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Population Biology

Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility genotypes

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ABSTRACT **Vegetative incompatibility is a self/nonself-recognition system in fungi that has often been used for describing phenotypic diversity in fungal populations. A common hypothesis is that vegetative incompatibility polymorphisms are maintained by balancing selection. However, understanding the evolutionary significance of vegetative incompatibility and the factors that maintain these polymorphisms has been limited by a lack of knowledge of the underlying genetics of vegetative compatibility (vc) types. Genotypes of 64 vc types, controlled by six unlinked vegetative incompatibility (*vic*) loci, have been identified in the chestnut blight fungus, *Cryphonectria parasitica*. By interpreting vc type survey data in terms of *vic* genotypes, we estimated *vic*-allele frequencies and analyzed the multilocus genetic structure of 13 populations in Europe and 3 populations in the U.S. European populations have less vc type diversity than the US populations because of a combination of lower *vic*-allele diversity and limited recombination. Genotypic diversity of 10 populations in Italy correlated to the abundance of sexual structures; however, significant deviations from random mating suggest that either sexual reproduction may not contribute many offspring in these populations or that *vic* genes (or *vic* genotypes) are under selection. Most *vic*-allele frequencies deviated from 0.5, the equilibrium frequency predicted under frequency-dependent selection, providing no evidence for selection acting on these loci.**

Vegetative incompatibility (also called heterokaryon or somatic incompatibility) is a self/nonself-recognition system in fungi that has been used extensively to describe fungal population structure and diversity (reviewed in refs. 1 and 2). The extent of polymorphism in vegetative incompatibility phenotypes and the relative ease with which they can be assayed have made these phenotypes popular for studying fungal populations. Nonetheless, the evolutionary significance of vegetative incompatibility and the factors maintaining these polymorphisms remain largely unknown. One hypothesis is that these polymorphisms are maintained by balancing selection because vegetative incompatibility restricts the transmission of parasitic nuclei or deleterious cytoplasmic elements such as viruses, plasmids, or debilitated organelles (3–6). Testing this hypothesis requires estimates of allele frequencies at vegetative incompatibility (*vic*) loci (or *het*, for heterokaryon incompatibility) or studies of *vic* gene genealogies. This approach has been applied in *Neurospora* where trans-species polymorphisms and intermediate allele frequencies at one *het* locus suggest that vegetative incompatibility may be under balancing selection (7).

Population-genetic and evolutionary inferences have been limited in studies of vegetative incompatibility because the genotypes underlying these phenotypes are generally not known. In most ascomycetes, vegetative incompatibility is controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at all *vic* loci (1, 2, 8). Conversely, individuals are vegetatively incompatible when alleles are different at one or more *vic* loci. Thus, vegetative incompatibility phenotypes, referred to as vegetative compatibility (vc) types, are genetically defined by the alleles at multiple *vic* loci, which collectively define the *vic* genotype. The number of known *vic* loci varies among species. For example, 17 *vic* loci have been identified in *Podospora anserina*, and 11 have been found in *Neurospora crassa* (reviewed in refs. 1, 2, and 8). In addition, segregation of large numbers of vc types in laboratory crosses has indicated polymorphisms at multiple *vic* loci in fungi for which detailed genetic analyses have not been done (e.g., ref. 9). Most *vic* loci have only two alleles, although multiple alleles have been found in some species (10, 11).

The diversity of vc types (*vic* genotypes) in a population is a function of allelic diversity and recombination among *vic* loci. Because ascomycetes are haploid, 2^k multilocus *vic* genotypes are possible, given two alleles at each of k polymorphic, unlinked *vic* loci. Therefore, potential genotypic diversity increases as k and allelic diversity increase. If *vic* genotypes were known for vc types, estimates of k and allelic diversity would be possible. Furthermore, inferences could be made from vc type survey data about recombination or clonality in natural populations (12, 13). Until recently, there had been no attempt to assign *vic* genotypes to vc types except for a few laboratory strains. Cortesi and Milgroom (14) determined *vic* genotypes for 64 ($= 2^6$) vc types, controlled by six unlinked *vic* loci, each with two alleles, in the chestnut blight fungus, *Cryphonectria parasitica*. Because all vc types of *C. parasitica* reported from Italy and Switzerland (15) are among these 64 vc types, analyses of population structure in these countries are possible simply by converting vc type survey data to *vic*-allele and -genotype frequencies.

