. Transmission was assessed under different degrees of vegetative incompatibility between the fungal virus donor and recipient, i.e. when the donor and recipient were heteroallelic at the vegetative incompatibility locus *vic1* (diamonds, dashed trend line), *vic2* (triangles, dotted trend line) and *vic6* (circles, solid trend line), respectively.

Discussion

The results of this study suggest that virulence has not only costs for transmission of the fungal virus CHV-1but also benefits. In the present experiment, more virulent viruses, which strongly debilitated the fungus, had higher rates of transmission per host-to-host contact (higher transmissibility). Unlike previously assumed (Cortesi et al. 2001), transmissibility was strongly influenced by the virus strain.

So far, most research on virus transmission in the *Cryphonectria*-hypovirus pathosystem has focused on the impact of vegetative incompatibility between fungal individuals (Liu and Milgroom 1996; Cortesi et al. 2001; Papazova-Anakieva et al. 2008). Cortesi et al. (2001) established a logistic regression model to predict the transmission of CHV-1. According to their model, the probability of virus transmission depends primarily on the *vic* loci at which the fungal donor and the fungal recipient are heteroallelic and to a minor extent also on the genetic background of the donor and the recipient strains. Our study confirmed the effect of the heteroallelic *vic* loci and of the genetic background of donor and recipient. In addition, we extended the model of Cortesi et al. (2001) by including virus strain as a factor. We investigated three virus strains that significantly differed in virulence, and assessed their transmissibility between different combinations of fungal donor and recipient strains (heteroallelic at one *vic* locus in all cases). The model selection showed that the factor virus had a significant impact on transmission probability, and that there was no interaction between virus and heteroallelic *vic* locus. The three virus strains tested not only significantly differed in virulence, but also in transmissibility: Higher virulence was strongly associated with increased transmission rate per host-to-host contact. This is the first time that the possibility that high virulence could be associated with higher transmissibility in CHV-1 has been considered.

The negative effects of virulence on the spread and persistence of CHV-1 have, however, been widely discussed (Milgroom 1995, 1999; Taylor 2002; Milgroom and Cortesi 2004; Robin et al. 2010). Infection with CHV-1 debilitates *C. parasitica* and reduces its growth and asexual sporulation. At the same time, CHV-1, like all fungal viruses, relies completely on fungal growth and sporulation for spread and transmission (Taylor 2002). In debilitating its host, CHV-1 should therefore directly reduce its own transmission potential. Based on these considerations, various mathematical models (Morozov et al. 2007; Brusini et al. 2011) and empirical observations (Robin et al. 2010) suggest that the ability of CHV-1 to establish and/or persist in populations decreases with increasing virulence. Our study, however, indicates that this scenario is too simplistic.

Our findings suggest that the positive effects of virulence in CHV-1 on virus replication in the fungus and on the infection rate of asexual fungal spores are not its only benefits. Virulence also seems to have positive effects on the virus transmission between hosts, and we found that virulent strains of CHV-1 had a higher transmission rate per host-to-host contact. These positive effects on transmission may counterbalance the known negative effects of high virulence on virus transmission between hosts, i.e. inhibiting fungal growth and the production of asexual fungal spores.

The correlation between virulence and transmissibility we detected may have to do with the process of virus transmission, which occurs after hyphal fusion between two fungal individuals (Nuss 2005). However, when vegetatively incompatible individuals (i.e. individuals that are heteroallelic at one or more *vic* loci) fuse, cell death is induced by the fungal self/nonself recognition system (Glass and Kaneko 2003). A comparative genomic study in *C. parasitica* recently confirmed the functional role of the *vic* loci for restricted virus transmission between incompatible individuals (Choi et al. 2012).

Virus transmission depends on the strength of the incompatibility reaction and negatively correlates to the rate at which cells die after fusion (Biella et al. 2002). CHV-1 moves rapidly within the fungal mycelium (Martin and Van Alfen 1991), and may thus cross hyphal bridges between fungal individuals before the fused cells collapse. Furthermore, it has been shown that infection with CHV-1 can influence the frequency of cell death after hyphal fusion (Biella et al. 2002). Given the finding that transmissibility increased with greater virulence, virulent viruses may interfere with the fungal self/nonself recognition and delay the initiation of cell death, thus, increasing their chances of being transmitted between incompatible individuals. The possibility that CHV-1 may interact with antiviral defence strategies in the fungus (such as the vegetative incompatibility reaction) is also mentioned by Choi et al. (2012). In addition, the chances of transmission of virulent viruses might be even further increased by their typically higher replication rate (Suzuki et al. 2003; Lin et al. 2007) and the resulting higher virus concentration in the hyphal tissue.

In our experiment, the transmission rate between vegetatively compatible hosts was 100% for all viruses, irrespective of their virulence. Between vegetatively incompatible hosts, however, the average transmission rate was 53% for the most virulent virus, 37% for the virus with intermediate virulence and 20% for the virus with lowest virulence (Table 3; Fig. 3). These differences in transmissibility between the virus strains were consistent across all combinations of vegetatively incompatible fungi tested. Virulent strains of CHV-1 may, thus, not only impair the general fitness of *C. parasitica* and negatively impact transmission, but also impede the incompatibility reaction and, thus, positively impact transmission.

This hypothesis corresponds with a more mechanistic view of virulence evolution, according to which the virulence-transmission relationship is determined by the mechanisms through which the parasite interferes with host functions (Frank and Schmid-Hempel 2008). A study in bacterial parasites of mice revealed that the same bacterial effector proteins that caused morbidity and mortality in mice were required for the transmission of bacteria (Wickham et al. 2007). Virulent bacterial strains, which possessed these effector proteins, were therefore favoured. Similar coupling of transmission with virulence was found in a nucleopolyhedrovirus of caterpillars (Szewczyk et al. 2006).

To explore the potential for the coupling of virulence and transmissibility in CHV-1, the differential impact of CHV-1 strains with different virulence on the frequency of cell death after hyphal fusion could be assessed with light microscopy (Biella et al. 2002). This was attempted recently in a yet unpublished study at the lab of Myron Smith in Ottawa, Canada. Smith et al. discovered that the highly virulent virus CHV-1/EP713 suppressed cell death more than the less virulent virus CHV-1/Euro7 (Myron Smith, personal communication). Further studies, both on the genomic and on the phenotypic level, should be conducted to understand how these different virulence factors are associated in CHV-1. The genomic regions that enable CHV-1 to affect the incompatibility reaction in *C. parasitica* may be determined by specific gene mutations (Choi and Nuss 1992b).

Findings from the above mentioned study on cell death and from our study both indicate that virulence factors, such as the reduction of growth and asexual sporulation of *C. parasitica,* are coupled with enhanced horizontal virus transmission in CHV-1. This would explain why no trend for virulence to be reduced was found when virus transmission barriers in fungal populations were high (Bryner and Rigling, submitted). Previously, it was assumed that transmission barriers (i.e. high levels of vegetative incompatibility in *C. parasitica* populations) had a stronger impact on virulent viruses, and would thus select against high virulence (Milgroom 1995, 1999). However, if the negative effects of high virulence on fungal growth and sporulation are counterbalanced by higher virus transmissibility across vegetative incompatibility barriers, there may be no selective disadvantage of highly virulent viruses. This would also agree with the observation of high variability in virulence within CHV-1 populations, irrespective of the presence or absence of transmission barriers (Bryner and Rigling, submitted).

These findings have positive implications for the biological control of *C. parasitica* with CHV-1. They suggest that strains of CHV-1 with increased virulence, which substantially reduces the pathogenic potential of *C. parasitica* towards the chestnut tree, are able to spread and persist in natural populations and exert an effective control of the chestnut blight disease. However, to exactly define the relationship between transmissibility and

virulence, further transmission experiments with more viruses that only gradually differ in virulence and transmissibility would be required. In any case, virulence might not increase infinitely. A minimum production of asexual fungal spores might be required for sufficient virus dispersal and for subsequent transmission from the spores to other fungal individuals (Taylor 2002).

Viruses of CHV-1 subtype I, the subtype with the lowest virulence, have successfully spread within and among populations, and, have thus become very abundant throughout Europe (Gobbin et al. 2003; Milgroom and Cortesi 2004). Viruses of the more virulent subtype D in Germany have been observed to also persist over several decades (F. Peters and B. Metzler, personal communication). However, viruses of subtype F1, the subtype with the highest virulence, could not be recovered from the area where they had artificially been released 20 years earlier in France (Robin et al. 2010). Nevertheless, viruses of subtype F1 have repeatedly been found in France (C. Robin, personal communication), and also in Spain (Montenegro et al. 2008) during surveys. Therefore, the range of virulence exhibited by viruses of subtype F1 may be just around the mentioned virulence threshold, which could explain why some viruses persist while others do not.

Our study highlights the importance of understanding if and how virulence factors are coupled in parasites. Coupling of virulence factors is likely to shift the cost-benefit relationship of virulence, and thus affect the optimal degree of virulence that will evolve. CHV-1 belongs to the family *Hypoviridae*, which consists of the single genus *Hypovirus* (Nuss and Hillman 2011; Rigling and Hillman 2012). Three other members of that genus – CHV-2, CHV-3 and CHV-4 – have been described. All hypoviruses are parasites of *C. parasitica,* but they differ in genome size and organization (Hillman and Suzuki 2004). In contrast to CHV-1, the other three hypovirus species have not been very successful as biocontrol agents, either due to their low virulence and limited ability to reduce the pathogenic potential of *C. parasitica* (CHV-3 and CHV-4), or due to a lack of ecological fitness and of spread (CHV-2) (Milgroom and Cortesi 2004). The hypothesized coupling of host debilitation with the ability to inhibit cell death upon hyphal fusion in CHV-1 could perhaps explain why CHV-1 has evolved much higher virulence than most other fungal viruses (including CHV-3 and CHV-4), and also why it has spread widely (unlike CHV-2). In the absence of such coupling, virulent viruses should be ecologically less fit than less virulent ones and may therefore not become widely established. This might be the case for the fungal viruses that have evolved low virulence and cause no symptoms in their host.

In conclusion, our study provides strong evidence for an association of high virulence with high transmissibility in the fungal virus CHV-1. This finding indicates that virulence

may have negative effects on one factor influencing virus transmission between hosts (the production of virus infected spores), but also positive effects on another factor influencing this transmission (transmission rate per host-to-host contact, i.e. transmissibility). We suggest that virulence is coupled with transmissibility in CHV-1; and that virulent viruses might interfere with the fungal self/nonself recognition system, and thus facilitate virus transmission between vegetatively incompatible fungi. Nevertheless, virulence may only increase up to a certain threshold above which dispersal, and eventually also transmission, are inhibited. Overall, the results of this study are in line with previous suggestions (Read 1994; Weiss 2002; Ebert and Bull 2003; Frank and Schmid-Hempel 2008), which indicate that the biological specificities of the host-parasite interaction, and the associated cost-benefit relationship in particular, are keys for understanding the evolution of parasite virulence.

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Supporting information

Table S1. Data of virus virulence assessments.

