Hypovirus Virulence and Vegetative Incompatibility in Populations of the Chestnut Blight Fungus

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ABSTRACT

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Cryphonectria hypovirus 1 hyperparasitizes the chestnut blight fungus *Cryphonectria parasitica* and acts as a biocontrol agent for 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. Therefore, virulent viruses are 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 European *C. parasitica* populations with high diversity of vegetative compatibility types and in four populations with low diversity. 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 versus 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.

Additional keywords: avirulence, biological control, fungal virus, host–parasite interaction.

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) with which a biocontrol agent affects the pest or disease organism is pivotal for the level of control achieved. In extensively managed ecosystems, such as forests and pastures, attempts have been made to establish biocontrol agents in the long term (21,42). In these systems, biocontrol agents need to be virulent enough to achieve sufficient disease control but also need to spread efficiently through the pathogen population (25). They should spread independently and persist in the ecosystem, exerting continuous control. Therefore, the evolution of virulence in biocontrol agents is crucial for the sustainability and effectiveness of the biocontrol. However, little is known about how virulence evolves (25).

Current theory suggests that the evolution of virulence relies on a trade-off between virulence costs and virulence benefits (2,6,18). The fitness of a parasite is the result of its multiplication within the host and its transmission from infected to noninfected hosts. Highly virulent parasites multiply rapidly and exploit their host efficiently but at the cost of increased host mortality or debilitation, which reduces the parasite's chances of being transmitted. Several models have been developed to describe the evolution of virulence in host–parasite interactions. These take into account the characteristics of the parasite (12,13), competition among parasites (17), the life history of the host (27), the host population structure (30), and the ecological and evolutionary dynamics of the host–parasite interaction (3,29). In general, these models suggest that the optimal degree of virulence maximizes the parasite's fitness with the transmission potential provided. Lenski and May (28) further suggest that such an optimal degree of virulence will decline over time due to an ecological feedback effect from the decreasing number of susceptible hosts available for successful infection.

For transmission, some parasites rely on relatively normal host function and, hence, lower virulence, while other parasites may inflict more damage to the host without inhibiting transmission (14). Therefore, the particular cost-benefit relationship of virulence in each host–parasite interaction is a key factor determining the optimal degree of parasite virulence and transmission. Vertically (to offspring) transmitted parasites, for example, depend on the reproduction of the infected host. The cost for them of host debilitation is much higher than for horizontally (to other hosts) transmitted parasites, which do not require their host to reproduce (7). Similarly, the cost of host debilitation is much higher for parasites that cannot survive outside the host and depend on the availability of a live host than for parasites that are able to survive outside the host (52).

Cost-benefit relationships should be taken into account when evaluating the sustainability of a biocontrol system (25). According to the theory described above, biocontrol agents with high costs of virulence for transmission would evolve toward avirulence, because virulent strains would not be able to spread and persist within the population. A loss of virulence in the biocontrol agent, however, leads to the erosion of disease control.

The *Cryphonectria*–hypovirus pathosystem is a famous biocontrol system in Europe, which has been rather successful thus far (21,37). The fungus *Cryphonectria parasitica* causes lethal bark cankers on chestnut trees (*Castanea* spp.) and is responsible for the serious tree disease commonly known as chestnut blight. It was introduced from Asia to both North America and Europe early in the 20th century (1). In North America, it destroyed the

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplementary tables.

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native chestnut forests (37). In Europe, however, the situation was not as dramatic. A few years after the first disease records, superficial cankers were observed but they did not result in dieback of the infected trees (21). These so-called "healing cankers" were found to be the result of the emergence of a viral disease of *C. parasitica* (9). *Cryphonectria hypovirus 1* (CHV-1) hyperparasitizes *C. parasitica* and reduces its pathogenicity. Infection with CHV-1 is persistent and does not kill *C. parasitica.* It does, however, inhibit its sexual reproduction and strongly attenuates the fungal growth and asexual sporulation of the fungus (45). The reduction in fungal growth and sporulation is commonly regarded as the major virulence determinant in CHV-1 (37,45,50). Following its emergence, the virus spread spontaneously throughout *C. parasitica* populations in Europe. Natural dissemination and active biocontrol efforts have led to a wide prevalence of virus infection, which has successfully controlled chestnut blight disease in most regions in Europe (21).