Two questions about the population biology of *C. parasitica* were addressed with *vic* genotype data. The first question is, why is vc type diversity lower in Europe than in North America (16, 17)? The two simplest hypotheses are that (*i*) founder effects resulted in fewer polymorphic *vic* loci and/or less *vic*-allele diversity in Europe and (*ii*) restricted recombination in European populations keeps *vic*-genotype diversity low. *C. parasitica* is native to east Asia and was introduced into North America and Europe, most likely from Japan (18). Restriction fragment-length polymorphism diversity in North America is lower than in Asia because of founder effects (19). By chance, introductions also could have resulted in different, and possibly lower, *vic*-allele diversity in Europe than in North America. Alternatively, *vic*-genotype diversity may be lower in Europe because of less recombination even if *vic*-allele diversities are comparable in Europe and North America. *C. parasitica* can reproduce asexually or sexually either by outcrossing or selfing (20, 21). Analysis of the genetic structure of

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Abbreviation: vc type, vegetative-compatibility type.

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one population of *C. parasitica* in Italy by using DNA fingerprinting showed that it is partially clonal, with evidence for some recombination (22). In contrast, populations in the eastern U.S. appear to be closer to panmixis (22, 23). By combining knowledge of *vic* genotypes and vc type surveys, we can analyze the population structure of additional populations.

The second question we addressed with *C. parasitica* is whether *vic* loci are under balancing selection. Hypoviruses in *C. parasitica* (24) may be exerting frequency-dependent selection on vegetative incompatibility. Hypovirus infection reduces the fitness of *C. parasitica*, thereby effecting biological control of chestnut blight (25–27). More importantly, virus transmission is restricted between vc types (28–30), such that rare vc types may have a selective advantage by escaping virus infection. We predict that, if frequency-dependent selection acts on *vic* genes, allele frequencies should be intermediate, and populations should approach their maximum potential vc type diversities as they approach equilibrium (4, 5).

The objective of this study was to test the following predictions about *C. parasitica.* (*i*) *vic*-allele diversities are lower in Europe than in North America; (*ii*) populations in Europe deviate from random mating; and (*iii*) *vic* alleles are at intermediate frequencies because of frequency-dependent selection.

MATERIALS AND METHODS

Analyses of population structure in Europe were based on vc type survey data from 11 populations in Italy (17) and two in Switzerland (15, 31). At least 46 individuals were sampled in all populations, except for Tonara, Italy, where only 33 were available; larger samples ($n > 100$) were analyzed in three populations in Italy. In Teano, Italy, we initially sampled 50 individuals (17) but later increased the sample size to 194 (22); vc type data for the expanded sample are presented in this report. Samples from three populations in the U.S. [Depot Hill, NY and Finzel, MD, denoted DU-90 and MD, respectively, in ref. 32, and Bartow, WV (33)] were assayed for vc types (see below). We defined a fungal individual as an isolate from a single chestnut-blight canker. All samples were collected without regard to virus infection. To avoid sampling clones on the same trees (32, 34), we sampled one isolate from each tree in Italy and in West Virginia. In Maryland, 57 isolates were collected from 48 trees; in New York, 60 isolates were collected from 32 trees. Including DNA fingerprint clones from the same trees (32) did not significantly affect any analysis in the present study, therefore, all isolates from Maryland and New York were used.

The *vic* genotypes for all vc types found in Italy and Switzerland were determined previously (14). We determined the *vic* genotypes for isolates in the U.S. by testing for vegetative compatibility with the 64 testers from Europe (14). Vegetative-compatibility testing was done by pairing isolates on agar medium amended with bromocresol green pH indicator dye, as described (14, 17). Pairs of isolates that formed a zone of dead cells (barrage) or discoloration in the medium between colonies were considered incompatible. Vegetative incompatibility assayed this way has been shown to correspond to heterokaryon incompatibility (35). Isolates from the U.S. that were compatible with European testers were assumed to have the same *vic* genotypes. This assumption is based on finding the predicted recombinant *vic* genotypes in a cross between isolates from Italy and Japan (14), showing that vc types on two continents have the same underlying genetic control. Isolates not compatible with any genotype tester were omitted from further analyses.