Fungal strain	Virus strain	Block	Virus effect on PDA	Virus effect on chestnut stems
baw	D	1	5%	-41%
baw	D	2	14%	-40%
baw	D	3	10%	-54%
baw	D	4	5%	-74%
baw	D	5	9%	-65%
baw	D	6	12%	-70%
baw	F1	1	-33%	-84%
baw	F1	2	-16%	-78%
baw	F1	3	-34%	-81%
baw	F1	4	-27%	-77%
baw	F1	5	-22%	-72%
baw	F1	6	-25%	-82%
baw	I	1	35%	-28%
baw	I	2	43%	15%
baw	I	3	40%	8%
baw	I	4	30%	-1%
baw	I	5	27%	92%
baw	I	6	41%	-27%
var	D	1	20%	-65%
var	D	2	13%	4%
var	D	3	3%	-87%
var	D	4	10%	-68%
var	D	5	24%	-71%
var	D	6	14%	-48%
var	F1	1	-13%	-79%
var	F1	2	-22%	-84%
var	F1	3	-26%	-87%
var	F1	4	-19%	-64%
var	F1	5	-24%	-81%
var	F1	6	-24%	-75%
var	I	1	40%	-6%
var	I	2	36%	-12%
var	I	3	28%	-45%
var	I	4	32%	22%
var	I	5	48%	-39%
var	I	6	28%	-25%

Note: The effect of infection with *Cryphonectria hypovirus 1* strains on the growth of *Cryphonectria parasitica* on potato dextrose agar (PDA; growth rate in colony diameter during the phase of linear growth) and on dormant chestnut stems (lesion diameter after 18 days of incubation) was assessed and given as a proportion (%) of the growth of the virus-free fungal clone.

Chapter 5

Invasion history and demographic pattern of *Cryphonectria hypovirus 1* across European populations of the chestnut blight fungus

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Abstract

We reconstructed the invasion history of the fungal virus *Cryphonectria hypovirus 1* (CHV-1) in Europe, which infects the chestnut blight fungus *Cryphonectria parasitica*. The pattern of virus evolution was inferred based on nucleotide sequence variation from isolates sampled across a wide area in Europe at different points in time. Phylogeny and time estimates suggested that CHV-1 was introduced together with its fungal host to Europe and that it rapidly colonized the central range along the south facing slopes of the Alps and the north-east facing slopes of the Dinaric Alps. These central populations were the source for two waves of simultaneous invasions towards the southern Balkans and Turkey, as indicated by migration rates. Our results showed that the evolutionary scenarios for CHV-1 and *C. parasitica* were spatially congruent. Since infection with CHV-1 reduces the pathogenicity of *C. parasitica* towards the chestnut tree, CHV-1 invasions of the newly established *C. parasitica* populations likely prevented the development of devastating chestnut blight epidemics in Europe. We demonstrated that in this, and supposedly in other pathosystems, geographic, vegetation-related, demographic, economic and political factors may help to explain the correlated invasion pattern of a parasite and its host.

Introduction

In modern time, global travel and trade have repeatedly facilitated the introduction of foreign species and their establishment in new environments (Perrings et al. 2002). In many cases, such introductions have promoted the emergence of new diseases (Anagnostakis 1987; Parker and Gilbert 2004). The population dynamics of parasites, in particular of obligate parasites such as viruses, depends largely on the dispersal of the host (Biek and Real 2010). The pattern of virus evolution can therefore be spatially and/or temporally congruent with the evolution of the host (Holmes 2004). The majority of DNA viruses are persistent, transmitted vertically (to offspring) or sexually, and their virulence is often low (Villarreal et al. 2000; Holmes 2008). They have an evolutionary rate similar to that of their host and their evolution is mostly congruent with that of their host on both the spatial and the temporal scale. The vast majority of RNA viruses, in contrast, cause acute (i.e. short-term) infections, are transmitted horizontally from host to host or by vectors and are usually highly virulent. RNA viruses have fast nucleotide substitution rates, largely exceeding that of their hosts, they evolve rapidly and may even jump species barriers (Villarreal et al. 2000; Holmes 2008). The evolution of acute RNA viruses is therefore rarely congruent with the evolution of their hosts on the temporal scale (Villarreal et al. 2000). Nevertheless, RNA viruses that depend on horizontal transmission between hosts often show an evolutionary pattern congruent with that of their host on a spatial scale (Real et al. 2005; Biek et al. 2007; Nadin-Davis et al. 2010; Torres-Pérez et al. 2011).

Cryphonectria hypovirus 1 (CHV-1) is an RNA virus that persistently infects its host, the fungus *Cryphonectria parasitica*. It is therefore an exception among RNA viruses causing short-term infections in their hosts, and also among fungal viruses. Unlike the vast majority of fungal viruses, CHV-1 is highly virulent and causes marked symptoms in the fungus (Nuss 2005). In accordance with its persistent nature, it does not kill its host. However, it inhibits sexual reproduction, attenuates growth and strongly reduces asexual sporulation of the fungus (Milgroom and Cortesi 2004). Spread of CHV-1 depends on a combination of vertical and horizontal transmission. CHV-1 is transferred into the asexual spores of the fungus (vertical transmission), dispersed in these spores and then transmitted from the outgrowing spores to other fungal individuals (horizontal transmission) by hyphal fusion (Hoegger et al. 2003; Milgroom and Cortesi 2004). However, asexual spores are only dispersed over short distances, unless they are carried by vectors such as insects, birds, mammals or by humans on chestnut plants or wood (Heiniger and Rigling 1994). Spread and dissemination of CHV-1 is therefore expected to depend largely on host movement and contacts between hosts.

The host of CHV-1, the fungus *Cryphonectria parasitica*, is native to East Asia and was introduced to North America and Europe early in the 20th century (Anagnostakis 1987). *C. parasitica* is a serious tree pathogen and causes lethal bark cankers on susceptible chestnut (*Castanea* spp.). After its introduction to Italy, either directly from Asia or via a bridgehead in North America (Dutech et al. in press), it spread rapidly throughout the chestnut growing regions and evoked a dramatic chestnut blight epidemic (Anagnostakis 1982). In North America, it destroyed the native chestnut forests, whereas in Europe chestnut blight incidence to date is high but maintained at low severity. The slowdown of the epidemic in Europe may have – at least in part – resulted from infection of *C. parasitica* with CHV-1 and the emergence of this debilitating viral disease in the fungal population. CHV-1 occurred naturally and spread spontaneously in European *C. parasitica* populations, and has thus been biologically controlling the disease. Infection with CHV-1 reduces the pathogenicity of *C. parasitica* to the chestnut tree, a phenomenon called hypovirulence (Van Alfen et al. 1975; Nuss 2005). Much research on chestnut blight has been directed towards the characterization of fungal populations based on the diversity of vegetative compatibility (vc) types (Cortesi et al. 1998; Milgroom and Cortesi 1999; Sotirovski et al. 2004; Braganca et al. 2007; Krstin et al. 2008; Milgroom et al. 2008; Montenegro et al. 2008; Robin et al. 2009; Dutech et al. 2010). *C. parasitica* vc types are determined by at least six bi-allelic genetic loci (Cortesi and Milgroom 1998). Milgroom et al. (2008) analyzed the genetic diversity of *C. parasitica* populations and found that populations in the central range were genetically diverse, while the more recently founded populations in the southern Balkans and in Turkey were mainly clonal. This corresponds with observations from other systems, where founder populations showed reduced diversity compared to their source populations (Goodwin et al. 1994; Allendorf and Lundquist 2003; Dlugosch and Parker 2008).

Since CHV-1 has been found in China and Japan it has been assumed that CHV-1 was introduced together with *C. parasitica* from Asia (Liu et al. 2007). Records of first detection of hypovirulent *C. parasitica* in different regions indicated that CHV-1 spread throughout Europe following the spread of the fungus with a lag in time (Heiniger and Rigling 1994; Robin and Heiniger 2001). The dependence of CHV-1 on its host for spread and transmission as described above may further support the suggestion of a tight relationship between spread of the fungus and spread of the virus. However, while many studies were directed at studying the fungal host population (Cortesi et al. 1998; Milgroom and Cortesi 1999; Sotirovski et al. 2004; Breuillin et al. 2006; Braganca et al. 2007; Krstin et al. 2008; Milgroom et al. 2008; Montenegro et al. 2008; Robin et al. 2009; Prospero and Rigling 2012), the genetic structure of CHV-1 populations in Europe has remained largely unexplored. Therefore, information about the dispersal of CHV-1 is based on first records of CHV-1 in the different European regions (Heiniger and Rigling 1994; Robin and Heiniger 2001) and little is known about the true population structure of

CHV-1. Four genetically distinguished subtypes of CHV-1 have been described in Europe, named subtype I, D, F1 and F2 (Allemann et al. 1999; Gobbin et al. 2003). The degree of genetic differentiation together with the estimated rate of nucleotide substitution suggested that these subtypes diverged several hundred years ago (i.e. before the introduction of the fungus) and that they were introduced at independent events to Europe (Gobbin et al. 2003). Among the four subtypes, subtype I is the most widespread and is prevalent all across Switzerland, Italy, the Balkans and Turkey, thus, providing the opportunity to study the invasion of this subtype across the expanding range of its fungal host.

To our knowledge, there is only one molecular study of CHV-1 in Europe, which investigated virus transmission within and between two *C. parasitica* populations in Italy (Carbone et al. 2004). Here, we generated a large data set of CHV-1 sequences from Europe by sampling eight populations from different geographic regions. By complementing this recent data set with CHV-1 sequences obtained from earlier samplings we were able to reconstruct the unexplored phylogenetic history of CHV-1 in Europe. We tested the hypothesis of evolutionary congruence between the virus and its fungal host, estimated the time to the most recent common ancestor using coalescent analysis and discussed the likely pattern of invasion.

Materials and methods

Samples of CHV-1

In 2008/2010, we sampled a total of eight virus-infected *C. parasitica* populations that were naturally infected with CHV-1 (Gobbin et al. 2003). All fungal populations were sampled within an area of <1 ha. Four of these populations were obtained from the central range (Switzerland and Bosnia-Herzegovina) where vc type diversity was known to be high and another four populations were obtained from the southern range (Macedonia, Greece and Turkey) where *C. parasitica* populations were dominated by a single vc type (Robin and Heiniger 2001; Milgroom et al. 2008). All sampling sites were coppice forests with 10 – 20 years old chestnut sprouts and a high incidence of chestnut blight. Bark samples were taken with a cork borer (5 mm diameter) from chestnut blight cankers at intervals of at least 5 m between trees. Only one canker per tree was sampled and the cork borer was sterilized with 70% ethanol and flaming between cankers. We obtained pure cultures of *C. parasitica* by isolation from the bark samples on water agar followed by culturing on potato dextrose agar (PDA, Difco Laboratories, Detroit; Robin et al. 2010). The vc type of each *C. parasitica* isolate was determined by pairing with vc type tester strains (Cortesi and Milgroom 1998). The viral double-stranded (ds) RNA was extracted from lyophilized mycelium by cellulose CF-11 chromatography as described by Alleman et al. (1999).

