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FULL RESEARCH PAPER

Horizontal transmission of hypoviruses between vegetative compatibility types of *Cryphonectria parasitica* in Macedonia

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Abstract Biological control of chestnut blight with hypovirulence depends on the successful transmission of hypoviruses between individuals of the chestnut blight fungus, *Cryphonectria parasitica*. Vegetative incompatibility inhibits horizontal virus transmission, but not completely. In an effort to assess the potential for the spread of hypoviruses in the Republic of Macedonia, we studied the transmission of *Cryphonectria hypovirus 1* (CHV-1) among the five observed vegetative compatibility (vc) types of *C. parasitica*. One fungal isolate of each vc type was infected with CHV-1 and was paired in vitro with isolates of all other vc types for a total of 20 combinations of virus donors and recipients, and 250 replicate trials per combination. Virus transmission was scored after 7 days as successful if the recipient isolate took on an unpigmented culture phenotype typical of virus infection. Transmission occurred at high frequencies between some pairs of vc types, but in <1% of the trials for 10 of the 20 combinations of donors and recipients. Asymmetric transmission was observed between some vc types that had different alleles at vegetative incompatibility loci *vic1* or *vic7*; i.e., transmission occurred at high frequencies in one direction, but very low frequencies between the same pair of isolates in the opposite direction. The expected virus transmission, calculated as the average transmission predicted for any two randomly chosen individuals from a population, was highly negatively correlated to vc type diversity. Results for isolates of *C. parasitica* from Macedonia were similar to those from Italy, but less transmission was generally observed. Differences in genetic background effects on transmission may vary among different populations even when isolates differ by the same *vic* alleles.

Keywords Biological control · Chestnut blight fungus · Hypovirulence · Mycovirus · Vegetative incompatibility · Virus transmission

Abbreviations

CHV-1	*cryphonectria hypovirus 1*
vc type	vegetative compatibility type
vic	vegetative incompatibility

Introduction

Fungal viruses are transmitted horizontally between individuals only by cell-to-cell contact, in which

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hyphae fuse (anastomosis) and cytoplasm from one individual containing virus mixes with that of the other; there is no known extracellular transmission of fungal viruses (Buck 1986; Wickner 1996). Because of the requirement for hyphal anastomosis, virus transmission is inhibited by vegetative incompatibility. Vegetative incompatibility is a self/nonself recognition system in filamentous fungi that, in most cases, prevents the formation of stable heterokaryons (Glass et al. 2000; Saupe 2000; Micali and Smith 2005). Vegetative incompatibility is controlled by multiple, independent vegetative incompatibility (*vic*) genes; two individuals with different alleles (heteroallelic) at one or more *vic* loci are vegetatively incompatible. Hyphae of conspecific individuals can anastomose upon contact, but subsequent recognition of nonself triggers a cascade of biochemical events that leads to programmed cell death and failure to form stable heterokaryons (Glass and Kaneko 2003). The inhibition of transmission of viruses or other deleterious genetic elements by vegetative incompatibility has been well documented in fungi and has led to the hypothesis that vegetative incompatibility has evolved as a defence mechanism to avoid parasitism and infection (Caten 1972; Hartl et al. 1975; Debets et al. 1994).

