Diversity and Multilocus Genetic Structure in Populations of *Cryphonectria parasitica*

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ABSTRACT

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Genetic diversity and multilocus genetic structures of four populations of the chestnut blight fungus, *Cryphonectria parasitica*, were analyzed. Two populations in Michigan had very low vegetative compatibility (vc) type diversity and also low diversity of DNA fingerprints and mitochondrial DNA (mtDNA) haplotypes. A population in Teano, in southern Italy, had low vc type diversity but higher levels of fingerprint and mtDNA haplotype diversity. In contrast, a population in Finzel, Maryland, had high diversity for all markers. Mating type was in a 1:1 ratio in one Michigan population and in Finzel but significantly deviated from 1:1 in the other populations. DNA fingerprints were more similar within vc types than between vc types in Teano but not in Finzel; lack of diversity for all markers precluded similar analyses for the Michigan populations. Based on tests for gametic disequilibrium and genotypic diversity, the multilocus structure in Finzel was consistent with a hypothesis of random mating. In contrast, the random mating hypothesis was rejected in Teano for the full sample but not within the dominant vc type, which comprised 75% of the sample. Recombinant vc types between the two common vc types in Teano were found only rarely in the field, and DNA fingerprints did not always correlate to mtDNA haplotypes and vc types, indicating that recombination occurs infrequently in Teano. These results demonstrate that vc diversity does not necessarily correlate to diversity of other genetic markers but may be related to the reproductive biology of *C. parasitica* in nature.

Additional keywords: biological control, *Endothia parasitica*, hypovirulence, population structure.

Sexual reproduction typically results in a population structure characterized by high genotypic diversity (relative to clonal populations) and random association of alleles at different loci (reviewed by Milgroom [35]). Therefore, analyses of multilocus population structure can be used to make inferences about the reproductive biology of fungal plant pathogens. Analyses of multilocus genetic structure and the mating system of the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, in one population in Virginia suggested that recombination occurred frequently, although there was also a significant amount of clonal reproduction (39,40). In one population in Connecticut, where a chestnut blight epidemic was monitored for 3 years, Anagnostakis and Kranz (6) concluded that relatively few individuals in limited numbers of vegetative compatibility (vc) types initially colonized the chestnut population and that vc type diversity later increased because of sexual reproduction and recombination. Although there are numerous other studies on the diversity of vc types in *C. parasitica* (5,10,15,24,25,27,28,32,41), few inferences can be made from them about reproductive biology, because vc types are not defined genetically.

The reproductive biology of *C. parasitica* is relevant to the success of biological control of chestnut blight with transmissible hypovirulence in several ways. First, the diversity of vc types correlates negatively with the success of biological control (2,5,29). Hypovirulence has been more successful in Michigan and Europe, where vc type diversity is low, than in the eastern United States, where diversity is relatively high. The transmission of hypoviruses, which cause hypovirulence (16), occurs much less frequently between isolates in different vc types than between isolates in the same vc type (1,4,18,27). Moreover, the probability of hypovirus transmission in laboratory studies is correlated to the genetic relatedness of the vc types (27). Therefore, the spread of viruses among individuals interacting in natural populations is likely to be greater when vc type diversity is low than when it is high.

Sexual reproduction by *C. parasitica* has the potential to generate and maintain vc type diversity by recombination of vegetative incompatibility (*vic*) genes. Vegetative incompatibility is controlled by at least five to seven independent *vic* loci (3,11). Individuals with the same alleles at all *vic* loci are vegetatively compatible; those that differ at one or more *vic* loci are incompatible. The number of polymorphic *vic* loci sets an upper limit on the number of possible vc types for any given population. The maximum number of vc types in any population is 2^n, for n polymorphic *vic* loci (only two alleles per *vic* locus have been found in *C. parasitica* [3,11,18]), whereas frequencies of vc types depend largely on *vic* allele frequencies and how much recombination occurs in each population.

The second way in which the reproductive biology of *C. parasitica* relates to the spread of hypovirulence is that hypoviruses

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inhibit the production of perithecia, preventing sexual reproduction (3). Thus, hypoviruses have the potential to reduce genotypic diversity in general, including vc type diversity. Furthermore, hypoviruses are not transmitted through ascospores when virus-infected isolates mate as conidial (male) parents (12,21). Therefore, the success of hypovirulence also may be correlated to the reproductive biology of *C. parasitica* in each population, as well as to vc type diversity.

The main objective of this study was to analyze the multilocus genetic structure of *C. parasitica* populations to determine the extent of recombination that occurs in each. Our hypothesis is that populations with a low diversity of vc types, and in which hypovirulence has been relatively successful, are primarily clonal in structure, whereas populations with a high diversity of vc types are closer to randomly mating (2,39,40). We investigated the structure of four populations: two populations in Michigan and one in Italy that were chosen to represent populations with low vc type diversity (10,32) and one population in Maryland, in the central Appalachian region, that was considered a population with high vc type diversity (27). Analysis of population structure was done by first testing for random mating and then by looking for evidence of recombination in those populations that deviated from random mating (35). A secondary objective was to compare estimates of diversity based on vc types with those from DNA fingerprints and mitochondrial DNA (mtDNA) haplotypes.