In addition to the eight CHV-1 subtype I populations described above, we extended our data set by including sequence data from two populations sampled in 1998 from the central host range (one from Switzerland and one from Bosnia-Herzegovina) and data for two populations sampled in 2000 from the southern range (Macedonia). We also added a few individual CHV-1 sequences from Europe dating back to the 1970s, including the reference sequences for CHV-1 subtype I (CHV-1/Euro7) and CHV-1 subtype F1 (CHV-1/EP713) and additional samples from each of the four CHV-1 subtypes published in an earlier study (Gobbin et al. 2003). Finally, we included Genbank sequences from China and Japan, the putative origin of CHV-1. An overview over all populations and individual samples used in this study is given in Table 1.

Sequencing of CHV-1

Complementary DNA (cDNA) was synthesized from the dsRNA with random hexamer primers using the Maxima First Strand cDNA Synthesis kit from Fermentas (St. Leon-Rot, Germany). PCR amplification and sequencing was performed as described in Gobbin et al. (2003) with the modification that EP721-4 (5'- GGAAGTCGGACATGCCCTG-3') was used

as reverse primer. This allowed us to sequence a variable 693 bp region of ORF A corresponding to positions 1473-2165 in the nucleotide sequence of CHV-1/Euro7 (Chen and Nuss 1999).

Table 1. *Cryphonectria hypovirus 1* samples used in this study.

Country	Year of sampling	Name, Location	Population	Number of samples	Reference [GPS coordinates]
Switzerland	2010	Pu, Pura	yes	42	this study [45.98 N,8.86 E]
Switzerland	2010	Go, Gnosca	yes	50	this study [46.24 N, 9.01 E]
Macedonia	2010	Ra, Radolista	yes	55	this study [41.16 N, 20.62 E]
Greece	2010	He, Anelio	yes	41	this study [39.43 N, 23.14 E]
Turkey	2010	Bu, Kurşunlu	yes	33	this study [40.36 N, 29.02 E]
Turkey	2010	Ya, Kurtköy	yes	17	this study [40.58 N, 29.22 E]
Bosnia-Herzegovina	2008	Ka, Kostajnica	yes	38	this study [45.22 N, 16.55 E]
Bosnia-Herzegovina	2008	Iv, Ivanjska	yes	30	this study [44.88 N, 17.04 E]
Switzerland	1998	CHF, Faido	yes	17	(Gobbin et al. 2003)
Bosnia-Herzegovina	1998	BOVr, Vrnograc	yes	18	(Rigling et al., unpublished data)
Macedonia	2000	MKVrat, Vratnika	yes	10	(Rigling et al., unpublished data)
Macedonia	1996	MKFra, Frangovo	yes	2	(Rigling et al., unpublished data)
Switzerland	1976	E-3	no	1	(Gobbin et al. 2003)
	1976	E-5	no	1	(Gobbin et al. 2003)
	1976	E-6	no	1	(Gobbin et al. 2003)
	1980	E-27	no	1	(Gobbin et al. 2003)
Italy	1978	CHV-1/Euro7	no	1	(Chen and Nuss 1999)
France	1970	CHV-1/EP713 (F1)	no	1	(Shapira et al. 1991)
	1970	E-55 (F1)	no	1	(Gobbin et al. 2003)
	1974	E-56 (F1)	no	1	(Gobbin et al. 2003)
	1975	E-57 (F2)	no	1	(Gobbin et al. 2003)
	1997	E-62 (F1)	no	1	(Gobbin et al. 2003)
Spain	1988	E-71 (D)	no	1	(Gobbin et al. 2003)
Germany	1992	E-72 (D)	no	1	(Gobbin et al. 2003)
China	1987 – 2002	CN	no	11	Genbank accession numbers HM246637 to HM246647
Japan	1992	JP	no	2	Genbank accession numbers HM246649 and HM246650

Haplotype determination and diversity measures

CHV-1 haplotypes were determined in DnaSP v5 (Librado and Rozas 2009) and haplotype richness (R_{Haplo}) was calculated for each population. The Shannon-Wieners index was used as an estimate for the diversity of haplotypes (H'_{Haplo}). Diversity ($H'_{Haplo[17]}$) and richness ($R_{Haplo[17]}$) of haplotypes expected for the smallest sample size being analyzed (n = 17) was determined by rarefaction analysis implemented by the vegan package in the software R 2.6.2 (R-Development-Core-Team 2008). DnaSP was further used to estimate nucleotide diversity (π) in each CHV-1 population. In addition, richness (R_{Vc}) and diversity (H'_{Vc}) of vc types were calculated as a measure for diversity of the fungal host populations and rarefaction analysis was employed to determine richness ($R_{Vc[44]}$) and diversity ($H'_{Vc[44]}$) expected for the smallest sample size analyzed (n = 44). After rarefaction, independent samples t-test in SPSS 19.0 (SPSS, Somers) was employed to detect significant differences in richness and diversity among virus and among fungus populations.

Bayesian coalescent analyses

We used the program BEAST v1.6.1 (Drummond and Rambaut 2007) to conduct coalescent-based genetic analyses. The program consists of a Bayesian Markov chain Monte Carlo inference package and a range of coalescent models. The input file was generated with help of the BEAUTi program implemented in the BEAST package and sequences were dated with the year of sampling (see Table 1). Our full set of samples obtained at different points in time allowed us to estimate the evolutionary rate and the time of divergence between evolutionary lineages, that is the time to the most recent common ancestor (TMRCA). We inferred the population dynamic of the virus by estimating the effective population size trough time (expressed as the product of N_e and generation τ) by using the implemented Bayesian skyline plot. All analyses were performed using the lognormal relaxed-clock model and the HKY85 substitution model (Hasegawa et al. 1985) with gamma correction for site rate heterogeneity as determined by MODELTEST (Posada and Crandall 1998). The Bayesian skyline plot model was selected as it can account for more complex demographic models than one of the *a priory* defined tree models. BEAST was run for 10^8 generations with three repeats to ensure convergence. The TRACER v1.4 program (Rambaut and Drummond 2007) was used to combine the BEAST output log-files for the final analysis.

Phylogenetic reconstruction and geographic population structure

The phylogenetic relationships were reconstructed with two different approaches. A Bayesian-based maximum clade credibility (MCC) tree was inferred by the BEAST

analyses as described above and a maximum likelihood (ML) tree was reconstructed using MEGA 5.05 (Tamura et al. 2011) and the implemented HKY85 substitution model (Hasegawa et al. 1985) with gamma correction for site rate.

Clustering of the European CHV-1 subtype I sequences from the eight populations sampled recently was also conducted on the amino acid level using a principal component analysis (PCA). The method is implemented in JALVIEW (Waterhouse et al. 2009) and generates the components by an eigenvector decomposition of the matrix formed from the sum of BLOSUM scores at each aligned position between each pair of sequences.

To further assess the geographic structure of CHV-1 subtype I, we used spatial analyses of molecular variance based on F-statistics as implemented in SAMOVA (Dupanloup et al. 2002). The program performs a simulated annealing procedure that aims at maximizing among group variance (F_{CT}) and minimizing among populations within group variance (F_{SC}). The goal is to define groups of populations that are geographically homogeneous and genetically maximally differentiated from each other. To avoid spurious results due to initial population configuration 100 initial conditions were used as recommended by Dupanloup et al. (2002).

Finally, a Mantel test with 1000 iterations was performed to determine the significance of isolation-by-distance (IBD) between populations using the Isolation-by-Distance Web Service 3.21 (Jensen et al. 2005). As suggested by Slatkin (1993), correlation was tested between matrices of linearized F_{ST} values and the logarithm of geographic distances.

Estimation of gene flow

Migration rates between populations were estimated with the maximum-likelihood approach of MIGRATE (Beerli and Palczewski 2010). Markov chain settings were 20 short chains (500 recorded steps), three long chains (1000 recorded steps) and a four step adaptive heating scheme. Results were averaged over five independent runs. Based on the phylogenetic reconstructions and results of the population differentiation analyses, direction and amount of migration was estimated between the pooled central populations (Go, Pu, Ka, Iv) and each of the four southern populations (Ra, He, Bu, Ya).

Results

Genetic diversity of virus and fungus populations

Table 2 displays the indices of genetic diversity in the eight virus and fungus populations. After rarefaction analysis, mean (± SD) richness of haplotypes in the virus populations was 15.25 (± 1.49), mean diversity of haplotypes was 2.67 (± 0.11) and mean nucleotide diversity was 0.00709 (± 0.00249). No significant differences ($p > 0.05$) in either of these indices were observed between the populations form the central range and the populations from the southern range. While a few haplotypes occurred more than once within populations, there were no shared haplotypes between populations. In the fungal populations, highly significant ($p \leq 0.001$) differences existed between the populations from the central and the populations from the southern range. Diversity and richness of vc types (see also Table S1) were substantially higher in the populations from the central range. The mean richness of vc types was 7.75 (± 7.31) and mean diversity of vc types was 1.05 (± 1.13).

Table 2. Indices of genetic diversity in virus and fungus populations.

		Virus						Fungus				
Name	Range	N_{Virus}	R_{Haplo}	$R_{Haplo[17]}$	H'_{Haplo}	$H'_{Haplo[17]}$	π	N_{Fungus}	R_{Vc}	$R_{Vc[44]}$	H'_{Vc}	$H'_{Vc[44]}$
Go	central	50	43	16	3.71	2.75	0.00820	97	14	10	1.91	1.63
Pu	central	42	38	16	3.61	2.83	0.01183	97	24	17	2.57	2.54
Ka	central	38	27	14	3.12	2.56	0.00488	46	17	17	2.37	2.26
Iv	central	30	25	15	3.14	2.64	0.00516	44	13	13	1.87	1.87
Ra	southern	55	51	17	3.91	2.75	0.00713	72	2	2	0.13	0.11
He	southern	41	29	13	3.00	2.51	0.00445	93	1	1	0.00	0.00
Bu	southern	33	32	17	3.45	2.75	0.00616	96	1	1	0.00	0.00
Ya	southern	17	14	14	2.59	2.59	0.00893	89	1	1	0.00	0.00

Note: The populations were sampled across Europe (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya). $R_{Haplo[17]}$ is the virus haplotype richness after rarefaction analysis for the smallest sample size (n=17). $H'_{Haplo[17]}$ is the Shannon-Wiener's index for haplotype diversity after rarefaction analysis for the smallest sample size (n=17). $R_{Vc[44]}$ is the fungal vegetative compatibility (vc) type richness after rarefaction analysis for the smallest sample size (n=44). $H'_{Vc[44]}$ is the Shannon-Wiener's index for vc type diversity after rarefaction analysis for the smallest sample size (n=44).