The most thoroughly studied example of horizontal virus transmission in fungi is that of hypoviruses in the chestnut blight fungus, *Cryphonectria parasitica*. Most of the hypoviruses, in the family *Hypoviridae*, markedly reduce the virulence of *C. parasitica* on chestnut trees in a phenomenon known as hypovirulence (Hillman and Suzuki 2004; Nuss et al. 2005). These viruses have received considerable attention because of the potential of hypovirulence for biological control of chestnut blight (Van Alfen et al. 1975; Anagnostakis 1982; Griffin 1986; MacDonald and Fulbright 1991; Nuss 1992; Heiniger and Rigling 1994; Milgroom and Cortesi 2004). The transmission of hypoviruses between isolates of *C. parasitica*, however, has been known for a long time to be inhibited by vegetative incompatibility (Grente and Berthelay-Sauret 1978; Anagnostakis and Day 1979). This inhibition led to the paradigm that vegetative incompatibility is a major constraint to biological control of chestnut blight, especially in North America where the diversity of vegetative compatibility (vc) types is relatively high (Anagnostakis et al. 1986). Further research into the effects of vegetative incompatibility on hypovirus transmission showed that the effect was variable and depended on the number and identity of heteroallelic *vic* loci (Anagnostakis and Waggoner 1981; Kuhlman et al. 1984; Liu and Milgroom 1996; Bissegger et al. 1997; Cortesi et al. 2001). Inhibition between vc types is not complete, i.e., it is ‘leaky’, and viruses can be transmitted between vegetatively incompatible isolates, although typically at a lower frequency than between compatible isolates.

In a detailed study on hypovirus transmission in *C. parasitica*, Cortesi et al. (2001) determined the effects of each of six known *vic* genes (Cortesi and Milgroom 1998) by replicating transmission trials on independent pairs of isolates heteroallelic at the same *vic* loci. The purpose of using independent, replicate pairs was twofold. First, this approach allowed them to construct a regression model to predict virus transmission based on knowledge of alleles at the six known *vic* loci (*vic* genotype). Second, they could estimate variation in transmission attributable to genetic background effects, i.e., genes other than *vic* affecting transmission. Cortesi et al. (2001) found enough variation among replicate pairs heteroallelic at the same *vic* loci to conclude that the genetic background has a considerable effect. Because of background effects, the regression model was not always a good predictor of transmission between field isolates. Furthermore, background effects are potentially greater in clonal populations because genes in the genetic background are not randomized with respect to *vic* alleles, as they would be in randomly mating populations (Cortesi et al. 2001).

In practice, we would like to be able to predict the potential for horizontal virus transmission and biological control of *C. parasitica* from knowledge of vc type distributions in field populations (Milgroom and Cortesi 2004). Recent studies of *C. parasitica* and hypoviruses in the Republic of Macedonia have shown that *Cryphonectria hypovirus 1* (CHV-1) is present in several locations (Sotirovski et al. 2006), and that vc type diversity is very low (Sotirovski et al. 2004). Populations of *C. parasitica* in Macedonia appear to be clonal based on the predominance of a single vc type (EU-12) and one mating type (*MAT-1*); together the EU-12/*MAT-1* genotype makes up 94% of the 786 isolates sampled in Macedonia and 100% of 379 isolates sampled in Greece (Sotirovski et al. 2004). Preliminary results from genotyping with molecular genetic markers have confirmed the clonal nature of *C. parasitica* populations (K. Sotirovski and M. G. Milgroom, unpublished data). Clonality may indicate good prospects for biological control of chestnut blight with hypovirulence. Our overall objective, therefore, was to estimate the potential for transmission of CHV-1 among vc types in Macedonia as a way of evaluating whether CHV-1 is likely to invade populations of *C. parasitica* as a biological control agent where more than one vc type is present. Transmission potential was estimated by addressing the following specific objectives: (1) to estimate the probabilities of transmission of CHV-1 between all combinations of vc types found in Macedonia; (2) to compare transmission results between Macedonian vc types with empirical results and model predictions from Italian isolates published by Cortesi et al. (2001); and (3) to estimate the expected or mean probability of transmission within populations of *C. parasitica* in Macedonia.

Materials and methods

Isolates of *C. parasitica*

One isolate of each of the five vc types found in Macedonian populations of *C. parasitica* was randomly sampled from our collection (Sotirovski et al. 2004) and used for assaying horizontal transmission of CHV-1. Isolates Vr38 (vc type EU-12), G25 (EU-1), Po72 (EU-2), G110 (EU-10) and Po5 (EU-22) were collected from field populations in the Tetovo region of Macedonia. Single isolates were used from each vc type because Macedonian populations appear to be highly clonal, with no evidence for recombination of *vic* genotypes (Sotirovski et al. 2004) or other genetic markers (K. Sotirovski and M. G. Milgroom, unpublished). Therefore, a single isolate was assumed to be representative of each clone.