MATERIALS AND METHODS

Population samples. Four populations were sampled in this study: two in Michigan and one each in Italy and Maryland. The two populations sampled in Michigan were County Line, in Manistee County, and Frankfort, in Benzie County. American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) were recovering from chestnut blight at both Michigan sites (13). Sixteen and nineteen single-conidial isolates were sampled by A. Michna from County Line and Frankfort, respectively, in 1986 (32). An initial sample of 50 isolates was collected from European chestnut trees (*C. sativa* Mill.) in Teano, in Campania in southern Italy, in 1995; another sample of 145 isolates was collected in 1996 to look for recombinant vc types. These isolates were collected in a 10- to 15-year-old coppice forest from an area of approximately 1 ha and were described previously in a study of vc type diversity in Italy (10). The sample from Maryland consisted of 57 mass hyphal isolates collected by A. Webb and E. Seligmann near Finzel in 1991 from cankers on stems of 48 blighted American chestnut trees from an area approximately 240 × 100 m. The Maryland sample was analyzed previously for hypovirus transmission among vc types (27) and spatial patterns of genotypes (38); the Michigan and Maryland samples also were included in an analysis of population differentiation in North America (37).

Vegetative compatibility tests. Vegetative compatibility tests for the Maryland sample were performed essentially as described by Anagnostakis (3); minor modifications in the vc testing method and vc types of 57 isolates were reported previously (27). Two small cubes of agar, ~3 mm on each side, were cut from the margins of 4- to 6-day-old colonies and placed next to each other on potato dextrose agar (PDA). Plates were incubated at 25°C in the dark for 6 to 7 days before scoring. The presence of a barrage was interpreted as evidence of incompatibility. Each test was performed at least three times. Vegetative compatibility testing for the Michigan samples was done originally by Michna (32) and was repeated for this study, using the same method as for the Maryland sample. For the Italian sample, vc testing was done essentially the same way, but a different medium (PDAg [42]) was used as described previously (10).

DNA fingerprinting and mtDNA haplotypes. Methods for preparation of DNA, agarose gel electrophoresis, Southern blotting, probing, and autoradiography have been described previously (36, 40). Probe pMS5.1, which usually hybridizes to 7 to 12 restriction fragments in each isolate, was used to determine DNA fingerprints (40). DNA fingerprint fragments segregate in simple Mendelian ratios for the presence and absence of fragments, and most loci are unlinked (40). mtDNA of *C. parasitica* is maternally inherited and highly diverse within populations (36). Haplotypes of mtDNA were determined by the hybridization pattern resulting from probing Southern blots of *Pst*I- or *Eco*RI-digested total DNA with ^{32}P-labeled purified mtDNA as described by Milgroom and Lipari (36). DNA fingerprints and mtDNA haplotypes were not determined for the 1996 Teano sample.

The Shannon index (19) was used to describe diversity for each type of genetic marker: $D = -\sum p_i \ln p_i$, where p_i is the frequency of the ith vc type or genotype. This index typically has been used to estimate vc type diversity in *C. parasitica* (5,7,10) and was used in this study to allow comparisons to other studies. However, the estimated diversity, D, was normalized to correct for differences in sample sizes (14): $D' = D/\ln N$, where N is the sample size. D' can range from 0, when all isolates have the same genotype, to 1, when every individual has a unique genotype.

Analyses of population structure: overview. Random mating was tested by three analyses. First, we determined the ratio of mating types in each population. The mating-type ratio is expected to be 1:1 for randomly mating populations (35). Second, we tested for gametic disequilibrium, i.e., the nonrandom association of alleles at different loci. Gametic disequilibrium was analyzed two ways: first, by testing for correlations between vc types and DNA fingerprints, which are independent sets of nuclear genetic markers and should be uncorrelated if there is random mating (33, 35,47); and second, by determining the index of association, which is a multilocus measure of gametic disequilibrium (8,31,35). Finally, we compared the observed genotypic diversity (17,46) to the expected genotypic diversity under the null hypothesis of random mating.

Mating-type ratios. To identify mating types, crosses were made between sampled isolates and mating-type testers as described previously (27). For the Michigan sample, we used cream-colored mating-type testers, isolates EP389 (ATCC 38980) and EP393

TABLE 1. Number of genotypes and diversity of vegetative compatibility (vc) types, DNA fingerprints, and mitochondrial DNA (mtDNA) haplotypes in four populations of *Cryphonectria parasitica*

C. parasitica population	No. of genotypes observed					Estimated diversity[a]		
	N	vc	vc_2[b]	Fingerprint	mtDNA	D'_{vc}	D'_{fp}	D'_{mt}
County Line	16	1	1	6	1 (1)[c]	0	0.41	0 (0)
Frankfort	19	2	1	3	7 (6)	0.07	0.41	0.48 (0.44)
Teano	50	3	2	36	...[d] (13)	0.16	0.86	... (0.57)
Finzel	57	31	12	53	20 (19)	0.79	0.97	0.83 (0.52)

[a] The diversity of each genotype is represented by the Shannon diversity index (19): $D = -\sum p_i \ln p_i$, where p_i is the frequency of the ith vc type or genotype. D was normalized to correct for differences in sample sizes (14): $D' = D/\ln N$, where N is the sample size.