Fungal viruses are obligate parasites that infect their hosts persistently (40). They lack an extracellular stage outside the host and, therefore, completely depend on their host for survival and fitness. Therefore, the fact that the vast majority of the fungal viruses known infect the host without any apparent symptoms (40) corresponds with the predictions of the models described above. CHV-1 is a rare exception among fungal viruses, causing comparatively severe damage to its host. This is particularly intriguing because CHV-1 also depends on vertical transmission to spread. CHV-1 is transferred into the asexual but not the sexual spores of its fungal host (8,46). In the field, an average rate of 69% virus transmission into the asexual spores was observed (46). CHV-1 is dispersed in these asexual spores and then transmitted horizontally from the outgrowing spores to virus-free fungal individuals upon hyphal fusion (24). Thus, new fungal genotypes can be infected through hyphal fusions but not through sexual reproduction. The cost of virulence should be very high for CHV-1 because virulence is associated with reduced fungal growth and reduced production of asexual spores. Therefore, virulence compromises the spread of the virus directly, and it was predicted that CHV-1 would evolve toward lower virulence over time (12,36).

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 (32). Fusion between cells of incompatible individuals results in programmed cell death, which hampers the transmission of viruses and other cytoplasmic elements. Between compatible individuals of *C. parasitica* (i.e., which are identical vegetative compatibility [vc] types), CHV-1 is transmitted in virtually 100% of host-to-host contacts (10). Transmission can also occur between different vc types but at much reduced rates (10,32,41). In *C. parasitica* populations with high vc type diversity, the transmission probability of CHV-1 is expected to be greatly limited (10,37). When such transmission barriers are present, virulent viruses, which reduce their own transmission probability by excessively debilitating their host, are expected to be strongly selected against (35).

The failure of biological control with CHV-1 in North America but not in Europe was assumed to be, at least in part, due to the much higher vc type diversity in North America (21,37). 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* (26,47). Given that virus transmission barriers in European *C. parasitica* populations will potentially increase, the evolution of avirulence in CHV-1 is of great concern. To date, in Europe, several *C. parasitica* populations with high and low vc type diversity have been found (21,37,38,49) that are infected with the same subtype of CHV-1 (19). These populations provide an opportunity to study the influence of the vc type diversity on the virulence of CHV-1 not only within the same hypovirus subtype but also in several geographically separated natural populations of *C. parasitica*.

Thus, in this study, we were able to test the hypothesis that the cost of high virulence for transmission of CHV-1 was higher in populations with large numbers of vc types than in populations with only one or two vc types. We assumed that virulence was reduced in host populations with high vc type diversity, where transmission barriers were present, than in populations with low vc type diversity and no transmission barriers present. 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 CHV-1 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 the Italian subtype of CHV-1 had established naturally (19). All populations were sampled within an area <1 ha. Four populations were obtained from regions where the *C. parasitica* vc type diversity (i.e., only one dominant vc type) was known to be low and four from regions with known high vc type diversity (38,49). All sampling sites were coppice forests with 10- to 20-year-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 in diameter) from chestnut blight cankers at intervals of 5 to 20 m between trees. Only one canker per tree was sampled and the cork borer was flame-sterilized with 70% ethanol between cankers.

Isolation of *C. parasitica*, determination of virus infection, and assessment of vc type diversity. We obtained a total of 634 *C. parasitica* cultures (Table 1) by first isolating them from the surface-sterilized bark samples on water agar and then by culturing them on potato dextrose agar (PDA) (Difco, Voight Global Distribution, Lawrence, MD) (50). Because culture morphology is an indicator of CHV-1 infection (9), *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 (45,50). The presence and identity of CHV-1 subtype I was verified by extraction of the viral double-stranded (ds) RNA and subsequent sequence analysis (19).