Allelic diversity was estimated for each *vic* locus as $\hat{h} = n(1 - \hat{p}^2 - \hat{q}^2)/(n - 1)$, where $\hat{p}$ is the estimated frequency of allele 1, $\hat{q} = 1 - \hat{p}$ is the estimated frequency of allele 2, and n is the sample size. Mean allelic diversity, $\hat{H}$, is the average of $\hat{h}$ over all six *vic* loci. Mean allelic diversities estimated in European populations were compared with $\hat{H}$ estimated from U.S. populations by a one-sided Mann–Whitney U test; this test was used instead of a t test because of unequal variances. In addition, allelic diversities were partitioned within and between European populations by estimating G_{ST}, the proportion of diversity attributed to differences between populations (36) with corrections for small and unequal sample sizes (37).

Multilocus genetic structure was analyzed by using three different tests. For each population, we estimated (*i*) the number of *vic* genotypes (N_{vc}), (*ii*) genotypic diversity ($\hat{G}$) (38, 39), and (*iii*) the index of association (I_A), a multilocus measure of gametic disequilibrium (40, 41). These estimates were compared with expectations under the null hypothesis of random mating. Given that the *vic* loci we examined are unlinked (14), null distributions of *vic*-genotype frequencies for each population were determined by randomly assigning *vic* alleles at each locus. Alleles were assigned to each of n individuals based on the observed allele frequencies in each population or by randomly permuting observed alleles at each locus. Loci with allele frequencies <0.05 and >0.95 were not used in these analyses. Estimates for the three parameters were calculated for each of 1,000 randomizations to generate null distributions. Expected N_{vc} and $\hat{G}$ were estimated as the median and mean of the null distributions, respectively. I_A was calculated as $s_k^2/\sigma_k^2 - 1$, where s_k^2 and σ_k^2 are the observed and expected variances in k, respectively, and k is the number of loci at which a pair of individuals had different alleles. The observed variance in k was calculated for all pairwise comparisons among individuals in a sample (40, 41). The expected variance, σ_k^2, was estimated as the mean from the null distribution of variances calculated for each of 1,000 randomizations. P values were estimated as the proportion of the null distribution that was less than or equal to the observed parameter estimate for N_{vc} and $\hat{G}$ and greater than or equal to s_k^2 for I_A.

To study the reproductive biology of *C. parasitica* in relation to deviations from random mating, we surveyed 10 populations in Italy for the proportion of chestnut blight cankers with sexual structures (perithecia). Perithecia are easily visible in fungal stromata on chestnut-bark samples with a dissecting microscope. In addition, we tested the mating type of 50 isolates from Zafferana, Italy, because no perithecia were observed in this population. Mating types were determined by making crosses with tester isolates as described (22).

Allele frequencies were tested for differences from 0.5 by calculating 99% confidence intervals: $\hat{p} \pm 2.58\sqrt{\hat{p}\hat{q}/n} + 1/2n$, where $1/2n$ is a correction for continuity. If the confidence interval did not include 0.5, the frequency was considered different from 0.5 at the 1% level of significance.

RESULTS

We determined vc types of additional isolates in Teano, Italy, and for samples in the U.S. In Teano, we found 8 vc types among 194 isolates, although 6 vc types were rare (Table 1). One vc type, EU-40, had not been found previously in the field in Europe (15). In the U.S., we found 26, 30, and 34 vc types among 57, 59 and 60 isolates from Maryland, West Virginia, and New York, respectively. In the U.S. samples, only 23, 22, and 20 vc types, respectively, were compatible with European testers and could be assigned *vic* genotypes (Table 1). Therefore, 3, 9, and 23 isolates, respectively, had vc types whose *vic* genotypes are unknown; these isolates were omitted from further analyses.

Allele frequencies at six *vic* loci were estimated for all populations (Table 2). Seven of 13 European populations had five polymorphic *vic* loci, whereas the others had fewer. In Europe, *vic3* was only polymorphic in two populations in southern Italy (Teano and Cittanova). In contrast, all six *vic*

Table 1. Vegetative compatibility type distribution in *C. parasitica* from Teano, Italy and three populations in the U.S.