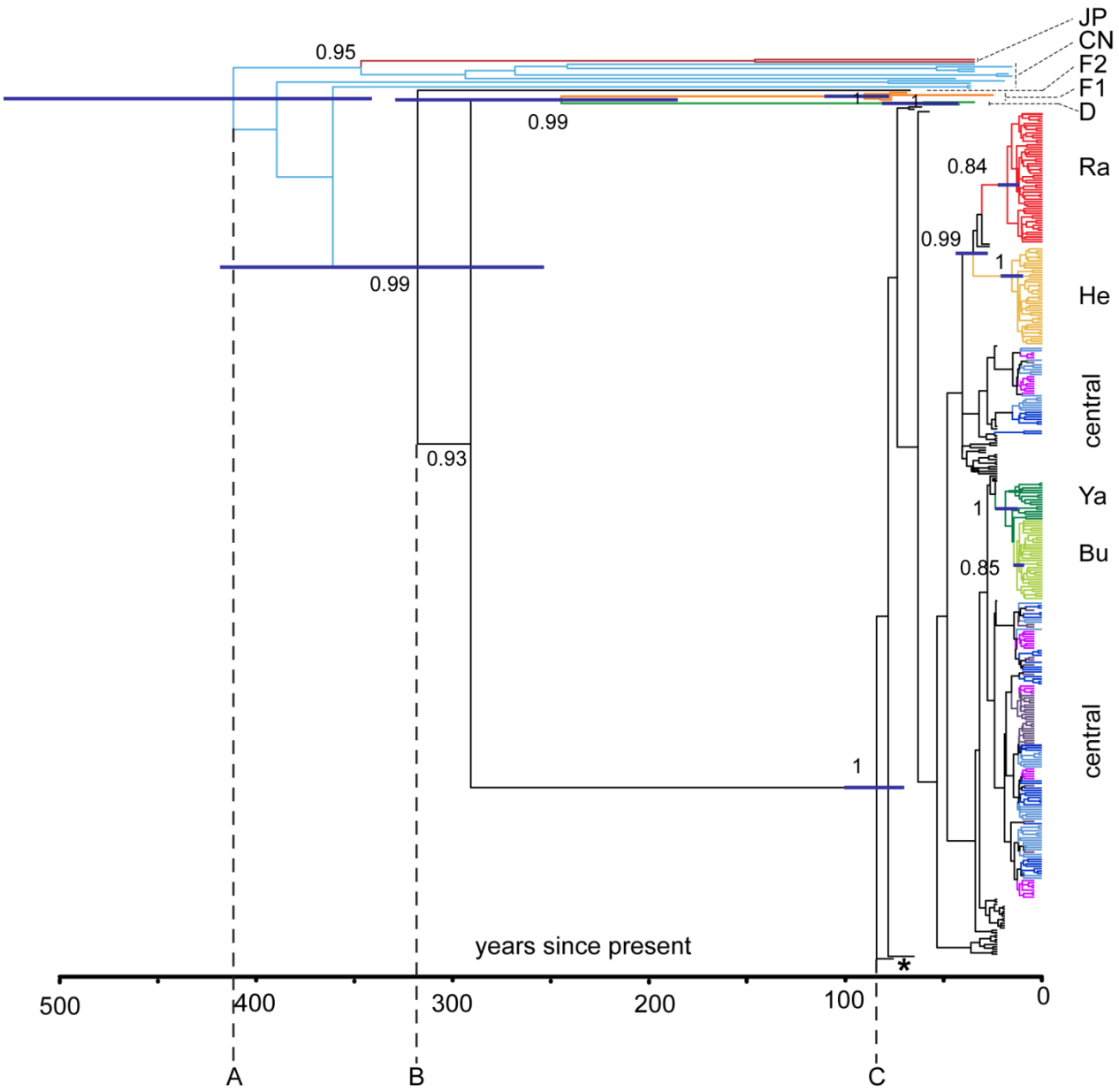

Fig.1. Bayesian-based coalescent phylogeny (maximum clade credibility tree) of *Cryphonectria hypovirus 1* (CHV-1)*;* deep phylogeny including samples from the European CHV-1 subtypes I, F1, F2 and D as well as samples from China and Japan. Node-bars are time estimates (95% HPD) and values at nodes are posterior probabilities for the main phylogenetic groups. The bottom axis represents time estimates (years since present). Node A is the root of the tree, node B represents the TMRCA for the European subtypes, and node C is the TMRCA for the European subtype CHV-1 subtype I. The asterisk indicates the position of the reference sequence CHV-1/Euro7. The populations Go, Pu, Ka and Iv cluster together and represent the central populations from Switzerland and Bosnia-Herzegovina. Their names are not indicated seperately. The populations Ra and He and Bu and Ya represent the southern populations from the southern Balkans and Turkey, respectively.

Phylogenetic tree and geographic clustering

Deep phylogenetic relationships among CHV-1 isolates were reconstructed by using both the MCC tree obtained from the BEAST analysis (Fig. 1) and the ML tree obtained from the MEGA analysis (Fig. S1). Both trees inferred the same pattern. The isolates from

China and Japan were all located at the base of the tree, supporting the Asian origin of the virus. The European CHV-1 isolates formed a distinct cluster divergent from the Asian isolates. All CHV-1 subtype I isolates formed a maximally supported monophyletic clade in both the MCC (1.0 posterior probability) and ML (100% bootstrap values) analyses. Analysis of the recently sampled CHV-1 subtype I populations revealed that the samples from the central populations Go, Pu, Ka and Iv where intermixed and did not represent distinct clades (Fig. 1). However, individual clusters were observed for the southern populations Ra, He, Bu and Ya. Furthermore, Ra and He diverged at a different node than Bu and Ya from the central populations.

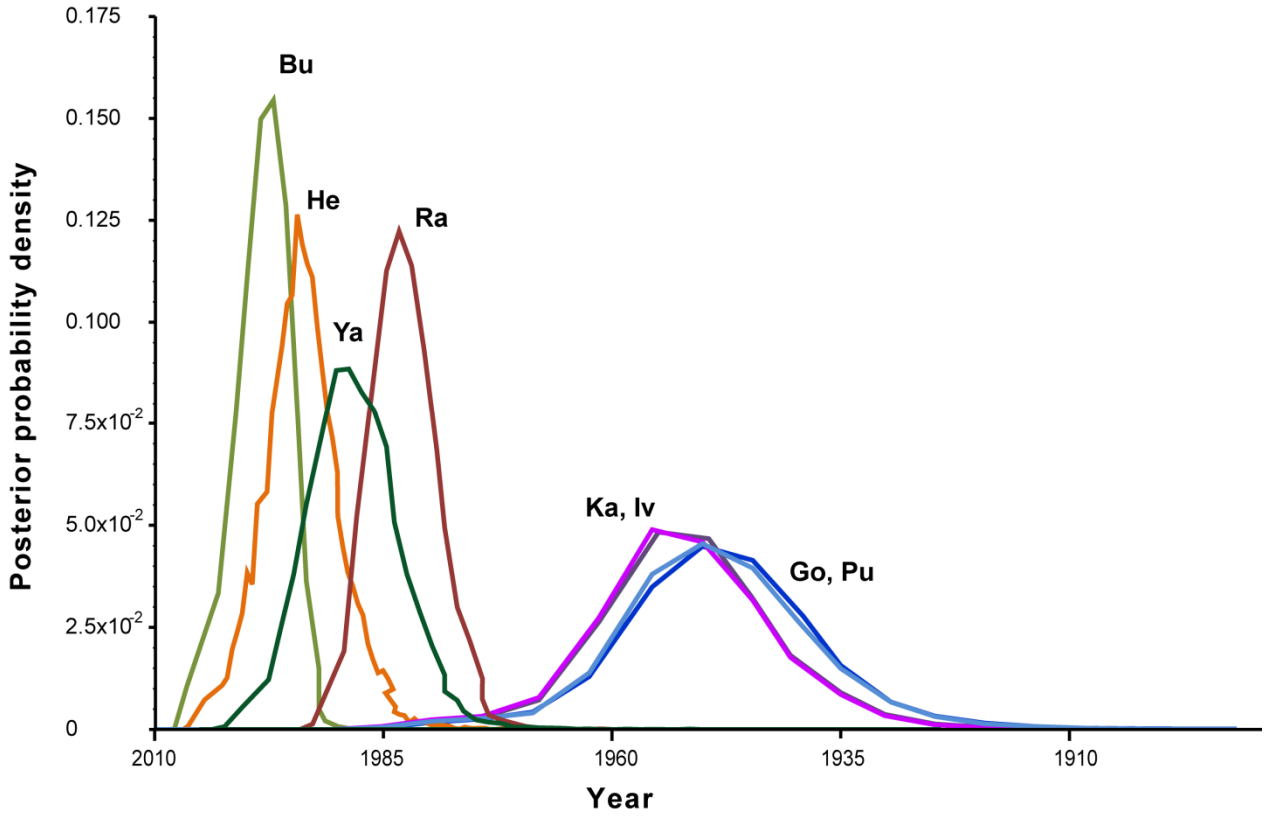

Fig. 2. Bayesian posterior density plot of the time of most recent common ancestors for the eight European *Cryphonectria hypovirus 1* subtype I populations in this study. The populations Go, Pu, Ka and Iv represent the central populations from Switzerland and Bosnia-Herzegovina, and the populations Ra and He and Bu and Ya represent the southern populations from the southern Balkans and Turkey, respectively.

Nucleotide substitution rate and time to the most recent common ancestor

The Bayesian estimates gave a mean evolutionary rate of 3.7×10^{-4} (lower 95% HPD 2.4×10^{-4}, upper 95% HPD 6.1×10^{-4}) substitutions/site/year. Given this evolutionary rate, the estimated TMRCA for the four European subtypes I, F1, F2 and D of CHV-1 was 322 (95% HPD 411 – 251) years (node B in Fig. 1). The TMRCA for the dominant European

subtype CHV-1 subtype I was 88 (95% HPD 102 – 72) years (node C in Fig. 1). Within the eight European populations of CHV-1 subtype I the TMRCA estimates supported the distinction of five genetic lineages (Fig. 2) as also suggested by the phylogenetic trees. The four populations forming the central population cluster had very similar TMRCA estimates with values of 64 years (1946) for both Swiss populations (Go, Pu) and 61 years (1949) for both Bosnian populations (Ka, Iv). Estimates for the four southern populations were clearly more recent, i.e. 28 years (1982) for Ra, 22 years (1988) for Ya, 15 years for He (1995) and 12 years (1998) for Bu. Although the TMRCA estimates of the southern populations were partially overlapping, the posterior probabilities of TMRCA support the genetic and geographic clustering results of one undifferentiated central population and four distinct southern populations described above.