The vc type of each *C. parasitica* isolate was determined by pairing with genetically defined vc type tester strains (11). Because the *vic*4 locus has previously been found not to restrict virus transmission (10), allelic status of isolates at this locus was ignored for all diversity calculations. Thus, vc types in this study were defined by the loci *vic*1, *vic*2, *vic*3, *vic*6, and *vic*7. To estimate the diversity of vc types in each population, the Shannon-Wiener's index of diversity was calculated based on the vc type frequency observed (H'_{obs}) and on the vc type frequency expected (H'_{44}) for the smallest sample size analyzed (n = 44). The expected diversity was determined by rarefaction analysis implemented by the vegan package in the software R 2.6.2 (48), which allows comparisons among populations without bias because of differences in population size. Furthermore, we calculated the Taylor and Stoddart's index of genotypic diversity and the evenness index according to Grünwald et al. (20). To estimate the potential of hypovirus transmission in each population, we used the logistic regression model described by Cortesi et al. (10) and calculated the mean probability of virus transmission according to the frequency distribution of the vc types observed in the population and the transmission restriction imposed by the specific allelic differences at the vegetative incompatibility loci between pairs of fungi.

Preparation of experimental isolates. To assess the CHV-1 virulence, we conducted an inoculation experiment. A subsample of 16 virus-infected isolates were selected from each population (Supplementary Table 1). To incorporate the vc type diversity

within populations, a modified stratified procedure was applied. One isolate from each vc type (defined by the loci *vic*1, *vic*2, *vic*3, *vic*6, and *vic*7; see above) was selected at random. The additional samples required to reach a sample size of 16 were then randomly selected from the entire population.

From each of the 16 isolates per population, we produced a virus-free culture. Because the virus transmission rate to conidia is <100% in vivo (46) and in vitro (44), virus-free cultures can be obtained through single conidial isolations. 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 color and the exudation of conidia (9). 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 CHV-1 virulence. Virulence was measured as the reduction of fungal growth due to virus infection, which has previously been shown to be correlated with the reduction in sporulation (5,45). These effects are common virulence measures of fungal viruses (40,43) and are relevant for the biocontrol of chestnut blight (21,37). Significant genotype–genotype interactions have been found between CHV-1 and *C. parasitica* (5,45), which made it impossible to use 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 (5). For each population, we inoculated two cultures of the 16 virus-infected and the 16 virus-free fungi (512 cultures in total) on dormant chestnut stems. Healthy *C. sativa* stems (50 cm in length, 5 to 10 cm in diameter) were cut in 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, 6 mm in diameter, were made and filled with the mycelia mats (5). The virus-free and the virus-infected cultures of the same fungal strain were always put into neighboring wounds on the same stem. Two replicates of each culture