[b] vc_2 is the number of vc types occurring more than once in each sample.

[c] Figures in parentheses are the number and diversity of mtDNA haplotypes determined only from *Pst*I digests.

[d] mtDNA haplotypes were determined only by restriction analysis with *Pst*I in Teano, Italy.

(ATCC 38984), which were provided by S. L. Anagnostakis. The mating types of EP389 and EP393 have been redesignated as *MAT*-2 (formerly *Mat*1-1) and *MAT*-1 (formerly *Mat*1-2), respectively (R. E. Marra and M. G. Milgroom, *unpublished data*), based on the presence of the HMG domain, which is conserved among *MAT*-2 mating types in other ascomycetes studied to date (48). Two isolates of each mating type were used as testers for the Italian sample: these were cream-colored isolates derived from crosses between wild-type isolates and EP389 or EP393 but were selected for greater fertility (R. E. Marra and M. G. Milgroom, *unpublished data*). Isolates from Maryland were tested with two resident isolates that had previously had opposite mating types (27). Each sample isolate was crossed with each of the mating-type testers in at least three replicates. Mating types were determined by the presence of fertile perithecia. For isolates from Michigan, segregation of the cream phenotype was used to confirm that outcrossing had occurred between the tester strains and sampled isolates; in the Finzel population, segregation of vc types was used to confirm outcrossing (27). Segregation data were not obtained for mating-type tests in the Teano sample.

Correlation between vc types and DNA fingerprints. To determine the correlation between vc types and DNA fingerprints in each population, similarities between DNA fingerprints among all isolates within and among vc types were estimated. Similarities between DNA fingerprints based on the proportion of shared fragments were estimated as $S_{xy} = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the numbers of fragments in isolates x and y, respectively, and N_{xy} is the number of fragments shared by the two isolates. Similarity, S_{xy}, can range from 0, when two isolates share no common fragments, to 1, when isolates have identical fingerprints. Correlations between similarity and vc types were analyzed by a matrix comparison technique (30). We tested the null hypothesis that the average similarity between isolates in the same vc type was not different from the average similarity between individuals with vc types assigned at random. Significance tests were done by randomization, in which P values were estimated by 1,000 randomizations as described previously (38,41).

Index of association. The index of association, I_A (8,31), is a multilocus estimate of the degree of deviation from random mating. I_A is based on a comparison of the observed and expected variances, S_k^2 and σ_k^2, respectively, in the number of heterozygous loci in all pairs of individuals in each population (34,35): $I_A = S_k^2/\sigma_k^2 - 1$. Significance testing was done by comparing the observed variance with the distribution of the variance expected under the assumption of random association, as determined from 100 randomizations of the observed genotype data (34). If the observed variance was greater than 95% of the null distribution, then the sample significantly deviated from random mating. Only those DNA fingerprint fragments with frequencies ranging from 0.1 to 0.9 were analyzed.

Genotypic diversity. Genotypic diversities based on DNA fingerprints were estimated within each population and compared to that expected under random mating (17,34,35,46). Genotypic diversity was estimated as $\hat{G} = 1/\sum p_i^2$, where p_i is the observed frequency of the ith multilocus genotype. $\hat{G}$ can range from 1, when all individuals have the same genotype, to N, the sample size, when all isolates have unique genotypes. Significance testing was done by randomization of the data to generate a null distribution of $\hat{G}$ expected under random mating to be compared to the observed $\hat{G}$ (17,40). As above, only DNA fingerprint frag-

TABLE 2. Mating-type ratios in four populations of *Cryphonectria parasitica*

C. parasitica population	N	Mating-type ratio (*MAT*-1:*MAT*-2)	χ^{2a}	P
County Line	16	9 : 7	0.25	>0.50
Frankfort	19	15 (1)[b] : 3	8.00	<0.005
Teano	50	34 (1)[b] : 15	7.37	<0.01
Finzel	55[c]	23 : 32	1.47	>0.10

[a] Chi-square goodness-of-fit test for a 1:1 ratio and associated P value.
[b] Figures in parentheses are the numbers of isolates that mated with both mating-type testers.
[c] Two isolates from Finzel did not mate with either tester.