Each isolate was infected with CHV-1 (‘converted’) in vitro by hyphal contact and anastomosis with isolate Vr16, which contains CHV-1 in the Italian subtype (Sotirovski et al. 2006). Conversion of each donor isolate was achieved either directly from Vr16, or through intermediate isolates that, after infection by CHV-1, served as donors to other isolates (Anagnostakis 1983; Peever et al. 2000; Cortesi et al. 2001). Isolate Vr16 has an unpigmented culture morphology typical of CHV-1 infection; successful transmission was evident in recipient isolates because of a change of colony colour from orange to white (Heiniger and Rigling 1994; Cortesi et al. 2001). Previous studies have shown that transmission is unaffected by different virus strains (Liu and Milgroom 1996; Cortesi et al. 2001).

Virus transmission

Transmission of CHV-1 between vc types was tested using a previously described method (Anagnostakis and Day 1979; Liu and Milgroom 1996; Cortesi et al. 2001). Briefly, cubes of agar (ca. 4×4 mm) cut from the margins of actively growing colonies were placed together on 20 ml of potato dextrose agar (PDA; Difco, Detroit, MI) in 9-cm Petri plates. Donor and recipient isolates were placed 2–3 mm apart, approximately 5 mm from the edge of the plate. Cultures were incubated for 7 days at 24°C, in the dark. Virus-infected donor isolates from each of the five vc types were paired with virus-free recipient isolates from the other four vc types for a total of 20 combinations. We tested each combination of vc types with 250 replications, each in a separate plate. Transmission between compatible isolates was tested with only 10 replications because previous studies have consistently shown 100% transmission between compatible isolates (Anagnostakis and Day 1979; Cortesi et al. 2001). Virus transmission was considered successful when the morphology and colour of the recipient isolate took on that of the donor, i.e., unpigmented mycelium. When no change of phenotype was observed in the recipient during the 7-day incubation, we scored a replicate as an unsuccessful transmission.

Data analysis

We compared the percent of successful transmissions for each pair of vc types with published transmission results from Italian isolates. Cortesi et al. (2001) estimated transmission rates as they were affected by specific alleles and combinations of alleles at six *vic* loci, not focusing on specific vc types as we have done in this study. For comparison between studies in Macedonia and Italy, we considered the differences in *vic* genotypes between the donor and recipient isolates (heteroallelic *vic* loci) instead of comparing vc types *per se*. Transmission results in Table 1 of Cortesi et al. (2001) show multiple pairs of Italian

Table 1 Transmission of hypoviruses between vc types of *Cryphonectria parasitica* in Macedonia