EU type*	Teano, Italy	Finzel, MD	Bartow, WV	Depot Hill, NY
EU-1	0	1	2	1
EU-2	1	0	0	0
EU-3	0	1	0	8
EU-5	1	2	0	0
EU-7	0	2	1	0
EU-8	0	1	1	0
EU-9	0	0	1	1
EU-10	36	1	8	1
EU-11	1	6	2	2
EU-12	151	2	0	2
EU-13	0	1	2	2
EU-14	1	1	0	2
EU-15	0	3	3	1
EU-17	2	7	3	4
EU-18	0	0	1	1
EU-19	0	0	1	0
EU-21	0	0	0	2
EU-22	0	1	2	0
EU-24	0	1	0	0
EU-25	0	3	0	0
EU-26	0	1	0	0
EU-28	0	9	7	1
EU-32	0	0	0	1
EU-33	0	1	4	2
EU-34	0	1	2	0
EU-35	0	0	1	0
EU-36	0	3	1	1
EU-39	0	1	0	1
EU-40	1	3	1	1
EU-41	0	0	1	0
EU-43	0	0	2	0
EU-45	0	2	0	1
EU-46	0	0	3	0
EU-47	0	0	1	0
EU-51	0	0	0	2
n	194	57	59	60
EU types, no.	8	23	22	20
Non-EU types, no.	0	3	8	14

*EU nomenclature for vc types was defined by Cortesi *et al*. (15).

loci were polymorphic in the three U.S. populations. Mean *vic* allele diversities ranged from 0.11 to 0.30 in Europe and from 0.36 to 0.38 in the U.S. (Table 2). Allelic diversity in Europe was significantly lower than in the U.S. for all six *vic* loci ($P = 0.005$) and when data for *vic3* were omitted ($P = 0.01$). Approximately 35% of the total allelic diversity in Europe was because of differentiation among populations ($G_{ST} = 0.35$, Table 3). Most of the differentiation among populations can be attributed to the populations in southern Italy (Teano, Cittanova, and Zafferana) that have significantly different vc type distributions from the other populations (17). Differentiation among the other 10 European populations was much lower than for all 13 populations ($G_{ST} = 0.07$, Table 3).

Analyses of multilocus genetic structure of *vic* genotypes showed that all populations in Europe deviate significantly from random mating for one or more analyses (Table 4). Only 6 of the 13 European populations had significantly ($P < 0.05$) fewer vc types present than would be expected for the observed *vic*-allele frequencies under random mating. However, 10 populations had significantly less genotypic diversity than expected under random mating; 2 more were marginally significant ($P < 0.08$). Finally, all European populations showed highly significant gametic disequilibrium, as estimated by I_A. A notable example of deviation from random expectations is in Teano, where there are two dominant vc types, EU-10 and EU-12 (Table 1), that differ by two *vic* genes (14). In this sample ($n = 194$), we found only two isolates in recombinant vc type EU-17 and one isolate in recombinant vc type EU-40. However, 32 and 28 isolates would have been expected in EU-17 and EU-40, respectively, under random mating. In contrast, none of the U.S. populations deviated from random mating for any analysis (Table 4).

Deviations from random mating could be caused by a number of factors, including limited sexual reproduction. However, we found sexual structures (perithecia) of *C. parasitica* in 9 of the 10 populations in Italy and in both populations examined in the U.S. (Table 4). Genotypic diversity in Italy was positively correlated ($r = 0.66$, $P = 0.04$) to the proportion of individuals (cankers) that had perithecia (Fig. 1); proportions of cankers with perithecia were not significantly associated with I_A ($r = -0.43$, $P = 0.22$). Only one mating type (*MAT*-1) was found among 49 isolates from Zafferana where no perithecia were found (one isolate did not mate with either tester), suggesting that sexual reproduction is not possible in this population. Furthermore, Zafferana had the lowest genotypic diversity of any population analyzed (Table 4).

Most *vic*-allele frequencies are significantly different from 0.5 (Table 2), the equilibrium frequency expected under frequency-dependent selection. However, six European populations and all three U.S. populations had allele frequencies at *vic2* that were not different from 0.5.

DISCUSSION

A combination of lower *vic*-allele diversity and limited recombination explains the lower vc type diversities observed in European populations of *C. parasitica* compared with North America. This analysis was possible only because we previously determined *vic* genotypes for 64 vc types (14), which include all vc types found in Italy and Switzerland (15) and the majority of those found in the U.S. (Table 1). We know of no other fungus for which extensive data are available for *vic* genotypes. By analyzing *vic*-allele and -genotype frequencies instead of frequencies of vc types (phenotypes), we could address questions about population structure and the evolution of vegetative incompatibility that were not previously possible from vc type survey data.