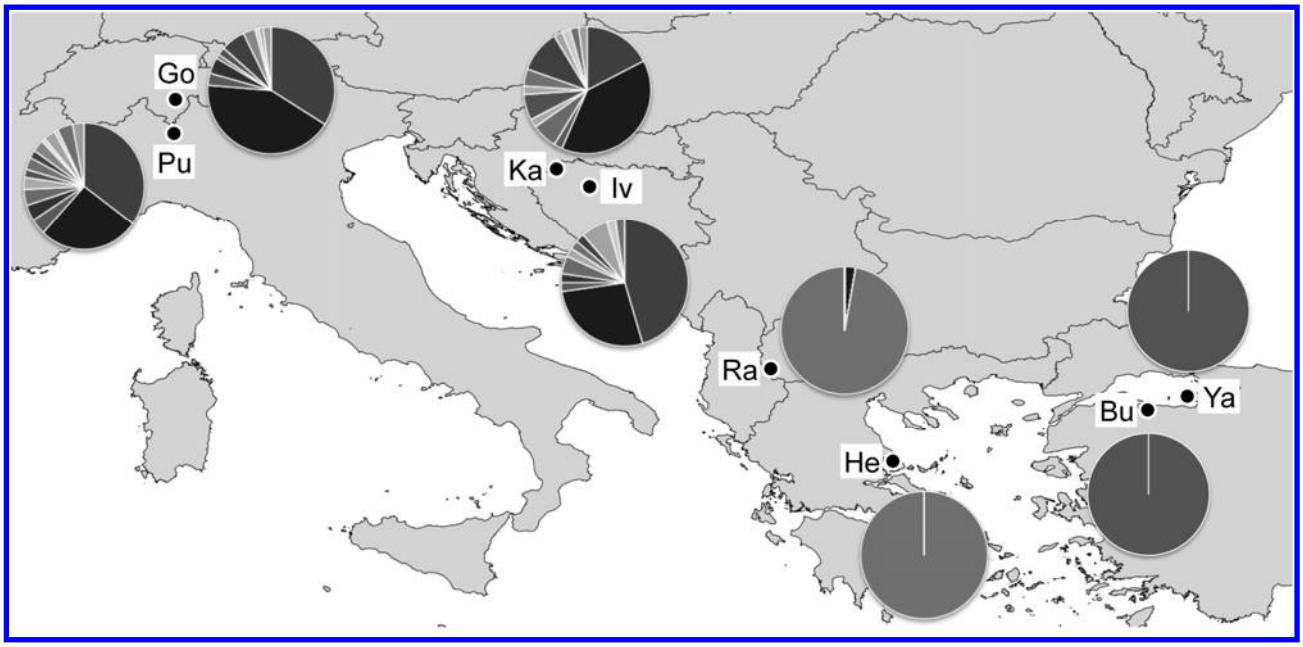

Fig. 1. Map of Europe showing the eight sampling sites. In 2010, Go and Pu were sampled in Switzerland, He in Greece, Ra in Macedonia, and Bu and Ya in Turkey. Ka and Iv were sampled in Bosnia in 2008. Pie graphs display the diversity of vegetative compatibility types (ignoring locus *vic*4) that we observed in each population of *Cryphonectria parasitica*.

TABLE 1. Diversity of vegetative compatibility (vc) types and potential for virus transmission in the eight *Cryphonectria parasitica* populations used in this study[a]

Population[b]	*N*[c]	Number virus-infected (%)[d]	S[e]	H'_{obs}[f]	H'_{44}[g]	G[h]	E_5[i]	Potential for virus transmission[j]
Go	97	50 (52)	11	1.55	1.52	3.30	0.62	0.45
Pu	97	42 (43)	15	2.04	1.87	4.82	0.57	0.40
Ka	46	39 (85)	13	1.99	1.96	4.79	0.60	0.35
Iv	44	31 (70)	11	1.64	1.64	3.43	0.58	0.48
He	93	41 (44)	1	0.00	n/a	1.00	n/a	1.00
Ra	72	55 (76)	2	0.13	0.12	1.06	0.43	0.93
Bu	96	33 (34)	1	0.00	n/a	1.00	n/a	1.00
Ya	89	18 (20)	1	0.00	n/a	1.00	n/a	1.00

[a] Vc types were defined by the loci *vic*1, *vic*2, *vic*3, *vic*6, and *vic*7. Locus *vic*4 was ignored in all indices calculated because it does not restrict virus transmission; n/a = not applicable. Vc type data, including *vic*4, are shown in Supplementary Table 1.
[b] Populations were sampled within an area <1 ha each in Switzerland (Go and Pu), Bosnia (Ka and Iv), Greece (He), Macedonia (Ra), and Turkey (Bu and Ya).
[c] Total number of collected isolates.
[d] Number of virus-infected isolates (percentage of total in parentheses).
[e] Vc type richness (i.e., the number of different vc types).
[f] Shannon-Wiener's index expressed in terms of the observed vc types: $H'_{obs} = -\Sigma p_i \ln p_i$, where p_i is the frequency of the *i*th vc type.
[g] Shannon-Wiener's index expressed in terms of the vc type distributions expected (H'_{44}) by rarefaction analysis for the smallest sample ($n = 44$).
[h] Stoddart and Taylor's index of genotypic diversity: $G = 1/\Sigma p_i^2$.
[i] Evenness index: $E_5 = (G - 1)/(e\text{^}H^{obs} - 1)$.
[j] Estimated mean potential of virus transmission within the population was calculated according to the observed vc type frequency distribution (10).