Heteroallelic loci[a]	Donor[b]			Recipient[b]			Transmission (%) between Macedonian isolates (95% CI)[c]	Transmission (%) between Italian isolates (range)[d]	Model prediction[e]
	Isolate no.	Vc type	*vic* genotype	Isolate no.	vc type	*vic* genotype			
vic2	Po 72	EU-2	2**1**12-22[f]	G25	EU-1	2**2**12-22	6.8 (3.7, 9.9)	12.7 (5.0, 46.7)	14.9
	G 25	EU-1	2**2**12-22	Po72	EU-2	2**1**12-22	13.6 (9.4, 17.8)	27.9 (5.0, 60.0)	31.1
vic7	Po 72	EU-2	2112-2**2**	Po5	EU-22	2112-2**1**	10.4 (6.6, 14.2)	41.1 (10, 100)	74
	Po 5	EU-22	2112-2**1**	Po72	EU-2	2112-2**2**	100	99.4 (97.5, 100)	98.5
vic1, *vic3*	G 110	EU-10	**2**1**2**2-11	Vr38	EU-12	**1**1**1**2-11	23.6 (18.3, 28.9)	84.1 (60, 100)	92
	Vr 16	EU-12	**1**1**1**2-11	G110	EU-10	**2**1**2**2-11	0.8 (0, 1.9)	11.1 (0, 20)	10.9
vic1, *vic6*	Vr 16	EU-12	**1**112-**1**1	Po5	EU-22	**2**112-**2**1	0	3.3 (0, 10)	0.3
	Po 5	EU-22	**2**112-**2**1	Vr38	EU-12	**1**112-**1**1	2.8 (0.8, 4.8)	5 (0, 10)	17.7
vic2, *vic7*	G 25	EU-1	2**2**12-2**2**	Po5	EU-22	2**1**12-2**1**	0.4 (0, 1.2)	4.4 (0, 6.7)	1.9
	Po 5	EU-22	2**1**12-2**1**	G25	EU-1	2**2**12-2**2**	4.8 (2.2, 7.4)	5.5 (0, 13.3)	14.4
vic3, *vic6*	G 110	EU-10	21**2**2-**1**1	Po5	EU-22	21**1**2-**2**1	0.4 (0, 1.2)	4.4 (0, 6.7)	4.3
	Po 5	EU-22	21**1**2-**2**1	G110	EU-10	21**2**2-**1**1	1.2 (0, 2.5)	4.4 (0, 13.3)	3.8
vic1, *vic6*, *vic7*	Po 72	EU-2	**2**112-**22**	Vr38	EU-12	**1**112-**11**	1.2 (0, 2.5)	nd[g]	2.3
	Vr 16	EU-12	**1**112-**11**	Po72	EU-2	**2**112-**22**	2.4 (0.5, 4.3)	Nd	0.6
Vic3, *vic6*, *vic7*	Po 72	EU-2	21**1**2-**22**	G110	EU-10	21**2**2-**11**	0.8 (0, 1.9)	Nd	0.2
	G 110	EU-10	21**2**2-**11**	Po72	EU-2	21**1**2-**22**	0.4 (0, 1.2)	Nd	4
vic1, *vic2*, *vic6*, *vic7*	G 25	EU-1	**22**12-**22**	Vr38	EU-12	**11**12-**11**	0	Nd	0.2
	Vr 16	EU-12	**11**12-**11**	G25	EU-1	**22**12-**22**	0.4 (0, 1.2)	Nd	0.1
vic2, *vic3*, *vic6*, *vic7*	G 25	EU-1	2**21**2-**22**	G110	EU-10	2**12**2-**11**	0.4 (0, 1.2)	Nd	0.1
	G 110	EU-10	2**12**2-**11**	G25	EU-1	2**21**2-**22**	0	Nd	0.2

[a] *vic* loci that were different between pairs of donor and recipient isolates.

[b] Donor isolates were infected with CHV-1; recipients were virus-free. vc type for each isolate was determined by Sotirovski et al. (2004); *vic* genotypes for these vc types were determined by Cortesi and Milgroom (1998). Note that all isolates were used both as donors and recipients.

[c] Percent of successful transmissions observed from 250 independent replicate pairings of the donor and recipient isolates, with the 95% confidence interval in parentheses.

[d] Percent of successful transmission between Italian isolates that differed by the same *vic* alleles as those in the Macedonian donor and recipient isolates (other *vic* alleles did not differ between donor and recipients but were not necessarily the same as those in the vc types tested in Macedonia). Data were combined from multiple pairs of isolates reported in Table 1 of Cortesi et al. (2001) to calculate an average transmission for each set of *vic* heteroallelic loci. The range of successful transmissions among replicate pairs of Italian isolates is in parentheses.

[e] Percent of successful virus transmission estimated from a regression model described in Cortesi et al. (2001).