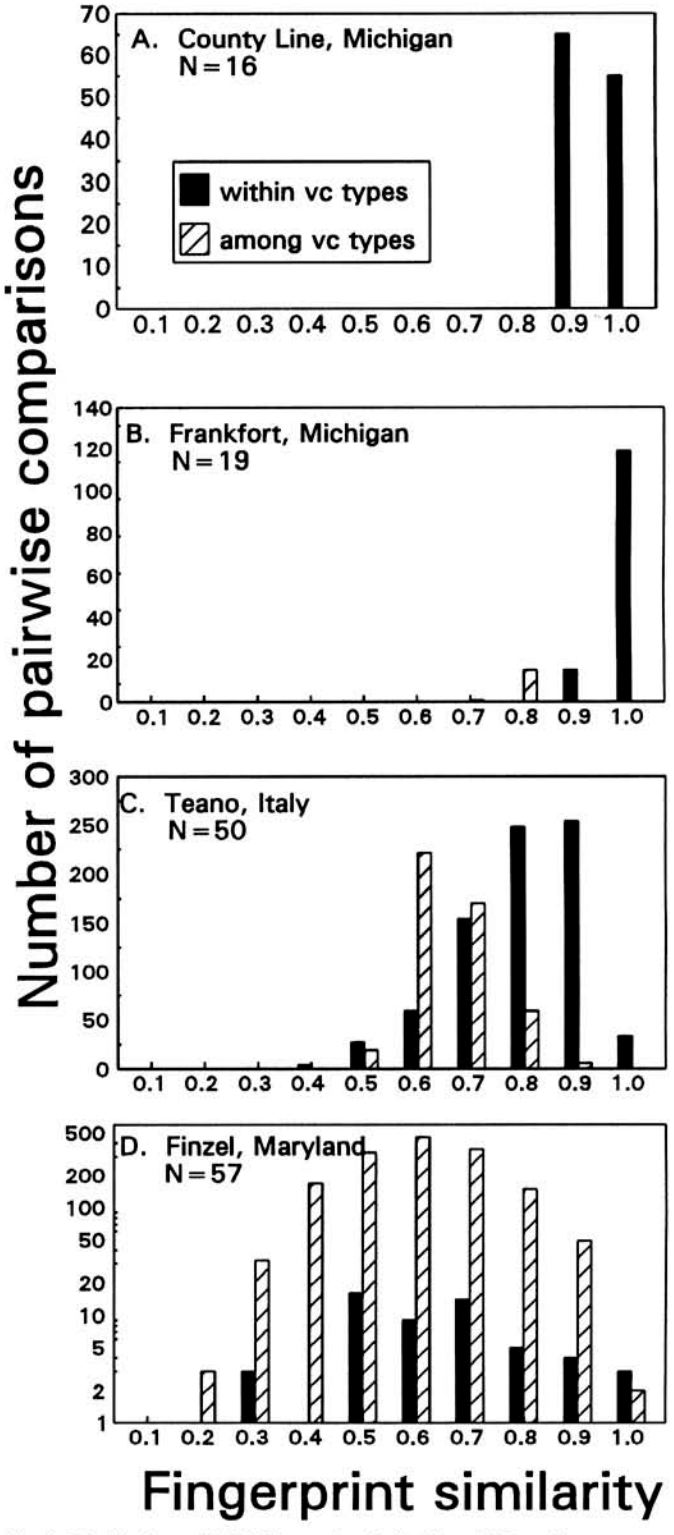

Fig. 1. Distributions of DNA fingerprint similarities within and among vegetative compatibility (vc) types for four populations of *Cryphonectria parasitica*. Fingerprint similarity was defined as $S_{xy} = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the numbers of fragments in isolates x and y, respectively, and N_{xy} is the number of fragments shared by the two isolates. Total numbers of vc types in each population and numbers of vc types occurring more than once are shown in Table 1.

ments with frequencies between 0.1 and 0.9 were used in these analyses.

Evidence for recombination. In populations that deviated from random mating, we assessed whether recombination had occurred by looking for recombinant genotypes among the DNA fingerprinting loci and for recombinant vc types when recombinant vc types were known. We also assessed whether isolates with the same DNA fingerprints had similar mtDNA haplotypes, as expected in primarily clonal populations.

RESULTS

Diversity within populations. In general, diversity for all markers was lowest in the two Michigan populations, intermediate in Teano, and highest in Finzel (Table 1). The diversity of fingerprint genotypes appeared moderate in the Michigan populations (D' = 0.41); however, only 6 of 15 and 6 of 12 fingerprint loci were polymorphic in County Line and Frankfort, respectively. In Teano and Finzel, 30 of 33 and 41 of 42 fingerprint loci were polymorphic, respectively. Furthermore, 17 of the 19 isolates in Frankfort had identical fingerprints.

Mating-type ratio. Both mating types were found in all populations in this study (Table 2). In County Line, the mating-type ratio did not deviate from 1:1. In Frankfort, 15 of 19 isolates were in *MAT*-1, which is significantly different from a 1:1 ratio. One isolate from Frankfort mated with both tester strains. In Teano, the mating-type ratio was significantly different from 1:1, with one isolate mating with both testers. Both mating types were found in the two dominant vc types in Teano in approximately equal ratios: the *MAT*-1:*MAT*-2 ratios were 8:3 for vc type I-10 and 25:12 for vc type I-12. The mating-type ratio (23:32) in Finzel was not significantly different from 1:1.

Correlations between fingerprint similarity and vc types. Although variation in DNA fingerprints was found among individuals in the same vc types in all populations, some populations varied more than others. The similarity of DNA fingerprints within vc types was high in the two Michigan populations, lower in Finzel, and intermediate in Teano (Fig. 1; Table 3). Only one vc type was found in the County Line population, in which individuals shared 85 to 100% of the DNA fingerprint fragments (Fig. 1A). In Frankfort, 18 of 19 isolates were of the same vc type. Fingerprint similarity was higher among individuals in the dominant vc type than between the two vc types (Fig. 1B), although statistical testing was not possible in the two Michigan populations because of the lack of vc type diversity (Table 1).