Populations of *C. parasitica* in Europe have fewer polymorphic *vic* loci and lower *vic*-allele diversity than populations in the U.S., despite the fact that a substantial number of vc types in the U.S. do not have known *vic* genotypes. Estimates of *vic*-allele frequencies in U.S. populations were made with the assumption that omitting vc types with unknown genotypes would not introduce excessive bias. vc types with unknown *vic* genotypes could be the result of polymorphism at additional *vic* loci and/or multiple alleles at one of the six known *vic* loci. If additional polymorphic *vic* loci are unlinked to known *vic* loci, then they should not bias allele frequency estimates (assuming random mating). Multiple alleles at one or more of the six known *vic* loci, however, would cause allelic diversity to be underestimated. Nonetheless, *vic*-allele diversity was significantly greater in the U.S. than in Europe.

Inferences on the reproductive biology of *C. parasitica* have been made previously from studies of vc type diversity. Increases in vc type diversity (42, 43) and lack of spatial autocorrelation of vc types between trees (34) have been interpreted as evidence for the prevalence of sexual reproduction in U.S. populations. These conclusions were later corroborated by more detailed analyses of population structure by using DNA fingerprinting, confirming the prevalence of recombination (22, 23). Our studies of *vic* genotypes now provide an extensive analysis of the multilocus population structure of *C. parasitica*, showing clearly that recombination is suppressed to some extent in all populations studied in Europe but not in populations in the U.S.

Table 2. Allele frequencies, number of polymorphic loci, and mean allelic diversities at six *vic* loci in populations of *C. parasitica*

		vic1†		*vic2*		*vic3*		*vic4*		*vic6*		*vic7*		Polymorphic	
Population*	*n*	1	2	1	2	1	2	1	2	1	2	1	2	*loci*, no.‡	$\hat{H}$§
Italy															
Donnaz, AO	50	0.00	1.00	0.60¶	0.40	1.00	0.00	0.08	0.92	0.02	0.98	0.00	1.00	2	0.11
Crevoladossola, VB	131	0.02	0.98	0.29	0.71	1.00	0.00	0.44	0.56	0.12	0.88	0.06	0.94	4	0.21
Ponte in Valtellina, SO	46	0.09	0.91	0.59	0.41	1.00	0.00	0.20	0.80	0.09	0.91	0.13	0.87	5	0.22
Bergamo, BG	158	0.09	0.91	0.50	0.50	1.00	0.00	0.18	0.82	0.08	0.92	0.08	0.92	5	0.21
Pigna, IM	48	0.04	0.96	0.77	0.23	1.00	0.00	0.17	0.83	0.02	0.98	0.02	0.98	2	0.13
Corniglio, PR	50	0.20	0.80	0.72	0.28	1.00	0.00	0.24	0.76	0.20	0.80	0.20	0.80	5	0.29
Pomina, FI	50	0.08	0.92	0.60	0.40	1.00	0.00	0.08	0.92	0.14	0.86	0.08	0.92	5	0.19
Tonara, NU	33	0.15	0.85	0.88	0.12	1.00	0.00	0.09	0.91	0.18	0.82	0.18	0.82	5	0.21
Teano, CE	194	0.79	0.21	0.99	0.01	0.81	0.19	0.01	0.99	0.99	0.01	0.99	0.01	2	0.12
Cittanova, RC	50	0.02	0.98	0.24	0.76	0.78	0.22	0.00	1.00	0.24	0.76	0.26	0.74	4	0.25
Zafferana, CT	50	0.86	0.14	1.00	0.00	1.00	0.00	0.00	1.00	0.86	0.14	0.86	0.14	3	0.12
Switzerland															
Lumino	86	0.06	0.94	0.41	0.59	1.00	0.00	0.56	0.44	0.12	0.88	0.13	0.87	5	0.25
Gnosca	61	0.16	0.84	0.62	0.38	1.00	0.00	0.34	0.66	0.16	0.84	0.23	0.77	5	0.30
USA															
Finzel, MD	54	0.39	0.61	0.39	0.61	0.78	0.22	0.20	0.80	0.78	0.22	0.87	0.13	6	0.36
Bartow, WV	50	0.24	0.76	0.42	0.58	0.56	0.44	0.32	0.68	0.76	0.24	0.94	0.06	6	0.38
Depot Hill, NY	37	0.22	0.78	0.35	0.65	0.76	0.24	0.30	0.70	0.92	0.08	0.57	0.43	6	0.37