[f] *vic* genotypes are abbreviated only by the alleles at each of six loci (Cortesi and Milgroom 1998). For example, vc type EU-2 has *vic* genotype *vic1-2*, *vic2-1*, *vic3-1*, *vic4-2*, –, *vic6-2*, *vic7-2*, which is abbreviated *2112-22*. Note that *vic5*, which was tentatively identified by Huber and Fulbright (1996), and has no effect on virus transmission, cannot be determined by our assay (Cortesi and Milgroom 1998). The allele(s) in bold indicate(s) those that differ between donor and recipient isolates.

[g] Not done by Cortesi et al. (2001).

isolates heteroallelic at the same *vic* loci. For our purposes, we calculated the average transmission rates across multiple pairs between isolates that differed at the same *vic* loci as the vc types in Macedonia; *vic* genotypes and heteroallelic loci for all pairs of vc types in Macedonia are shown in Table 1. We also compared our observed transmission results between vc types in Macedonia with predictions from a regression model based on *vic* genotypes of donors and recipients. This model was constructed using results from transmission experiments between Italian isolates (Cortesi et al. 2001).

The expected probability of transmission was defined as the probability of transmission between two randomly sampled isolates from a population (Milgroom and Cortesi 2004). This was estimated for each population in Macedonia that had more than one vc type (Sotirovski et al. 2004) by calculating the mean transmission probability for all possible pairs ($n \times n$) of donors and recipients among sampled isolates, assuming each isolate was a donor and a recipient. We used the observed transmission frequency between vc types from Table 1 and 100% transmission for pairs of isolates with the same vc type.

Results

Table 1 shows virus transmission results between all pairs of vc types found in Macedonia. Heteroallelism at single *vic* loci (*vic2* and *vic7*) had variable effects on virus transmission. Heteroallelism at *vic2* inhibited transmission to 6.8% in one direction, EU-2 to EU-1, and to 13.6% in the other direction, from EU-1 to EU-2. In contrast, heteroallelism at *vic7* showed marked asymmetry with 10% transmission from vc type EU-2 to EU-22 (allele *vic7-1* in the recipient), but 100% transmission in the opposite direction (allele *vic7-2* in the recipient). Heteroallelism at two or more loci reduced transmission to <3% in most cases, with the exceptions of transmission from EU-10 to EU-12 (23.6%), and from EU-22 to EU-1 (4.8%; Table 1). In both of these cases, higher rates of transmission were associated with asymmetry caused by heteroallelism at *vic1* (*vic1-1* in the recipient) or *vic7* (*vic7-2* in the recipient), respectively, as observed previously (Cortesi et al. 2001). Transmission was consistently 100% between isolates in the same vc type (data not shown).

Observed transmission, based on 250 independent trials for each pair of Macedonian isolates, is correlated with the average transmission observed for multiple pairs of Italian isolates heteroallelic at the same *vic* loci ($r=0.85$, $n=12$; Fig. 1a), but the observed values are typically lower than those in Italy, especially when transmission is intermediate. A similar trend holds when comparing observed transmission in Macedonia with predicted transmission from the regression model ($r=0.77$, $n=20$; Fig. 1b). Neither the observed values of Italian isolates nor those from the regression model, therefore, appear to be good predictors for Macedonian isolates except at the extremes of transmission close to 0 or 100%. However, results from Macedonian isolates are in accord with those from Italy because in all but one comparison (from EU-10 to EU-12), the observed percent transmission was within the range of transmission observed for replicated pairs of vc types heteroallelic at the same *vic* loci in Italy (Table 1).