In Teano, fingerprint similarities ranged from 0.40 to 1.0 within vc types compared to 0.50 to 0.88 between vc types (Table 3; Fig. 1C). The average similarity between individuals in the same vc type was significantly greater than the average similarity when vc types were assigned at random ($P < 0.001$). The correlation between fingerprints and vc types in Teano is clearly illustrated in a phenogram based on fingerprint similarities, in which all but two isolates (TE1 and TE77) clustered with other members of the same vc type (Fig. 2). Similarly, vc types appeared to cluster with mtDNA haplotypes (Fig. 3) with few exceptions (TE7 and TE68); however, the correlation between mtDNA haplotype similarity and vc type was not significant ($P = 0.41$).

In Finzel, unlike Teano, the distribution of similarities within vc types almost completely overlapped the distribution of similarities among vc types (Fig. 1D). There was no significant difference (P =

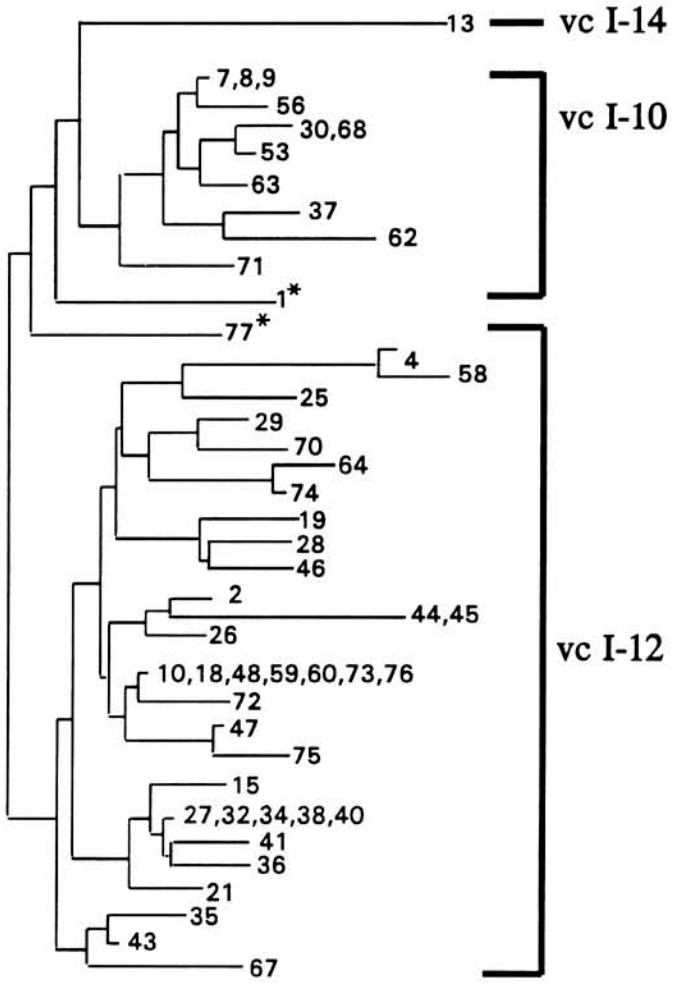

Fig. 2. Relationships among DNA fingerprint genotypes and vegetative compatibility (vc) types in a sample of *Cryphonectria parasitica* from Teano, Campania in southern Italy. This tree was constructed by neighbor-joining analysis (44) in the program NJTREE (22) on DNA fingerprint similarity data (discussed in text). Isolate numbers are indicated at the end of each branch. All isolates clustered with their respective vc types, except for isolates TE1 in vc type I-10 and TE77 in vc type I-12 (indicated by asterisks), which were not placed in discrete clusters.

TABLE 3. DNA fingerprint similarities within and among vegetative compatibility (vc) types in four populations of *Cryphonectria parasitica*

C. parasitica population	*N*	No. of vc	Fingerprint similarity[a]		*P*[b]
			Within vc	Between vc	
County Line	16	1	1.0 (0.85–1.0)	…[c]	…[d]
Frankfort	19	2	1.0 (0.87–1.0)	0.77 (0.66–0.77)	…[d]
Teano	50	3	0.80 (0.40–1.0)	0.66 (0.50–0.88)	<0.001
Teano (censored)[e]	36	3	0.75 (0.40–0.94)	0.62 (0.50–0.88)	<0.001
Finzel	57	31	0.62 (0.23–1.0)	0.60 (0.16–1.00)	0.21
Finzel (censored)[e]	53	31	0.61 (0.23–0.88)	0.58 (0.16–0.94)	0.37

[a] Median similarity, with range in parentheses.
[b] *P* value is for the null hypothesis that similarity is the same within and between vc types. Significance testing was done by randomization, as described in text (41).
[c] Not applicable.
[d] Significance tests were not conducted in County Line or Frankfort because only one vc type occurred more than once in each population.
[e] Isolates with identical DNA fingerprints (clonemates) were censored (explained in text).