pair were used and inoculated onto different stems. Within each set of replicates, the different culture pairs were randomly assigned to stems and to positions on stems. The stems were incubated in opaque plastic containers inside a climate chamber (5). 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 lesion diameter on the chestnut stems. Two diameters of each lesion were measured, one along the longitudinal and a second along the lateral axis of the stem. Because the shape of the lesions resembled an ellipse, we calculated the geometric mean diameter of an ellipse to assess the fungal growth on chestnut stems (5).

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 (5). The virus effect indicates what percentage of fungus's growth is changed due to infection with the virus, and is the measure we used to represent how virulent a virus is toward its natural host. Two replicates per CHV-1 isolate were assessed to obtain a more precise virulence measurement. The mean of the two replicates was calculated and used for statistical analyses. 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 versus 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 whether Type had an effect on the within-population variation. Furthermore, we analyzed the effect of the frequency of infected vc types on the virus effect. A GLM with the fixed factors "frequency of the vc type" "Common versus Rare", and population ("Pop") was used. A vc type was considered rare if the vc type (see above) occurred at a frequency of <5% within the population. If the frequency was >5%, it was considered common. The residuals of all GLMs were normally distributed and displayed constant error variances.

RESULTS

Vc type diversity. All isolates sampled in the eight populations could be assigned to a genetically defined EU vc type. Diversity estimates were calculated for each population based on the five *vic* loci (i.e., *vic*1, *vic*2, *vic*3, *vic*6, and *vic*7) that have previously been found to influence virus transmission (10). 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 for virus transmission (Table 1). Between 11 and 15 different vc types (ignoring *vic*4) per population were detected in the high-diversity populations (Go, Pu, Ka, and Iv) and the Shannon-Wiener's index of diversity H'_{obs} was 1.55 to 2.04. The evenness of vc types was very similar in all high-diversity populations. 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 2 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) was 0.35 to 0.48 in the high-diversity populations and 0.93 to 1.00 in the low-diversity populations.

CHV-1 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 (Figs. 2 and 3; Supplementary Table 2). Because 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. Order of mean virus effect did not distinguish between the populations of low and high vc type diversity (Fig. 3). Both the strongest (highest virulence)