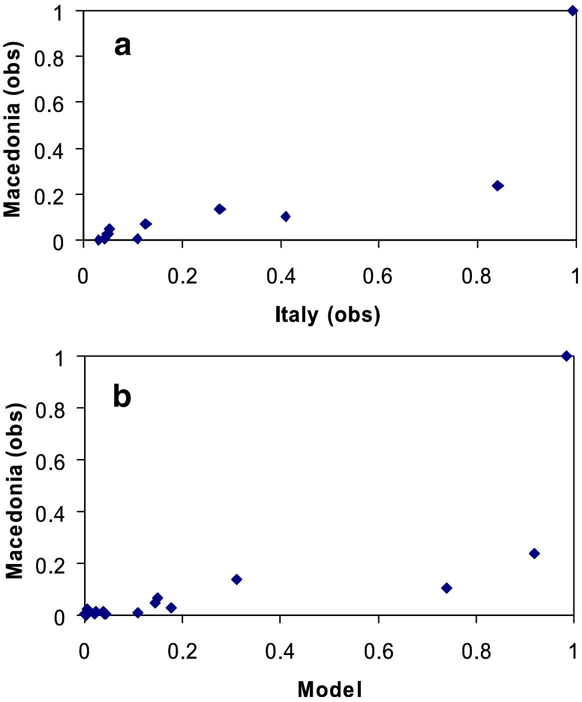

Fig. 1 Observed virus transmission between different vc types of *Cryphonectria parasitica* from Macedonia compared with those between individuals differing at the same *vic* loci from (**a**) Italy and (**b**) model predictions (Cortesi et al. 2001). Each data point for transmission between Macedonian isolates represents the proportion of 250 replicates in which virus was successfully transmitted

The expected probability of transmission for populations in Macedonia were >80% for four populations, but <60% for the two most diverse populations (Table 2). Expected probabilities were assumed to be 100% for 14 populations with only one vc type (Sotirovski et al. 2004). As found with other populations of *C. parasitica* (Milgroom and Cortesi 2004), expected transmission was significantly negatively correlated to vc type diversity ($r=-0.98$, $n=7$; Fig. 2).

Discussion

Virus transmission between vc types of *C. parasitica* in Macedonia was highly variable, with high transmission between some vc types, and no transmission between others. Transmission was also asymmetric between some vc types, depending on which isolate was the donor and which was the recipient. Such

Table 2 Distribution of vc types (Sotirovski et al. 2004) and expected probabilities of transmission for populations of *Cryphonectria parasitica* in Macedonia with more than one vc type

Region	Population	Number	vc type[a]					Expected probability[b]	vc type diversity (H')[c]
			EU-12	EU-1	EU-2	EU-10	EU-22		
Tetovo	Glogi	46	34	8	0	1	3	0.579	0.789
	Poroj	63	59	0	1	0	3	0.880	0.272
	Vratnica	49	46	0	0	0	3	0.884	0.230
Gostivar	Galate	57	52	0	4	0	1	0.839	0.341
	Vrutok	44	43	0	1	0	0	0.955	0.109
Kichevo	Osoj	72	49	1	22	0	0	0.556	0.684

[a] Distribution of vc types from Sotirovski et al. (2004). Only populations with two or more vc types are shown.

[b] Expected probability is the probability of transmission between two randomly selected individuals from each population. See text for details.

[c] Shannon–Weiner diversity estimates from Sotirovski et al. (2004); $H' = -\sum_i [p_i \cdot \ln(p_i)]$, where p_i is the frequency of the ith vc type.

marked asymmetry was particularly evident with heteroallelism at locus *vic7*, although it was also associated with *vic1*, as found previously (Cortesi et al. 2001). In general, these results are similar to those found for virus transmission among Italian isolates in different vc types (Cortesi et al. 2001), although estimates of transmission between isolates from Macedonia are generally lower, especially when transmission was intermediate. Therefore, results from Italy, from observed transmission and model predictions, were qualitatively similar but did not predict transmission between vc types well for Macedonian populations.

Regardless of any quantitative differences in transmission between this study and that of Cortesi et al. (2001), the same overall relationship was found for the correlation of expected transmission and vc type diversity (Fig. 2; Milgroom and Cortesi 2004). The expected transmission is the estimated probability of transmission between any two randomly chosen individuals in a population, and, therefore, should be a more precise predictor of the ability of viruses to spread within a population than vc type diversity alone. However, the high correlation between vc type diversity and expected transmission (r=−0.98) could also be interpreted as meaning that vc type diversity is an adequate predictor for potential virus transmission, and that intimate knowledge of *vic* genotypes is unnecessary if virus transmission can be estimated for all possible combinations of vc types. Complete evaluation of virus transmission among all combinations is possible in Macedonia because only five vc types were found throughout the country in an extensive survey (Sotirovski et al. 2004). Such a complete analysis would not be feasible in other parts of Europe or in North America where vc type diversity is greater (Milgroom and Cortesi 1999; Robin et al. 2000). In these cases, the regression model proposed by Cortesi et al. (2001) may be helpful even though it does not predict well for all pairs of vc types.