0.21) between the average similarity within vc types and the average similarity expected when vc types were assigned randomly (Table 3). One weakness of this test in Finzel is that only 12 of 31 vc types occurred more than once, and 10 of these had only two or three isolates each.

Index of association and genotypic diversity tests. Genotypic diversity and the index of association were not estimated for County Line and Frankfort because the frequencies of all DNA fingerprint fragments were outside the range of 0.1 to 0.9. Similarly, there was not enough polymorphism detected with single-locus restriction fragment length polymorphisms (RFLPs) (37) to conduct any further analyses in these populations.

In Teano, analysis of multilocus association revealed there was significant deviation ($P < 0.01$) from the genetic structure expected under random mating (Table 4). The observed genotypic diversity also was significantly less than expected for a randomly mating population (Table 4). Similar results were obtained for both the full sample of 50 isolates and the reduced sample with clonemates censored. In contrast, the random mating hypothesis could not be rejected for Finzel by either the index of association or the genotypic diversity test (Table 4).

Because of the striking correlation between vc types and fingerprint similarities in Teano (Fig. 2), we tested the hypothesis of random mating within the dominant vc type (I-12), which comprised 37 of 50 isolates. Neither the index of association nor the genotypic diversity test rejected the null hypothesis of random mating (Table 4). However, because reduction in sample size alone can result in less statistical power (26,31,35), we took 20 random samples of 37 isolates from the full data set and repeated the same tests. Random samples (without replacement) were drawn in two ways: first, as stratified random samples, such that the ratio of isolates in vc types I-10 and I-12 was the same as the original sample; and second, completely random samples. The random mating hypothesis was rejected by both the index of association and the genotypic diversity tests for all 20 samples (data not shown). Therefore, failure to reject the random mating hypothesis within vc type I-12 was not an artifact of reduced sample size. We conducted additional analyses within vc type I-12 to test whether failure to reject the random mating hypothesis was caused by reduction of the number of loci analyzed (with fragment frequencies ranging from 0.1 to 0.9) for the smaller sample size. The random mating hypothesis was not rejected by either the index of association or the genotypic diversity test within vc type I-12 when the analysis was done with 11 loci (with fragment frequencies ranging from 0.08 to 0.92; Table 4). This result revealed that reduction in the number of loci analyzed was not the cause of failure to reject the random mating hypothesis within vc type I-12.

Additional evidence of recombination. Based on the above analyses, we concluded that Teano appears, in part, to be clonal in structure. However, there appears to be some evidence of recombination between vc types I-10 and I-12: fingerprints for isolates TE1 and TE77 do not cluster with the rest of the isolates in their vc types and are possible recombinants (Fig. 2). To investigate further whether recombination occurred in Teano, we made a cross between isolates in vc types I-10 and I-12 and found two recombinant vc types among the progeny. Neither recombinant vc type was found in the 1995 sample; therefore, we collected an additional 145 isolates in 1996. Among the 145 isolates, we found only three isolates in the two recombinant vc types. We also looked for recombination among fingerprint loci. All four possible two-locus fingerprint genotypes were observed within vc type I-12 for 9 of 28 possible combinations among the 8 loci that were polymorphic in the entire sample (frequencies between 0.1 and 0.9). There was much less polymorphism for fingerprints within vc type I-10 ($N = 12$), but 4 of the 10 possible two-locus combinations (only five polymorphic loci) had all four genotypes.

Additional evidence for recombination involving vc type I-10 isolates can be seen in the lack of complete correlation between DNA fingerprints and mtDNA haplotypes. Isolates TE7, TE8, and TE9 all have identical fingerprints, but the mtDNA haplotype for TE7 is a haplotype found in a cluster of vc type I-12 isolates (Fig. 3). Similarly, TE30 and TE68 have identical fingerprints, but TE68 has the same mtDNA haplotype as TE7 (Fig. 3). Because mtDNA is maternally inherited (36), these results suggest that some recombination has occurred to produce identical fingerprints from different maternal isolates. Additional support for recombination among vc type I-12 isolates is that isolates with the same fingerprints (Fig. 2) frequently had mtDNA haplotypes that did not cluster together (Fig. 3).

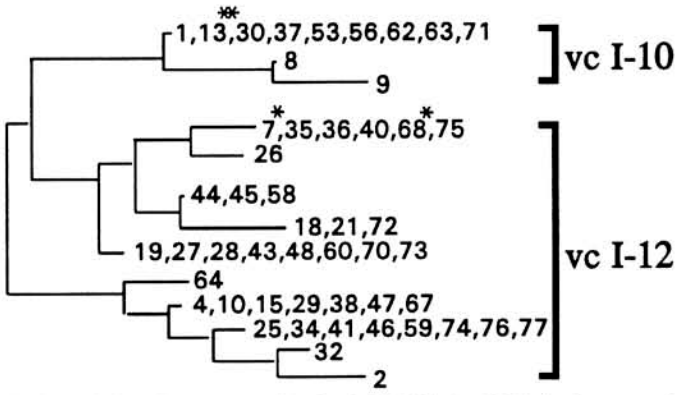

Fig. 3. Relationships among mitochondrial DNA (mtDNA) haplotypes and vegetative compatibility (vc) types in a sample of *Cryphonectria parasitica* from Teano, Campania in southern Italy. This tree was constructed by neighbor-joining analysis (44) in the program NJTREE (22) on mtDNA haplotype similarity data as was done for DNA fingerprints (discussed in text). Isolate numbers are indicated at the ends of each branch. Most isolates clustered with their respective vc types, except for TE7 and TE68, which are in vc type I-10 (indicated by asterisks); isolate TE13 is the only isolate in vc type I-14, but it clustered with vc type I-10 (indicated by two asterisks).