*vc type data are from Cortesi *et al*. (15, 17), Bissegger *et al*. (31), and Table 1.
†*vic* genotypes were determined for EU types by Cortesi and Milgroom (14); "1" and "2" refer to the two alleles found at each *vic* locus.
‡A locus is considered polymorphic if the frequency of the less common allele is >0.05.
§Allelic diversity was calculated for each locus according to Nei (36) as $\hat{h} = n\,(1 - \hat{p}^2 - \hat{q}^2)/(n - 1)$, where $\hat{p}$ is the frequency of allele 1, $\hat{q} = 1 - \hat{p}$ and n is the sample size. Mean diversity ($\hat{H}$) is the average of $\hat{h}$ over all six loci.
¶Underline indicates allele frequencies not significantly different from 0.5 ($\alpha = 0.01$).

Although 64 *vic* genotypes were found in laboratory crosses from field isolates collected in Europe (14), only 31 were found in the field (15), suggesting a marked lack of recombination. However, genetic differentiation among populations is another contributing factor, especially because allele *vic3-2* was only found in two populations in southern Italy (Table 2). Even if populations were freely recombining, most populations would only be expected to have 16 or fewer vc types for the observed allele frequencies and sample sizes in this study (Table 4). Strong geographic differentiation ($G_{ST} \approx 0.35$) indicates that little gene flow occurs among subpopulations, at least between southern Italy and subpopulations further north. Alternatively, subdivision could be caused by differences in selection on *vic* genes in different populations, but a mechanism to explain such selection is not clear. More likely, marked subdivision was caused by founder effects and has persisted because of restricted gene flow.

Sexual structures (perithecia) have been reported previously in populations of *C. parasitica* in Europe (reviewed in ref. 27) but were not described quantitatively. In this study, we found perithecia in 0–86% of the cankers in 10 populations in Italy; 83% of the cankers in both populations in the U.S. had perithecia. Only one mating type was present in Zafferana, the only population where perithecia were not found. Lack of a second mating type appears to prevent sexual reproduction in this population. Populations in Italy with more perithecia tended to have greater genotypic diversity (Fig. 1). However, *C. parasitica* may also reproduce asexually, and a significant proportion of perithecia may result from self-fertilization, which produce offspring genetically identical to their parents (20, 21). Both asexual reproduction and selfing can contribute to deviations from random mating.

The biological significance of outcrossed sexual offspring may not be accurately reflected by the occurrence of sexual structures. In theory, it takes relatively little recombination to give a population the appearance of panmixis (44–46). Therefore, even if ≈70% of the perithecia in Italy are the result of outcrossing (unpublished results), there should be sufficient recombination to result in panmixis if sexual reproduction is a significant mode of reproduction in these populations.

An alternative explanation for deviations from random mating is that *vic* genes are under selection. Selection may cause gametic disequilibrium among *vic* genes if some vc types have greater fitness than others. Hypoviruses are possible selective agents in *C. parasitica* because infected individuals do not produce perithecia, and asexual sporulation may be nearly completely inhibited (26, 47). *vic* genes restrict horizontal transmission of viruses between vc types (28–30), which could

Table 3. Gene diversity analysis for six *vic* loci in European populations of *C. parasitica*

	All 13 European populations			Southern Italian populations omitted		
Locus	H_S	H_T	G_{ST}	H_S	H_T	G_{ST}
vic1	0.17	0.32	0.46	0.158	0.163	0.03
vic2	0.36	0.47	0.22	0.435	0.482	0.10
vic3	0.05	0.06	0.17	0.000	0.000	—
vic4	0.25	0.30	0.17	0.321	0.363	0.12
vic6	0.20	0.37	0.46	0.197	0.200	0.02
vic7	0.20	0.37	0.47	0.191	0.198	0.04
All loci	0.21	0.31	0.35	0.217	0.234	0.07

Gene diversity $G_{ST} = (H_T - H_S)/H_T$, where H_S is the mean *vic* allele diversity within subpopulations and H_T is the total allelic diversity over all subpopulations (36, 37).