We are confident that our estimates of transmission probabilities between vc types in Macedonia are robust for laboratory conditions because each estimate is based on 250 independent replicate trials. This large number of replicates allows us to make accurate estimates, with relatively small 95% confidence intervals (Table 1). This number of replications is

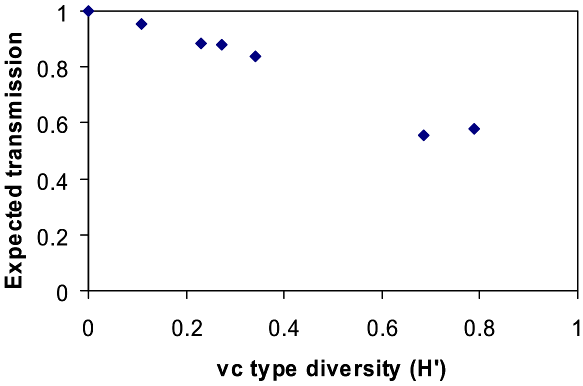

Fig. 2 Expected virus transmission in Macedonian populations as a function of vc type diversity (Sotirovski et al. 2004). Expected transmission is the probability of transmission between two randomly sampled individuals in a population and was calculated as described by Milgroom and Cortesi (2004; see text and Table 2)

useful for estimating rare events. For example, we found <1% transmission in 10 of the 20 combinations of donors and recipients; only three showed no transmission at all in 250 replicates.

Inherent in making inferences to the population level is the assumption that laboratory assays are good predictors of virus transmission under field conditions. Few studies have addressed this issue directly, although transmission appears to occur at higher rates in the field than in the laboratory (Bisiach et al. 1988). For example, some chestnut blight cankers were controlled by hypovirulence under field conditions even though virus transmission did not occur between the same two isolates in the laboratory (Double 1982). Carbone et al. (2004) analyzed nucleotide sequence variation in CHV-1 to estimate migration rates between dominant vc types in two populations of *C. parasitica* in Italy as a way of assessing how much horizontal transmission had occurred in nature. In general, they found that significant amounts of migration had occurred despite the low probabilities of transmission predicted from laboratory studies. They concluded that the long-term dynamics of transmission (estimated by migration rates) were better estimates of virus spread within a population because laboratory assays involve only short-term interactions between isolates, in artificial environments. Therefore, based on these previous studies, it appears that laboratory assays may underestimate horizontal virus transmission.

The consistent underestimate of transmission between Macedonian isolates compared to those in Italy raises an interesting hypothesis. Genes other than *vic* may be causing some effect on virus transmission, and these genes differ in Macedonia and Italy. Such genetic background effects were shown to be large between replicate pairs of vc types in Italy that were heteroallelic at the same *vic* loci. In fact, transmission estimates for Macedonia were almost all within the range of transmission observed among replicate pairs in Italy that differed at the same *vic* loci, making the case that estimates for Macedonia are within a normal range, and not unusually low. Further genetic analysis may be needed to test how much heritable effect there may be from background genes in *C. parasitica* affecting virus transmission.

CHV-1 has been found in several populations of *C. parasitica* in western Macedonia (Sotirovski et al. 2006). Based on the low diversity of vc types found in Macedonian populations of *C. parasitica*, coupled with estimates of virus transmission among vc types, we predict that CHV-1 will continue to spread and increase in incidence in the coming years, and that hypovirulence may eventually be a significant factor in the recovery of some chestnut stands.

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