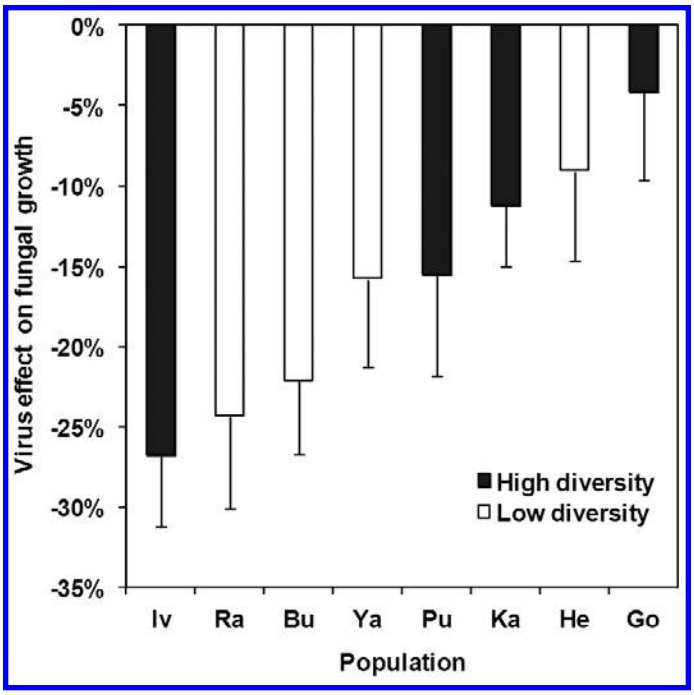

Fig. 3. Effect of virus infection on the growth of *Cryphonectria parasitica* on chestnut stems after 22 days of incubation. The virus effect is the difference in growth (lesion diameter) between the virus-infected and the virus-free clone as a proportion (%) of the virus-free clone. For each population (Go, Pu, Ka, Iv, He, Ra, Bu, and 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.

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 to reveal the lesions more clearly.

and the lowest (lowest virulence) mean virus effect 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 (Table 2, Type). The population had a significant ($P = 0.027$) effect (Table 2, Pop[Type]). 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 the virus effect was not significantly ($P > 0.05$) affected by whether the virus was infecting a common or a rare vc type (Table 2B, Common versus Rare). The virulence of the CHV-1 strains infecting common vc types was neither significantly lower nor higher than the virulence of the strains infecting rare vc types. The population again had a significant ($P = 0.029$) effect (Table 2B, Pop).

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, taking into account only the *vic* loci that restrict virus transmission. Based on all diversity indices applied, the eight populations fall into two groups (i.e., high versus low vc type diversity populations). The virus transmission probability was considerably restricted in the populations with high vc type diversity ($H'_{obs} = 1.55$ to 2.04) but unrestricted in the populations with low diversity ($H'_{obs} = 0.00$ to 0.13). The vc type diversities that we observed had been at the same levels for >30 years in all populations (49). This indicates that there has been no or only very little migration between high- and low-diversity populations (38), so that they had been able to coevolve over an extended period of time. However, the presence or absence of these transmission barriers seemed to have no effect on the average virulence of virus populations or on the variation of virulence within populations. Hypovirus virulence did not correspond with any of the diversity indices calculated. Furthermore, the highest virulence of CHV-1 was observed in a population with high vc type diversity. We further compared the virulence of viruses infecting rare versus common vc types and found that the virulence of the viruses infecting rare vc types was not lower than the virulence of viruses infecting common vc types. These findings suggest that transmission barriers between different vc types did not exhibit a discernible selective pressure on CHV-1 virulence.

In all eight populations of CHV-1, we found high variation in virulence, which is the raw material for evolution. The apparent lack of selection may indicate that the selective advantage or disadvantage of viruses with low versus high virulence was not significant. *C. parasitica* and CHV-1 were both introduced into Europe during the past century. The first official records of *C. parasitica* were made in 1938 and of healing cankers in 1951. Only in 1964, however, were these healing cankers identified as being associated with the dsRNA of CHV-1 (21). A coevolution of 60 to 70 years is not long in evolutionary terms. However, parasites—and RNA viruses in particular—are known to evolve very rapidly (39,52). Furthermore, hypoviruses follow the properties of single-stranded (ss) RNA viruses more closely than those of true dsRNA viruses. It is generally thought that the dsRNA is most likely an artifact of the replication cycle, during which the nascent and template RNA strands anneal and copurify (15). Therefore, CHV-1 is fundamentally an ssRNA virus, which evolves even faster than dsRNA viruses (23). A classic example of rapidly evolved virulence comes from the biological control of introduced rabbits in Australia with the myxoma virus (16). Within only 1 year of coevolution, the mortality rate of infected rabbits decreased from 100 to 90%. Therefore, overall, it is not apparent why virulence in CHV-1 had not evolved after half a century of coevolution—unless the selective pressure was just not strong enough.