Table 4. Analyses of multilocus population structure in *C. parasitica* based on *vic* genotypes

Population	*n*	No. of polymorphic loci*	No. of *vic* genotypes (N_{vc})†			Genotypic diversity ($\hat{G}$)‡				Index of association (I_A)§				Cankers with perithecia¶	
			Observed	Expected	*P*	$\hat{G}_{obs}$	$\hat{G}_{exp}$	%	*P*	s_k^2	σ_k^2	I_A	*P*	*n*	%
Italy															
Donnaz, AO	50	2	4	4	0.757	1.32	2.23	59	<0.001	0.43	0.38	0.14	<0.001	23	77
Crevoladossola, VB	131	4	10	12	0.126	3.66	4.73	77	0.005	0.94	0.76	0.23	<0.001	46	100
Ponte in Valtellina, SO	46	5	8	12	0.028	2.79	4.99	56	0.003	2.08	0.91	1.29	<0.001	86	50
Bergamo, BG	158	5	16	17	0.500	3.96	4.59	86	0.074	1.14	0.84	0.35	<0.001	49	100
Pigna, IM	48	2	4	4	0.665	1.79	2.14	83	0.128	0.62	0.43	0.44	<0.001	58	57
Corniglio, PR	50	5	12	16	0.022	1.56	7.61	20	<0.001	4.94	1.13	3.39	<0.001	25	55
Pomina, FI	50	5	7	11	0.024	2.99	3.95	76	0.067	1.49	0.81	0.84	<0.001	48	50
Tonara, NU	33	5	5	10	<0.001	1.69	4.04	42	<0.001	3.00	0.92	2.28	<0.001	—‖	
Teano, CE	194	2	4	4	0.593	0.80	2.15	37	<0.001	0.82	0.43	0.89	<0.001	36	77
Cittanova, RC	50	4	4	12	<0.001	1.68	5.78	29	<0.001	3.50	0.93	2.78	<0.001	20	82
Zafferana, CT	50	3	2	6	<0.001	0.10	2.28	4	<0.001	1.67	0.55	2.04	<0.001	0	74
Switzerland															
Lumino	86	5	14	16	0.269	4.82	6.56	74	0.010	1.23	0.93	0.32	<0.001	—	
Gnosca	61	5	15	18	0.109	5.77	9.04	64	0.010	1.63	1.12	0.45	<0.001	—	
U.S.															
Finzel, MD	54	6	23	23	0.525	12.68	13.18	96	0.442	1.47	1.35	0.09	0.132	—	
Bartow, WV	50	6	22	22	0.503	13.16	14.40	91	0.316	1.42	1.31	0.09	0.073	83	78
Depot Hill, NY	37	6	19	19	0.575	11.31	12.55	90	0.335	1.27	1.33	−0.04	0.729	83	72

*A locus was considered polymorphic for these analyses if the frequency of the less common allele was >0.05.
†Number of *vic* genotypes is based only on polymorphic *vic* loci (as defined above).
‡Genotypic diversity $\hat{G}$ was calculated as $1/\Sigma p_1^2$, where p_i is the frequency of the *i*th genotype (38, 39). Expected values were estimated as the mean of the null distribution generated from 1,000 randomizations under the assumption of random mating. Percentage of the expected genotypic diversity observed was calculated as $100 \times G_{obs}/G_{exp}$. *P* values were estimated as the proportion of the null distribution with values of *G* greater than equal to the observed diversity (see text).
§Index of association (I_A) was estimated as $s_k^2/\sigma_k^2 - 1$, where s_k^2 and σ_k^2 are the observed and expected variances in *k*, the number of heterozygous loci between pairs of individuals (40, 41). *P* values were estimated as the proportion of the null distribution with values of s_k^2 greater than equal to the observed variance (see text).
¶Percent of cankers with sexual structures (perithecia) of *C. parasitica* were determined for 10 populations in Italy. *n* are the number of canker samples examined.
‖No data available for these populations.

result in frequency-dependent selection where rare vc types are favored compared with more common ones (4, 5). Although we would expect to find intermediate *vic*-allele frequencies under equilibrium conditions, few observed frequencies were close to 0.5. In *N. crassa*, Wu *et al.* (7) found three alleles at the *het-c* locus, all in roughly equal frequencies within a single population. However, in *C. parasitica*, allele frequencies do not support the hypothesis of frequency-dependent selection on *vic* loci.