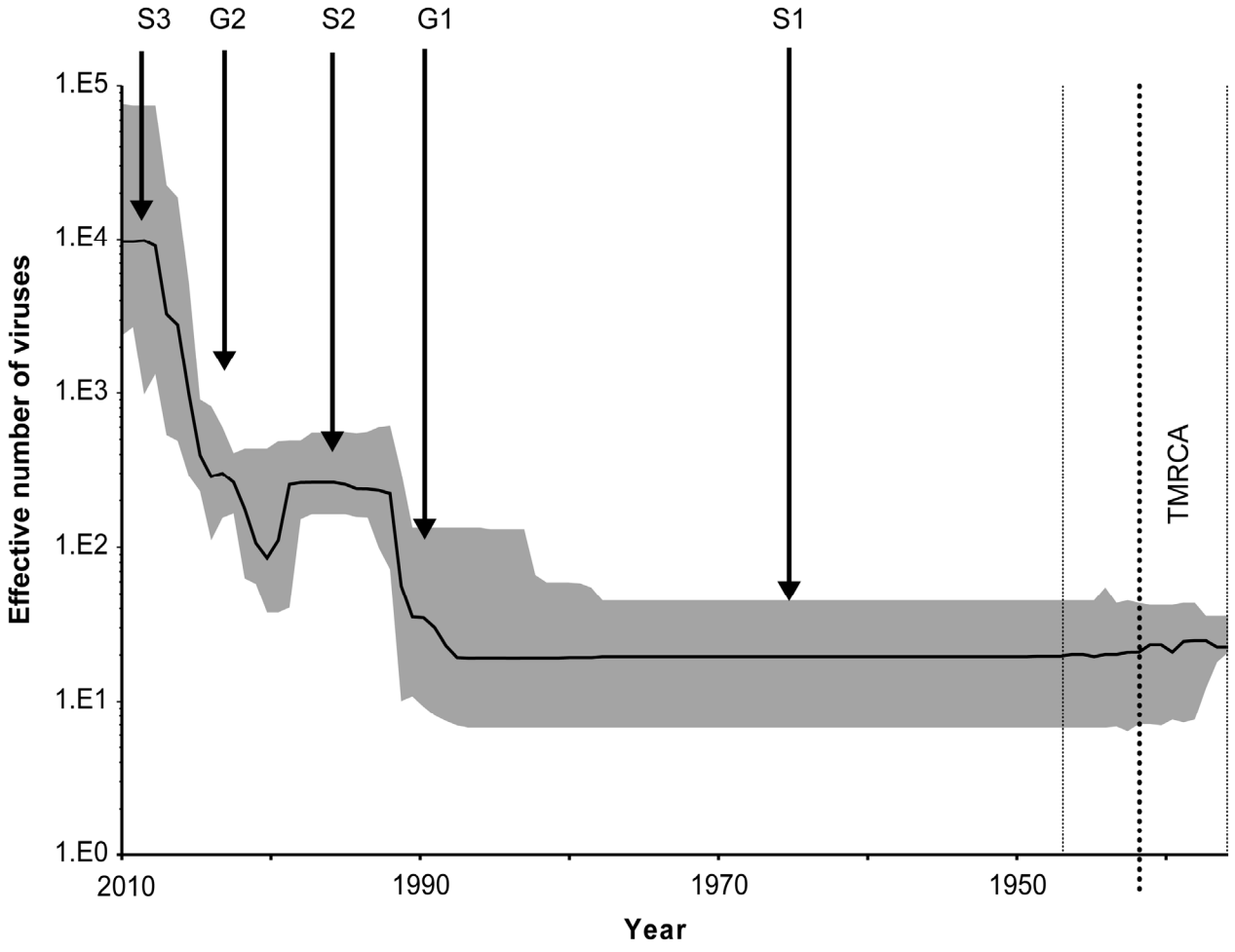

Fig. 3. Effective population size (N_e*τ) change of the eight European *Cryphonectria hypovirus 1* subtype I populations analyzed in this study represented as a Bayesian skyline plot through time. The grey area represents 95% highest posterior density (HPD) intervals. Dotted lines are estimates of the time to the most recent common ancestor (TMRCA and 95% HPD). Arrows indicate periods of growth (G) and stasis (S), respectively.

The reconstruction of the demographic history of CHV-1 is depicted in (Fig. 3). The skyline plot suggested that the effective population size of the virus increased in distinct periods of growth (G) and stasis (S) and not continuously.

Principal component analysis of population differentiation

In agreement with the phylogenetic analysis and the TMRCA estimates, the PCA of amino acid sequence differentiation among European CHV-1 subtype I isolates suggested the presence of one large undivided population cluster consisting of the four populations Go, Pu, Ka, Iv referred to as the central populations and of four individual populations that differentiate from the central cluster in two directions (Bu and Ya versus Ra and He) (Fig. 4). In Fig.4 the PCA of the CHV-1 subtype I amino acid sequence differentiation was contrasted against the vc type diversity of the respective fungal populations. The vc diversity was high in all central populations while there was only one dominant vc type in each of the southern populations. Furthermore, the populations Bu and Ya were dominated by a different vc type than the populations Ra and He.

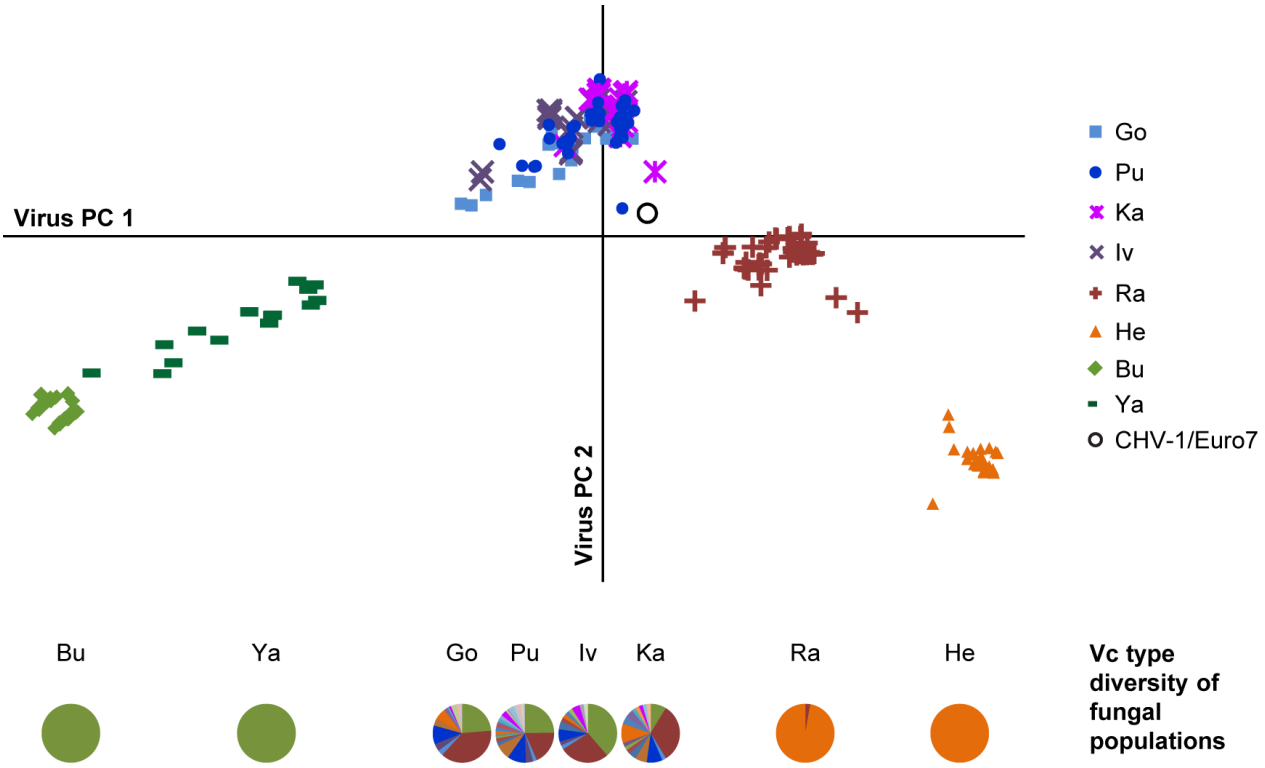

Fig. 4. Principal component analysis (PCA) of amino acid differentiation among isolates of the eight *Cryphonectria hypovirus 1* subtype I populations using the sum of pairwise BLOSUM scores for eigenvector decomposition; Bu and Ya from Turkey, Go and Pu from Switzerland, Iv and Ka from Bosnia-Herzegovina, Ra from Macedonia and He from Greece. The sequence of CHV-1/Euro7 from Italy was added as a reference. Pie graphs displaying the fungal vegetative compatibility (vc) type diversity in these eight populations were aligned to the PCA graph.

Geographic population structure

The SAMOVA result was congruent with the population clustering detected in the phylogenetic trees and the PCA. The best partitioning of the genetic diversity by SAMOVA was obtained when populations were divided into *K = 5* geographic groups (F_{CT} = 0.4759, p = 0.014, Table 3). The four central populations (Go, Pu, Ka, Iv) fell within a single cluster, although the geographic distance between them was up to 918 km (Table S2). The four southern populations (Bu, Ya, Ra, He), on the other hand, were each inferred as separate groups.

The Mantel test for the pairwise correlation of linearized F_{ST} calculated from molecular distances of pairwise haplotype differences and geographical distances was not significant (Fig. S2; r = 0.0113, p = 0.52), suggesting no IBD among the European populations of CHV-1 subtype I.

Table 3. Spatial analysis of molecular variance (SAMOVA) for eight European populations of *Cryphonectria hypovirus 1* subtype I.

Source of variation	df	Sum of squares	Variance components	Percentage of variation (%)	P
K = 2 (Go, Pu, Ka, Iv, Bu, Ya, Ra) vs. (He)					
Among groups	1	201.379	1.610	26.25	0.109
Among populations within group	6	474.900	2.059	33.57	<0.001
Within populations	298	734.668	2.465	40.19	<0.001
K = 3 (Go, Pu, Ka, Iv, Ra) vs. (He) vs. (Bu, Ya)					
Among groups	2	449.551	2.581	41.90	0.006
Among populations within group	5	226.729	1.113	18.07	<0.001
Within populations	298	734.668	2.465	40.03	<0.001
K = 4 (Go, Pu, Ka, Iv) vs. (He) vs. (Ra) vs. (Bu, Ya)					
Among groups	3	584.940	2.547	45.57	<0.001
Among populations within group	4	91.340	0.577	10.33	<0.001
Within populations	298	734.668	2.465	44.11	<0.001
K = 5 (Go, Pu, Ka, Iv) vs. (He) vs. (Ra) vs. (Bu) vs. (Ya)					
Among groups	4	614.387	2.655	47.59	0.014
Among populations within group	3	61.892	0.459	8.23	<0.001
Within populations	2	734.668	2.465	44.18	<0.001

Note: The populations were sampled from different regions (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya).

Gene flow and migration

The estimates of gene flow between the main clusters of CHV-1 subtype I are displayed in Table 4. Given the clustering results in this study, the populations Go, Pu, Ka and Iv were pooled into one central population cluster. All gene flow estimates were directed from this central cluster towards the southern populations. There was no indication of gene flow back to the central populations. The estimates of gene flow from the central populations was always higher for one of the two geographically proximate populations (Ra > He, Ya > Bu). The pairwise estimates between the two geographically proximate southern populations showed a clear directional bias from Ra into He and from Ya into Bu. No gene flow was observed between the populations from the southern Balkans (Ra, He) and from the populations from Turkey (Ya, Bu).

Table 4. Pairwise estimates of gene flow between European *Cryphonectria hypovirus 1* subtype I populations with 95% confidence intervals in parentheses.

	Receiving population				
	central	Ra	He	Ya	Bu
central	-	9.7 (4.5-17.9)	2.1 (0-4.7)	18.3 (8.5-33.8)	0 (0-0)
Ra	0 (0-0)	-	13.6 (6.3-25.2)	1.7 (0-4.2)	0 (0-0)
He	0 (0-0)	0 (0-0)	-	0 (0-0)	0 (0-0)
Ya	0 (0-0)	0 (0-0)	0 (0-0)	-	31.0 (14.4-57.2)
Bu	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	-

Note: The populations were sampled from different regions (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya). Given the clustering results in this study, the populations Go, Pu, Ka, and Iv were pooled into one central population cluster.