In our study, the prevalence of CHV-1 in populations with high vc type diversity was not lower than in populations with low vc type diversity. Therefore, the restriction on transmission imposed by the vc type diversity should be questioned (37), despite its wide acceptance in the *Cryphonectria*–hypovirus literature. A population genetics study by Carbone et al. (8) also suggested that a migration of CHV-1 among vc types occurs within (but insignificantly between) natural populations. These results imply that, in natural populations, the virus transmission probability is likely to be higher than expected from observations in short-term laboratory experiments (10,41) or from modeling (31). An earlier hypothesis was that, in the long term, CHV-1 could eventually be transmitted into every vc type in natural populations, when given enough time and some leakiness of the vc type barrier (51). Thus, the relevance of the vc type barrier for virus transmission in nature needs to be better quantified. In addition, the number of vc types in European *C. parasitica* populations might not be high enough to exert a selective pressure on virulence. Vc type diversities considered high in Europe are only moderate in comparison with vc type diversities in Asia, the center of origin of *C. parasitica* (1), where a survey of *C. parasitica* populations revealed that almost every isolate sampled had a unique vc type (33).

Furthermore, modeling the relationship between vc type diversity, CHV-1 virulence, and CHV-1 prevalence indicated a stable system. The model of Brusini et al. (4) suggests that the system is robust against change at moderate levels of both vc type diversity and 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 is moderate compared with other (less prevalent) subtypes present in Europe (5,19,50). Therefore, the results of our empirical study, which found no feedback effects, correspond with the model predictions.

The impact of the host population structure on parasite virulence is of particular importance in biocontrol systems, where the release of a biocontrol agent is specifically intended to affect the host population (25). If certain characteristics of host populations were to promote the evolution of avirulence in the biocontrol

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

Source	df	Mean squares	*F*	*P*
Effect of population type[a]				
Type	1	0.037	0.841	0.361
Pop (type)	6	0.110	2.483	0.027
Error	120	0.044	…	…
Effect of vc type frequency[b]				
Common vs. rare	1	0.069	1.651	0.204
Pop	3	0.132	3.153	0.031
Error	59	0.042	…	…

[a] Effects of the fixed factors population type (Type; low versus high vc type diversity) and population nested within population type (Pop [type]) on virus effect 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 of eight populations: four of low vegetative compatibility (vc) type diversity and four of high vc type diversity.

[b] Using the four populations with high vc type diversity only, the effect of the fixed factors vc type frequency (common versus rare) and population (Pop) on virus effect were tested.

agent, biocontrol would become ineffective in these populations. Therefore, in order to design sustainable disease management strategies, it is crucial to understand the interactions and interrelations of the host and parasite populations in each biocontrol system. The probability of parasite transmission has been shown to differ among host populations, and such differences may affect the evolution of parasite virulence. Herre (22) 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 plays an indispensable role in the biological control of chestnut blight in Europe, where it has kept *C. parasitica* populations under control. The incidence of chestnut blight is very high but infections of *C. parasitica* with CHV-1 are preventing severe damage, including dieback of the trees (21,37). The stability of this natural biological control system has been the subject of concern because it has been predicted that CHV-1 would loose its virulence due to a potential increase in vc type diversity (34). New vc types may be introduced or could emerge through sexual reproduction of *C. parasitica*. Fortunately for the biological control, we found no indication that the virulence of CHV-1 in *C. parasitica* populations with high vc type diversity was diminishing. This suggests that the biocontrol of *C. parasitica* with CHV-1 in Europe will not erode 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 become well established within a system. Thus, in the case of fungal pathogens, the vc type diversity of the pathogen population may be less important than previously thought.

In conclusion, the vc type diversity of *C. parasitica* populations was not found to have a significant effect on the virulence of CHV-1. Furthermore, virulent viruses were able to persist in populations with high vc type diversity, which has very promising implications for the sustainability of the biological control system. We found no sign of evolution toward reduced virulence in the biocontrol agent due to differences in the parasite transmission probability of the host population. These findings suggest that the biocontrol of chestnut blight in Europe may be more sustainable and effective in the long term than previously thought.

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