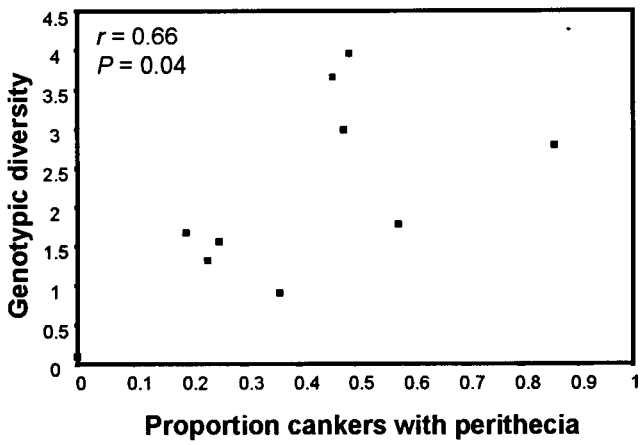

FIG. 1. Correlation between genotypic diversity and the proportions of cankers caused by *C. parasitica* with sexual structures (perithecia) in 10 Italian populations ($r = 0.66$, $P = 0.04$).

Rejection of the frequency-dependent-selection hypothesis may have occurred for several reasons. First, *vic*-allele frequencies may not yet have reached equilibrium since *C. parasitica* was introduced into Europe and North America ≈60 and 100 years ago, respectively (18, 27). Approximately one generation occurs in *C. parasitica* per year; therefore, relatively few generations have occurred since introduction. Second, under some conditions, polymorphisms at *vic* loci may be maintained by selection even though allele frequencies may not reach stable equilibria (4), e.g., stable oscillations of *vic*-allele frequencies are theoretically possible because of frequency-dependent selection (5). In this situation, a static sample of allele frequencies is not a sufficient test for frequency-dependent selection. However, the period of oscillations may be too long, making empirical observation impractical. A third reason that we might have failed to support the balancing-selection hypothesis is because populations in Europe are partially clonal. In clonal populations, viruses may not necessarily select for intermediate allele frequencies at individual *vic* loci because the entire *vic* genotype is under selection as a unit. Selection by viruses could theoretically result in approximately equal vc type frequencies (5), although the uneven vc type distributions observed in Europe do not support this hypothesis (15, 17, 31). Even in European populations with the greatest genotypic diversity (e.g., Swiss populations and Bergamo, Italy)—presumably because of recombination—allele frequencies deviate from 0.5. Furthermore, selection on clones of *C. parasitica*, independent of selection on *vic* genotypes, also may alter *vic*-genotype frequencies by hitchhiking selection.

Alternatively, lack of support for frequency-dependent selection may indicate that selection on *vic* genes is weak, perhaps because inhibition of virus transmission between individuals by vegetative incompatibility is incomplete (28–30). Therefore, viruses may be transmitted between vc types often enough to obviate frequency-dependent selection. Furthermore, predictions that all *vic*-allele frequencies should be intermediate under frequency-dependent selection may be too simplistic. Alleles at some *vic* loci have no effect on virus transmission, whereas others have intermediate or strong effects (unpublished data). Interestingly, alleles at *vic2* strongly restrict virus transmission in the laboratory (unpublished results) and are found at intermediate frequencies in nine populations (Table 2), suggesting that frequency-dependent selection may be operating at this locus. More detailed analyses of effects of *vic* genes on virus transmission in the laboratory and the field are needed before the factors affecting *vic*-gene polymorphisms can be fully understood.

Understanding the dynamic interaction of virus and fungus populations has been a major motivation for undertaking extensive vc type surveys and genetic analysis of *vic* genotypes in *C. parasitica*. In particular, it is an open question whether viruses fail to establish in populations with high vc type diversity or whether virus introduction selects for greater vc type diversity, causing viruses to decline because of the reduction in horizontal transmission associated with high vc type diversity (48). The decrease in virus infection in the Dutch elm disease fungus as epidemics progress may be an example of the latter scenario of frequency-dependent selection (49). However, *vic* alleles in the US are not at intermediate frequencies, as expected under frequency-dependent selection, making it an unlikely explanation for the virtual absence of deleterious hypoviruses in *C. parasitica* in the U.S. (33, 50) despite repeated introductions for biological control (25). The hypothesis of frequency-dependent selection by viruses on *vic* genes will require further investigation, perhaps by analyzing trans-species polymorphisms in *vic* alleles, as in *Neurospora* (7).

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