Discussion

Evolutionary congruence between parasite and host on the spatial scale is expected, if the parasite depends on the host for dispersal (Holmes 2004, 2008). This is largely the case for the fungal virus CHV-1 and has also been observed in other RNA viruses (Real et al. 2005; Biek et al. 2007; Nadin-Davis et al. 2010; Torres-Pérez et al. 2011). Ecological and demographic factors that influence the spread of the host have therefore also a great impact on the dispersal and evolution of the virus. In this study, we conducted a large scale sampling across Europe, analyzed the population structure of the fungal virus CHV-1 and reconstructed its phylogenetic history using a coalescent approach. Our results strongly suggest that the evolution of CHV-1 is spatially, but not temporally, congruent with the evolution of its host *C. parasitica*. This is the first investigation of CHV-1 genome sequences obtained from a wide area in Europe. Furthermore, it is the first study in the *Cryphonectria*-hypovirus pathosystem that allowed the estimation of the dates of introduction and population emergence based on genetic sequence information.

The phylogenetic analyses performed in our study support an Asian origin of the European CHV-1 isolates. Previous analyses (Liu et al. 2007) of Asian and European CHV-1 discovered that the genetic diversity among Asian isolates was higher than among European isolates, indicating that the European CHV-1 populations had emerged more recently. The four European subtypes of CHV-1 (I, F1, F2 and D) were more closely related to each other than to any of the Asian isolates. Estimates of the TMRCA for the four European CHV-1 subtypes indicated that the subtypes diverged more than 300 years ago, i.e. prior to the first records of *C. parasitica* in Europe. The finding that the emergence of the subtypes predates the introduction of *C. parasitica* to Europe is in agreement with the results of a previous study by Gobbin et al. (2003) and suggests that multiple introductions of CHV-1 to Europe have occurred.

As expected, all isolates from the eight recently sampled European populations mapped to the CHV-1 subtype I cluster and formed a well supported monophyletic clade. We detected five distinct viral lineages within this clade. Our results suggested the existence of one large undivided population cluster of CHV-1 subtype I and four individual small clusters. This cluster included the two populations sampled in southern Switzerland (Go and Pu) and the two populations sampled in north-western Bosnia-Herzegovina (Ka and Iv), and thus extended over a wide geographic area. These populations were referred to as the central populations, in accordance with the denomination used in a previous study on the fungal host populations in these regions (Milgroom et al. 2008). The populations from Macedonia (Ra) and Greece (He) and the two populations from Turkey (Bu and Ya), on the other hand, were inferred as four distinct southern populations that diverged from

the central populations more recently at two different nodes. It is remarkable that the Swiss and the Bosnian populations form a homogenous population cluster, even though the geographic distance between them is large (approx. 800 km). The geographic distances between the populations Ra and He (approx. 300 km) and between Bu and Ya (approx. 30 km) are much shorter and, nevertheless, they each represent a distinct phylogenetic group. The lack of significant IBD among the CHV-1 populations suggested that the emergence of CHV-1 was not shaped by a continuous dispersal from the central range towards the southern range.

All analyses performed in this study, i.e. the phylogenetic analyses using a Bayesian-based coalescent approach and a ML approach, estimates of the TMRCA, the PCA of amino acid differentiation and the SAMOVA, inferred the same pattern for the evolution of the CHV-1 subtype I populations. According to the TMCRA estimates, the populations from Switzerland were the oldest among the eight CHV-1 subtype I populations investigated in this study, but only a few years older than the populations from Bosnia-Herzegovina. The estimated date of TMRCA of the Swiss populations coincides with the first report of *C. parasitica* in Switzerland in 1948 (Robin and Heiniger 2001) and strongly suggests that CHV-1 was introduced together with *C. parasitica*. However, the presence of CHV-1 may have remained undetected for several years, since CHV-1 was not identified in Switzerland before 1975. *C. parasitica* was first found in Europe in northern Italy in 1938 and the first signs of virus infection were already observed in 1951 (Biraghi 1953). If CHV-1 was introduced to Italy together with *C. parasitica*, as our findings indicate, it colonized the central range in spreading north to southern Switzerland and east through the neighbouring countries Slovenia and Croatia to north-western Bosnia-Herzegovina.

Estimates of TMRCA and the skyline plot suggested that the establishment of CHV-1 in the central range was characterized by a long period (approx. 35 years) of stasis during which the effective number of viruses did not change significantly. This long period of stasis was followed by a period of rapid population increase, which roughly coincided with a first wave of invasion from the central to the southern range and the subsequent establishment of new populations. During this time period (1980 – 1990), the populations Ra (southern Balkans) and Ya (Turkey) were founded almost simultaneously by two independent invasions. After the establishment of these populations, the effective number of viruses reached a plateau until a second wave of invasion was initiated. Again roughly during the same time period (1995 – 2000), the populations He (southern Balkans) and Bu (Turkey) were founded by invasions from geographically proximate populations (represented in our study by Ra and Ya, respectively).

Figure 5 summarizes the evolutionary scenario proposed for CHV-1. This scenario is also supported by the pairwise estimates of gene flow between the main genetic clusters of CHV-1 subtype I. Three main conclusions can be drawn. First, the central populations are solely acting as a source of migrants; that is, all gene flow is directed towards the southern sink populations, but not back. Second, no gene flow was observed between the populations in the southern Balkans and Turkey, indicating independent invasions to these two regions in the southern range. Third, gene flow was always higher from the central populations into the older (as estimated by the TMRCA) of the two geographically proximate southern populations. In addition, the estimates of gene flow between the geographically proximate populations suggested that the older population acted as a source for the younger population in each region. This is consistent with our hypothesis of two waves of simultaneous invasions taking place during different time periods.

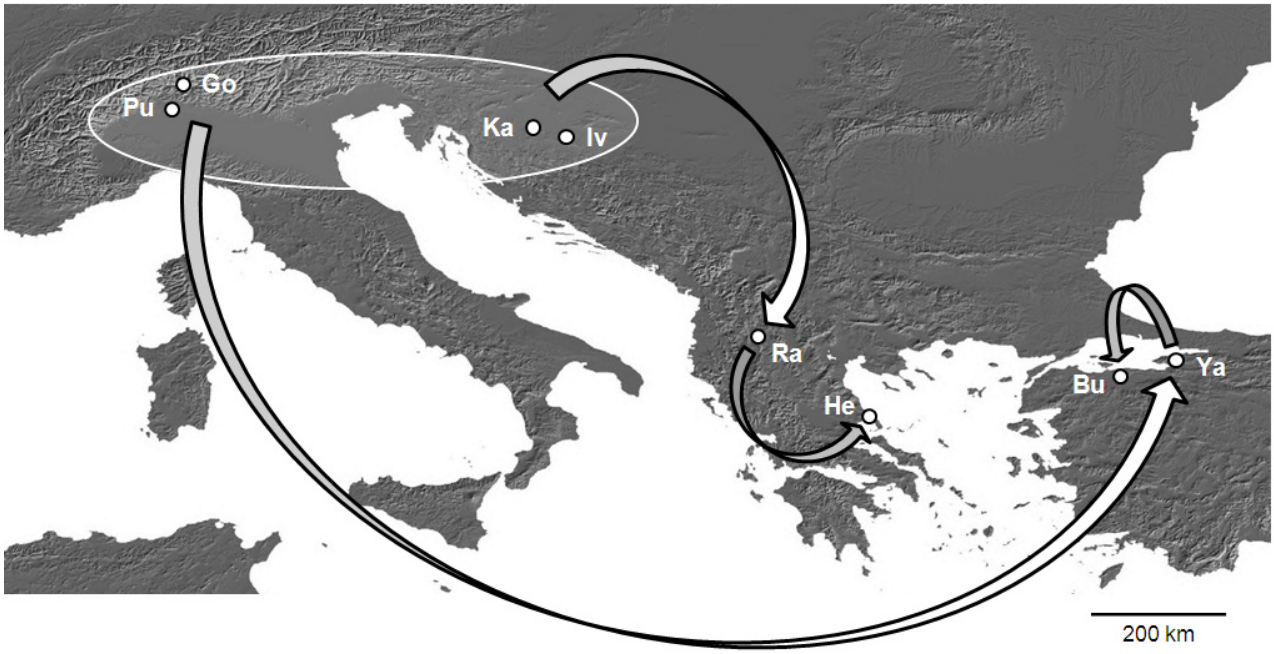

Fig. 5. Graphical illustration of the evolutionary scenario inferred for *Cryphonectria hypovirus 1* subtype I in Europe. The framed area comprises the central populations. Arrows indicate waves of introductions towards the southern range. The locations of the eight populations sampled in this study are indicated by white dots (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya).

The skyline plot indicates a steep drop in population size during a short period at around the time of the second wave of invasion. This could be a sign for population bottlenecks due to new founding events. The bottleneck was followed by an extended period of population growth, suggesting that the southern CHV-1 populations quickly recovered from the genetic bottlenecks. The high genetic diversity in all CHV-1 populations investigated, in the central as well as in the southern populations, further supports this

assumption. This quick recovery from the genetic bottlenecks contrasts with the situation in the populations of the fungal host *C. parasitica*. The clonal population structures in the southern range (evident from the presence of only one dominant vc type) reflect strong genetic bottlenecks in *C. parasitica* resulting from recent founder events. The reason for the incongruence in genetic diversity between the fungal and the viral populations may be differences in their evolutionary rate. We estimated a mean nucleotide substitution per site per year of 3.7×10^{-4} for CHV-1, which falls in the range of 10^{-2} to 10^{-5} reported for RNA viruses (Duffy et al. 2008 and references therein). This rate, however, exceeds the evolutionary rate of approx. 10^{-9} reported for ascomycete fungi (Kasuga et al. 2002) such as *C. parasitica* by several orders of magnitude. Our observation agrees with other observations of virus-host coevolution. It was described previously that, on the temporal scale, the evolution of RNA viruses is usually not congruent with the evolution of their hosts, due to differences in nucleotide substitution rates (Holmes 2004, 2008).

On the spatial scale, the evolutionary scenario inferred for CHV-1 in our study matches the scenario inferred for the *C. parasitica* populations. The first records of *C. parasitica* in Europe suggested that it was introduced on chestnut wood or plants through the port of Genoa in northern Italy around 1938 (Heiniger and Rigling 1994). After establishment, the *C. parasitica* populations most likely expanded from northern Italy to the nearby areas (Milgroom et al. 2008; Dutech et al. 2010; Jezic et al. 2012; Prospero and Rigling 2012). Milgroom et al. (2008) characterized *C. parasitica* population structures from the central and the southern range in Europe based on vc types and sequence characterized amplified region (SCAR) haplotypes. The study revealed a high genetic diversity and very similar structures of the fungal populations in the central range. The populations in the southern range, however, were mainly clonal. Different clones dominated the *C. parasitica* populations in the southern Balkans and the populations in Turkey, in accordance with our observations. Studies from Slovenia (Krstin et al. 2011), Croatia (Krstin et al. 2008; Jezic et al. 2012) and Bosnia-Herzegovina (Trestic et al. 2001) further suggested that the division between the genetically diverse central populations and the clonal southern populations runs through Bosnia-Herzegovina. The *C. parasitica* populations in Slovenia, Croatia and north-western Bosnia-Herzegovina shared the characteristics with the other central populations. The *C. parasitica* populations in southern Bosnia-Herzegovina, however, were dominated by the same clone as the populations in the southern Balkans. Milgroom et al. (2008) therefore concluded that the clonal population structures of the fungus in the southern Balkans and in Turkey were the results of two independent invasion events.