DISCUSSION

Multilocus structures varied among different populations of *C. parasitica*. The population structure in Finzel, Maryland, was not different from that expected for random mating; populations in Michigan had little genetic diversity; and the population in Teano, Italy, was partially clonal, with some evidence of recombination.

The diversities of vc types, fingerprint genotypes, and mtDNA haplotypes roughly correlated for the four populations studied. The low diversity for these markers in County Line and Frankfort is consistent with results from single-locus RFLPs, in which the Michigan populations were monomorphic or nearly monomorphic for all six loci assayed (37). In contrast, Finzel had relatively high diversities for all markers assayed, including single-locus RFLPs (37). The diversities of different markers were not entirely consistent in Teano, where there were only three vc types in the original sample, but there were relatively high diversities of DNA fingerprints and mtDNA haplotypes. These results demonstrate that vc type diversity may not necessarily be a good predictor of diversity for other markers; this result is important for trying to make inferences from previous studies of vc type diversity about genetic variation in general.

The low diversity of all genetic markers in the Michigan populations is probably the result of founder effects and genetic drift. These populations are outside the natural range of American chestnut trees (13) and, therefore, are disjunct populations of both the tree and fungus. We speculate that only a fraction of the genetic diversity present in the source populations was represented by the individuals in the founder population and that subsequent random genetic drift in small populations further reduced genetic diversity. Differences in dominant vc types (32), RFLP alleles (37), and double-stranded RNAs infecting *C. parasitica* (T. L. Peever and

M. G. Milgroom, *unpublished data*) in the two populations could be explained either by separate founder events (even though they are only 16 km apart) or by divergence due to restricted gene flow and drift. Mutation probably plays a role in maintaining some diversity. For example, most DNA fingerprint genotypes within County Line or Frankfort were different from each other by only one or two fragments (Fig. 1A and B) and may represent clonal lineages within these populations.

Testing for the occurrence of recombination in the Michigan populations was problematic. It is possible that these populations frequently undergo recombination, but we cannot detect it because there is insufficient polymorphism with any of the markers used, and furthermore, our sample sizes were too small. The 1:1 ratio of mating types in County Line may suggest that sexual reproduction occurs in the population; however, few perithecia were found in these populations, and hypovirulent strains were common (32). Hypovirulent strains seldom produce perithecia (3), and therefore, hypovirulence may affect population structure by inhibiting recombination.

High diversity of all markers in Finzel is consistent with previous reports of genetic diversity in the eastern United States. Relatively high levels of vc type diversity have been found in other populations of *C. parasitica* in the eastern United States (5,24,25,28,41). Similarly, *C. parasitica* is diverse for various molecular markers in this region (36,37,39,40). Population structure in Finzel was consistent with expectations of random mating, and therefore, high levels of diversity of multilocus genotypes are most likely maintained by frequent recombination. Only 4 of 57 isolates (7%) were part of the clonal fraction of the population (determined by DNA fingerprinting) compared to 15% found in Mt. Lake, VA (40). However, differences in plot size and sampling methods may have affected these estimates (38). The effect of mutation on fingerprint diversity in Finzel cannot be entirely discounted and probably contributes to the polymorphisms observed. However, recombination appears to have shuffled mutant fingerprint fragments with respect to *vic* genes; this is evident in the distribution of fingerprint similarities within and among vc types, which are symmetrically distributed around the mean (Fig. 1D), whereas those in other populations are skewed (Fig. 1A through C).

The most intriguing population we studied was from Teano, combining features of both clonal and sexual populations. In Teano we found low vc type diversity but intermediate diversities of fingerprints and mtDNA haplotypes (Table 1); a correlation between fingerprint similarities and vc types (Fig 2; Table 3); and a mating-type ratio that deviated from 1:1 (Table 2). We rejected the random mating hypothesis for the full sample but could not reject it within the dominant vc type (I-12) (Table 4). Recombinant vc types between the two main vc types were found only at low frequencies, suggesting recombination between these vc types is rare. If vc types I-10 and I-12 were spatially restricted, there may have been little chance of mating and recombination between the two vc types (33). No data are available on the spatial patterns of vc types in Teano; however, other studies of spatial patterns of vc types in similar populations of *C. parasitica* in Europe have failed to show significant aggregations (7; P. Cortesi and M. G. Milgroom, *unpublished data*).