The congruence between the invasion histories inferred for *C. parasitica* and for CHV-1 is striking. As explained earlier, the spread of CHV-1 is completely host-dependent. CHV-1 disperses in virus-infected mycelial particles or in virus-infected asexual spores (conidia)

of the fungus over very short distances. The dispersal of CHV-1 is therefore linked to ecological factors influencing the spread of the fungal host. It was shown in other RNA viruses such as rabies virus of fox (Real et al. 2005), of raccoon (Biek et al. 2007) and of bat (Nadin-Davis et al. 2010) and in Andes virus of rats (Torres-Pérez et al. 2011) that their phylogenetic history was tightly connected to host demography and geographic barriers limiting host movement and contacts. CHV-1 is not transmitted to the sexual spores (ascospores) of the fungus (Carbone et al. 2004), which are also transported by wind, and thus would enable the spread of fungal particles over longer distances. The data available on *C. parasitica* to date suggest that vector-aided transport of virus-infected mycelium or virus-infected conidia of *C. parasitica* was responsible for the long-distance dispersal of both the fungus and the virus. An important vector may, thus, have been infected chestnut plants, timber or firewood (Prospero et al. 2006) traded and transported across Europe.

The rapid colonization of the central range by CHV-1 may be explained by the rapid expansion of the fungal population across the forests on the south facing slopes of the Alps in Switzerland, Italy, Slovenia and Croatia and on the north-east facing slopes of the Dinaric Alps in north-western Bosnia-Herzegovina. The geography and the relatively continuous forest belt in the central range with widespread occurrence of chestnut might have been favourable for the short distance dispersal of (virus-infected) mycelial particles and conidia. In Bosnia-Herzegovina, however, the Dinaric Alps, which rise well above the timber line, cross the country from west to east, and thus impose a major barrier for any southward spread of the fungus and virus. This is consistent with the observed division between the genetically diverse populations of *C. parasitica* in north-western Bosnia-Herzegovina and the clonal populations in southern Bosnia-Herzegovina (Trestic et al. 2001). As discussed by Biek and Real (2010), spatial heterogeneity affects the population dynamics of the host as well as that of a host-dependent parasite. In the southern Balkans, the occurrence of chestnut forests is patchy and the spread of virus-infected *C. parasitica* has to occur over long distances by vectors such as infected plant material transported by humans. The quarantine measures that were taken after the breakout of the European chestnut blight epidemic, however, may have been effective in containing *C. parasitica* (and also CHV-1) within the central range for many years. According to our TMRCA estimates, the Macedonian CHV-1 population investigated in this study was founded in the early 1980s. Interestingly, this falls within the time of political instability in the state of former Yugoslavia after the death of president Tito in 1980. At that time, civil commotions increased and Yugoslavia started to fall apart, which may have been associated with increased movements of people and material across the state. In 1991, Macedonia and Bosnia-Herzegovina, which belonged to former Yugoslavia, became independent. Subsequently, a dispute arose between Greece and Macedonia about the name and flag of the new Macedonian republic, which culminated in a trade

embargo against Macedonia. The embargo was relieved in 1995 and trade between the independent Macedonian republic and Greece increased. It is striking that 1995 is also the TMRCA estimate of the CHV-1 population in Greece. These coincidences of political events and the estimated emergence of new CHV-1 populations may be accidental. However, it is likely that the political situation in the southern Balkans at the end of the 20th century facilitated long-distance dispersal of *C. parasitica* and CHV-1 due to alterations in the volume and direction of fluxes of trade and travel across the region.

Similarly, political tensions between Greece and Turkey and restrictions in trade between these two countries at the time may explain why Greece was (despite geographic proximity) clearly not the source for the CHV-1 and *C. parasitica* populations in Turkey. Turkey was most likely invaded by CHV-1 and *C. parasitica* on diseased plant material that had been shipped from Italy, the European centre of chestnut production and breeding (Castellini et al. 2009). Shipment of high quality chestnut germplasm from Italy to other European countries has not been uncommon. The hypothesis that CHV-1 invaded Turkey by boat is supported by the TMRCA estimates. The population Ya, which is closer to the port of Istanbul, emerged 10 years prior to the population Bu, which is located further west. Forests are patchy in this region and the two populations are not connected by a continuous forest belt. Hence, the population Bu was likely founded by a rare long-distance dispersal event that explains the time-lag between the emergence of the two populations as well as the persistent population differentiation (lack of regular gene flow).

CHV-1 plays an important role as a biocontrol agent of chestnut blight in Europe. In extensively managed or natural ecosystems, such as forests, the long-term establishment of the biocontrol agent is desirable (Payne et al. 1988). Ideally, a biocontrol agent is spreading independently and persists in the ecosystem, exerting a continuous control. The spatial and temporal evolution of biocontrol agents is therefore crucial for the sustainability and effectiveness of the disease control. However, little is known about these evolutionary processes in natural biocontrol systems (Hufbauer and Roderick 2005). CHV-1 occurred naturally in *C. parasitica* populations and spread independently across Europe (Heiniger and Rigling 1994). In our study, CHV-1 was prevalent in all *C. parasitica* populations investigated. Our results indicate that CHV-1 successfully invaded newly established populations of the destructive pathogen *C. parasitica*, and thus likely contributed to prevent the development of devastating chestnut blight epidemics, such as observed in North America (Anagnostakis 1982; Milgroom and Cortesi 2004). Furthermore, our study showed that the newly founded CHV-1 populations had quickly overcome genetic bottlenecks and reached a high genetic diversity due to the rapid evolutionary rate of CHV-1. The results of another study conducted in the same populations (Bryner and Rigling, submitted) revealed that

variation in CHV-1 virulence towards *C. parasitica* was present in all populations and that there was no evidence for reduced disease control in neither the older nor the newly founded populations. In conjunction, these results are promising for the preservation of the European chestnut forests. Studies investigating the spatial and temporal evolution of biocontrol agents in other ecosystems would be useful to define the characteristics and requirements of successful and sustainable biocontrol agents in general. Our study indicates that CHV-1 has characteristics that are ideal for a sustainable disease control in (semi-) natural ecosystems.

In conclusion, our analysis of genome sequences obtained from CHV-1 populations across Europe suggests that the evolution of CHV-1 was spatially congruent with the evolution of its host *C. parasitica*. We were able to show that CHV-1 had most likely been introduced together with *C. parasitica* and that its spread across Europe was not continuous. The use of a coalescent approach allowed us to estimate the dates of population emergence in the different European regions and to reconstruct the invasion history of CHV-1. We discussed the consistence of geographic, vegetation-related, demographic, economic and political factors with the correlated invasion pattern inferred for CHV-1 and *C. parasitica* in Europe.

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Supporting information

Table S1. Vegetative compatibility (vc) types observed in the eight populations of *Cryphonectria parasitica*.

Vc Type	Go	Pu	Ka	Iv	Ra	He	Bu	Ya
EU-1	23	24	4	17			96	89
EU-2	37	18	15	12	2			
EU-3	3	2	1	1				
EU-4	4	4		1				
EU-5	10	10	4	3				
EU-6	4	8	3					
EU-7		2	2	2				
EU-8		2	1	1				
EU-9		1	1					
EU-10			1					
EU-11		1						
EU-12	6	2	5	1	70	93		
EU-13		2	2	1				
EU-14		2						
EU-16				1				
EU-17	2	1	2					
EU-18	1	2	1					
EU-19			1					
EU-21		2						
EU-22	1	3	1	2				
EU-23	3	1						
EU-25		1		1				
EU-26		3	1					
EU-27	1		1					
EU-28	1	1						
EU-29	1	3						
EU-31		1		1				
EU-43		1						

Note: The populations were sampled from different European regions (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya).

Table S2. Pairwise values for linearized F_{ST} (above diagonal) and geographic distance (in km, below diagonal).

	Go	Pu	Ka	Iv	Ra	He	Bu	Ya
Go	0	0.00560	0.01843	0.01136	0.00463	0.03925	0.00426	0.01413
Pu	33	0	0.01715	0.01041	0.00368	0.03847	0.00329	0.01317
Ka	847	860	0	0.02359	0.01640	0.05237	0.01621	0.02683
Iv	906	918	65	0	0.00936	0.04517	0.00905	0.01935
Ra	1403	1408	623	559	0	0.03705	0.00230	0.01206
He	1732	1736	951	886	333	0	0.03738	0.04938
Bu	2311	2320	1475	1411	939	661	0	0.01178
Ya	2326	2336	1488	1425	959	686	31	0

Note: These values were used in the Mantel test for significance of isolation-by-distance (IBD) between populations of *Cryphonectria hypovirus 1* subtype I from different European regions (Switzerland: Go, Pu; Bosnia-Herzegovina: Ka, Iv; Macedonia: Ra; Greece: He; Turkey: Bu, Ya).