The failure to reject the random mating hypothesis within vc type I-12 does not necessarily mean random mating occurs (35). Assuming that vc types I-10 and I-12 are not spatially isolated, random mating is unlikely to occur within vc types but not between vc types, because *vic* loci are independent of mating compatibility in *C. parasitica* (3). Relatively low levels of recombination can have marked effects on population structure (9). Similarly, high mutation rates and rapid population expansion sometimes can result in a population structure lacking gametic disequlibrium (45). Therefore, the failure to reject random mating within vc type I-12 should not be interpreted as frequent recombination occurring within this subpopulation.

Variations in both mtDNA and DNA fingerprints were found among individuals of *C. parasitica* in the same vc type, especially in Finzel, where there was no correlation among markers. This is in marked contrast to populations of primarily clonal fungi (20,23). The diversity of vc types has been used as a quantitative measure of genetic diversity for several fungi (5,23,43). However, we have shown that vc type is not a sensitive marker to determine diversity in populations of *C. parasitica*. Isolates within vc types may be clones or clonal lineages in some *C. parasitica* populations but not in others. When there is sexual reproduction, associations among markers break down, and vc types may comprise genetically heterogeneous individuals. Furthermore, in a previous study, RFLPs and DNA fingerprints were more sensitive markers than vc types for detecting genetic differences in *C. parasitica* (39). vc types are determined by five to seven *vic* loci (3,11), yet they are treated as distinct phenotypes, losing most of their resolution for population genetic studies. Therefore, we conclude that vc types in *C. parasitica* are not necessarily uniform genetic entities, such as clones or clonal lineages, and interpretation of previous population studies based on vc types should be made with caution.

A significant limitation on the use of vc types for analyzing population structure in *C. parasitica* is that the genetics of vegetative incompatibility are known for only a few laboratory isolates (3,11,18). Inferences about recombination can be made with certainty from vc types only by directly observing segregation of re-

TABLE 4. Tests for random mating in two populations of *Cryphonectria parasitica*

C. parasitica population	*N*	No. of loci[a]	Index of association[b]				Genotypic diversity[c]		
			σ_k^2	s_k^2	I_A	*P*	G_{exp}	$\hat{G}$	*P*
Teano	50	8	1.74	3.77	1.16	<0.01	20.20	7.23	0.001
Teano (censored)[d]	36	8	1.77	3.31	0.87	<0.01	19.15	9.82	0.006
Teano (vc type I-12 only)[e]	37	3	0.61	0.51	−0.16	0.91	2.48	3.05	0.95
	37	11[f]	1.62	1.92	0.19	0.12	9.18	7.82	0.37
Finzel	57	15	3.14	3.49	0.11	0.13	48.98	45.76	0.25
Finzel (censored)[d]	53	15	3.19	3.36	0.05	0.25	47.01	49.28	0.78

[a] Number of polymorphic loci with fingerprint fragment frequencies in the range of 0.1 to 0.9.

[b] σ_k^2 and s_k^2 are the expected and observed variances, respectively, in the number of heterozygous loci in all pairwise comparisons among individuals in the sample. $I_A = s_k^2 / \sigma_k^2 - 1$ (8,31). *P* is the probability of observing s_k^2 under the null hypothesis of random mating, as determined from 100 randomizations (discussed in text).

[c] G_{exp} and $\hat{G}$ are the expected and observed genotypic diversities, respectively. $\hat{G} = 1/\sum p_i$, where p_i is the observed frequency of the *i*th multilocus genotype. G_{exp} is the mean diversity of 1,000 randomizations under the null hypothesis of random mating (discussed in text). *P* is the probability of observing $\hat{G}$ under the null hypothesis of random mating, as determined from 1,000 randomizations (discussed in text).

[d] Isolates with identical DNA fingerprints (clonemates) were censored (explained in text).

[e] vc = vegetative compatibility.

[f] Analyses were done with fingerprint fragments with frequencies ranging from 0.08 to 0.92.

combinant vc types sampled from field-collected perithecia (6, 33,39) or searching for known recombinant vc types in the field (as we did in Teano). Inferences cannot be made about recombination from vc type data unless the genetics of vc types are known or recombinant vc types are found in laboratory crosses. In theory, a population consisting of a single vc type could be randomly mating, but this would not be detected by vc type data.

Previous studies on chestnut blight have stressed the correlation between the success of hypovirulence and low vc type diversity, hypothesizing that vegetative incompatibility is a major impediment to the spread of hypovirulence in North America (2,5,29). Our results extend this correlation by showing that low vc type diversity may not necessarily correlate to low genetic diversity for all markers but does correlate to a clonal or partially clonal population structure in which there is restricted recombination. The reproductive biology of *C. parasitica* is confounded with vc type diversity in its effects on hypovirulence transmission because hypoviruses inhibit sexual reproduction (3), hypoviruses generally are not transmitted through ascospores (12,21), and recombination of *vic* genes by sexual reproduction can generate and maintain vc type diversity. Therefore, it is difficult to ascribe cause and effect to the observed relationship between hypovirulence and low vc type diversity. It is an open question whether populations are clonal because of the prevalence of hypoviruses preventing recombination or whether hypoviruses can invade clonal populations because they can spread more easily among individuals in the same vc types (18,27). More rigorous tests of these hypotheses are needed before cause and effect can be inferred.

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