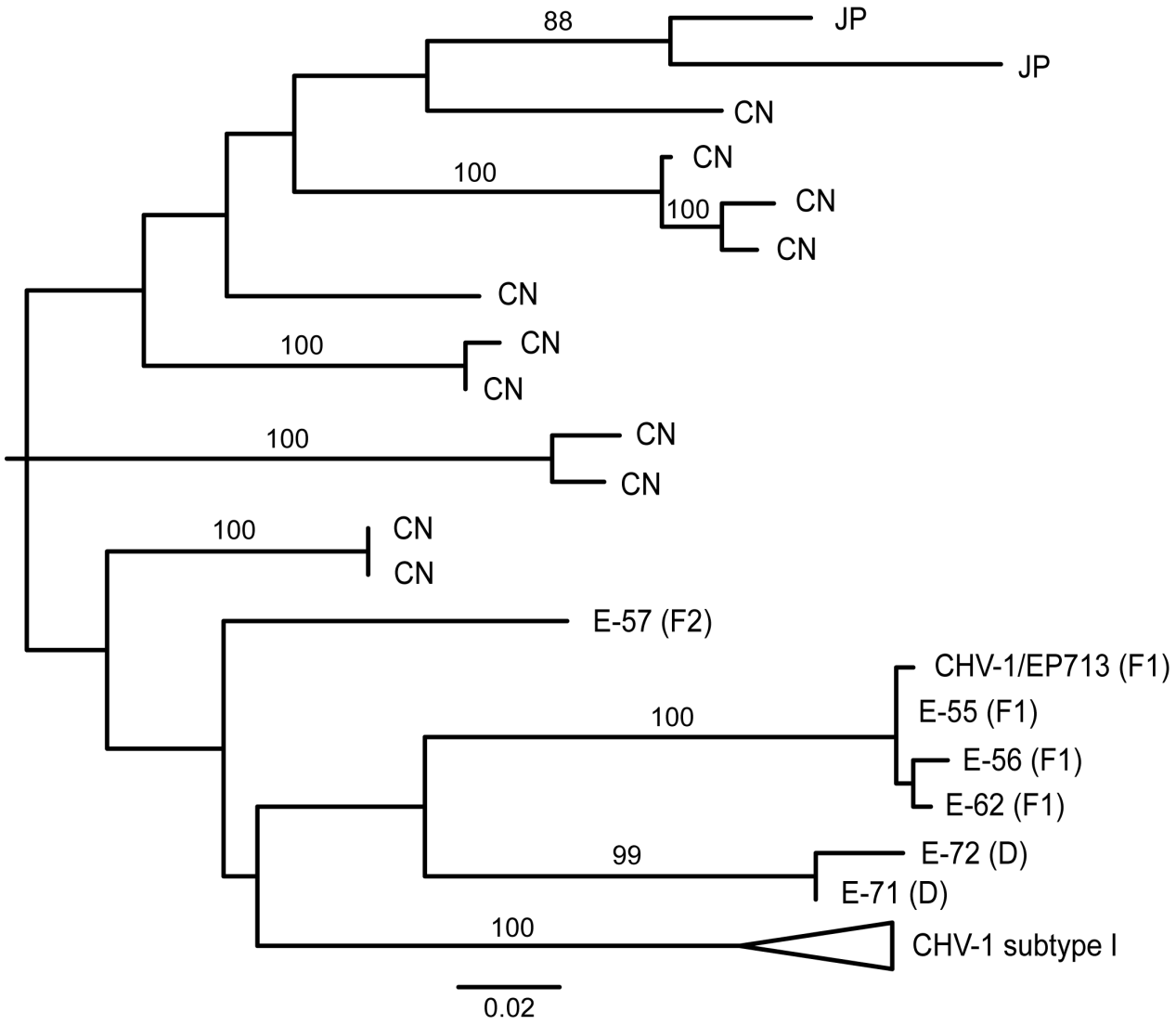

Fig. S1. Maximum-likelihood tree of phylogenetic relationships of *Cryphonectria hypovirus 1* (CHV-1); deep phylogeny including samples from the European CHV-1 subtypes I, F1, F2 and D as well as samples from China and Japan. Branch lengths are drawn to scale, with the scale bar indicating the number of nucleotide substitutions per site. Bootstrap support values >80% are indicated on branches.

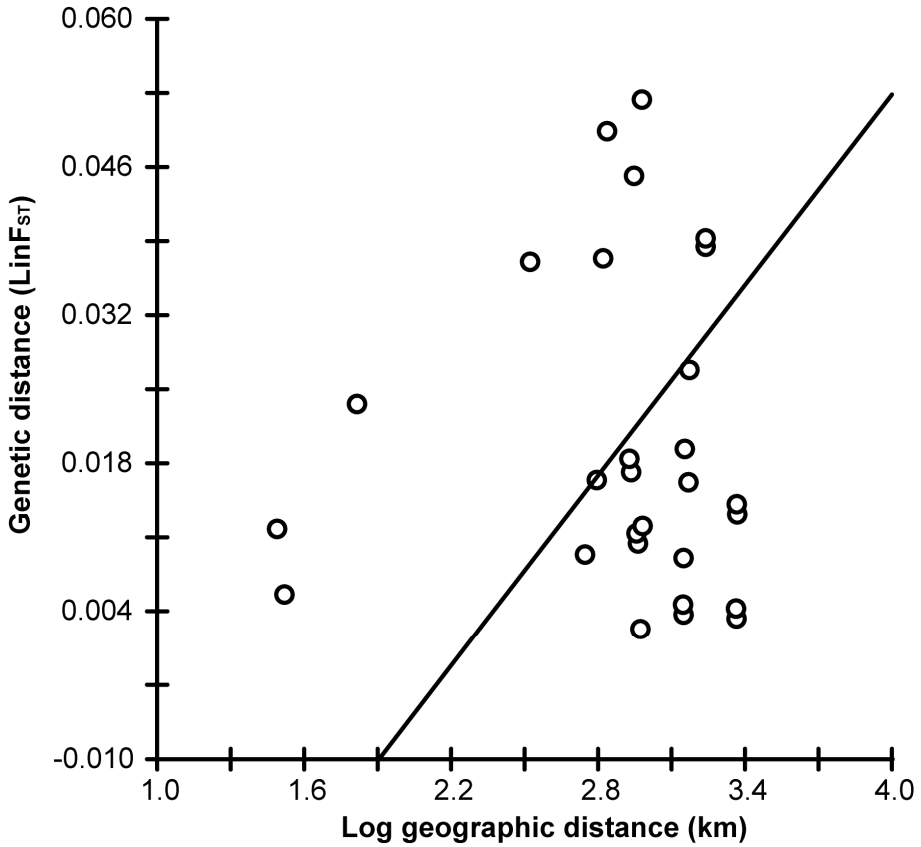

Fig. S2. Genetic distance (linearized F_{ST}) is plotted against the logarithm of geographic distances (in km). No isolation by distance was inferred from matrix correlation between genetic distance (linearized F_{ST}) and log geographic distance (km). Line is the reduced major axis (RMA) regression. The correlation between genetic distance and geographic distance was not significant (Z = 99.6662, r = 0.2378, P = 0.94) based on 1000 randomizations using Mantel test.

Chapter 6

General conclusions

Sarah F. Bryner

Conclusions

The results of this thesis draw a complex picture and demonstrate the impact of different interacting factors on virulence expression (i.e. harm to the host) in the fungal virus CHV-1. Changes in the virus, the fungus and/or the environment can affect the host-parasite interaction, and thus affect the biological control of chestnut blight.

The finding of significant genotype-by-genotype-by-environment interactions presented in Chapter 2 of this thesis indicates that environmental factors may change virulence expression in interactions between particular fungus and virus genotypes. Biocontrol treatments with CHV-1 on different populations of *C. parasitica* and/or in different climatic regions are therefore of unpredictable outcome. In most European regions, chestnut blight incidence is high but does not endanger the chestnut forests due the prevalence and effectiveness of CHV-1 in *C. parasitica* populations (Heiniger and Rigling 1994; Milgroom and Cortesi 2004). However, significant genotype-by-genotype-by-environment interactions suggest that climate change could disturb the subtle interaction between CHV-1 and *C. parasitica* and result in a re-emergence of the chestnut blight epidemic. Environmental differences may even lead to divergent selection of host-parasite combinations in the long term and ultimately to the evolution of genetically different host and parasite populations under different climatic conditions. To my knowledge, this study presented the first report of a genotype-by-genotype interaction in a host-parasite system that is modified by the abiotic environment. The results provide evidence that the environment impacts the coevolutionary trajectory of host-parasite interactions. Seen in this light, the successful establishment of CHV-1 in Europe as a biocontrol agent may have been – at least in part – the result of a combination of favourable conditions, e.g. high suitability of the introduced virus strains to control the fungal populations under the climatic conditions provided. However, conditions may become unfavourable in the future, thus, putting the biological control of chestnut blight at risk.

As outlined in Chapter 3, increased virus transmission barriers within fungal populations were suspected of promoting the erosion of biological control (Milgroom 1995; Taylor 2002). Vegetative incompatibility in *C. parasitica* has been shown to restrict virus transmission between fungi, and thus presents an important transmission barrier (Cortesi et al. 2001; Milgroom and Cortesi 2004). Highly virulent strains of CHV-1 strongly debilitate their host, and thus reduce their own transmission probability (Taylor 2002). Virulent viruses were therefore expected to face a selective disadvantage and to be strongly selected against in *C. parasitica* populations with high levels of vegetative incompatibility (Milgroom 1995). It is not unlikely that vc type diversity in *C. parasitica*

populations will increase due to new introductions or sexual recombination of *C. parasitica* in the future (Jezic et al. 2011), and thus challenge the sustainability of the biological control. However, the results presented in Chapter 3 do not support these hypotheses. No differences in virus virulence were observed among *C. parasitica* populations with high and low vc type diversity. Virulent strains of CHV-1 seemed to be able to persist in populations with high vc type diversity. These results are promising for the sustainability of biological control in Europe. However, they also raise the question whether high virulence not only negatively affects transmission of CHV-1 as previously assumed but also benefits transmission of CHV-1 between fungi.

This question was approached by the study presented in Chapter 4 of this thesis. The results of the study provide evidence for an association of high virulence with increased transmissibility in CHV-1. In fact, this indicates that virulence has benefits for virus transmission that potentially counterbalance virulence costs for virus transmission. High virulence negatively affects the production of virus infected spores, thus reducing the transmission potential of the virus (Milgroom 1995; Taylor 2002). However, this might be compensated by positive effects of high virulence on the virus transmission rate per host-to-host contact. I hypothesize that increased transmissibility is a virulence factor coupled with other virulence-related effects of CHV-1 on *C. parasitica*. Virulent viruses might interfere with the fungal self/nonself recognition system and inhibit cell death after hyphal fusion, thus, permitting virus transmission between vegetatively incompatible fungi. Therefore, virulent strains of CHV-1 may not face a selective disadvantage over less virulent ones in *C. parasitica* populations with high levels of vegetative incompatibility. Furthermore, coupling of host debilitation with the ability to inhibit cell death upon hyphal fusion in CHV-1 could explain why CHV-1 has evolved much higher virulence than most other fungal viruses (including its close relatives CHV-3 and CHV-4) and why it has spread widely (unlike CHV-2).

Most likely, these particular features have enabled the successful spread of CHV-1 throughout the *C. parasitica* populations in Europe. Phylogenetic and coalescent analyses of European CHV-1 populations presented in Chapter 5 of this thesis indicated that CHV-1 was introduced together with its host from Asia. Phylogeny and time estimates revealed that CHV-1 rapidly colonized the central range along the south facing slopes of the Eastern Alps and the north-east facing slopes of the Dinaric Alps. These central populations were the source for two waves of simultaneous invasions towards the southern Balkans and Turkey, as indicated by migration rates. Furthermore, these results suggested that the evolutionary scenarios for CHV-1 and *C. parasitica* were spatially congruent. CHV-1 invaded new regions at about the same time as its fungal host, which may have prevented the development of devastating chestnut blight epidemics in Europe. I demonstrated that in this, and likely in other pathosystems, geographic,

vegetation-related, demographic, economic and political factors may help to explain the correlated invasion pattern of a viral parasite and its host.

Overall, the studies presented in this thesis have increased our understanding of the host-parasite interaction between *C. parasitica* and CHV-1 and have shed some light on the factors that shape the evolution of virulence in CHV-1. The results obtained revealed the complexity of the *Cryphonectria*-hypovirus pathosystem and have important implications for the biological control of chestnut blight. To predict the development of chestnut blight epidemics, various interacting factors have, thus, to be considered. Pathogenicity of *C. parasitica*, virulence and prevalence of CHV-1, susceptibility of the local chestnut trees and environmental conditions mutually influence each other and ultimately determine severity and emergence of the disease. In general, these findings highlight the fact the biological details of the host-parasite interaction and the cost-benefit relationships involved are key for understanding the ecology and evolution of parasite virulence.

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