Diversity of *Cryphonectria parasitica* Hypovirulence-Associated Double-Stranded RNAs within a Chestnut Population in New Jersey

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ABSTRACT

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The effects on colony morphology, sporulation, laccase production, and virulence of hypovirulence-associated double-stranded (ds)RNAs of the chestnut blight fungus, *Cryphonectria parasitica*, from a population of chestnut trees in eastern New Jersey were examined. Size and number of dsRNAs were determined by gel electrophoresis, and their genetic variability was determined by polymerase chain reaction (PCR) and nucleotide sequencing. The New Jersey hypovirulent isolates were similar in cultural phenotype and electrophoretic characteristics of their dsRNAs. PCR amplification primed by oligonucleotides specific for a conserved region of a New Jersey-derived Cryphonectria hypovirus dsRNA (CHV2-NB58) for which the entire nucleotide sequence is known resulted in amplification of a single band of predicted size from all but one of the New Jersey dsRNAs. Neither the European-derived dsRNA, CHV1-EP713, nor a Rhode Island dsRNA amplified with these primers. The sequences of 400 nucleotides of PCR-amplified products primed by two oligonucleotides (200 residues from each primer) were determined. Alignments of these sequences with CHV1-EP713 and CHV2-NB58 and phylogenetic trees drawn from these alignments indicated that all New Jersey isolates were closely related and easily distinguishable from CHV1-EP713. In the region sequenced, none of the dsRNAs from fungal isolates from different trees were identical; however, CHV2-NB58 dsRNA isolated in 1992 was identical to the original 1988 CHV2-NB58 dsRNA from the same tree. This suggests that there is significant drift among dsRNA sequences even within a small *C. parasitica* population but that there is stability within a particular fungal thallus.

Additional keywords: fungal virus.

The chestnut blight fungus (*Cryphonectria parasitica* (Murrill) Barr) virtually wiped out the native American chestnut (*Castanea dentata* (Marsh.) Borkh.) in the early- to mid-1900s. The introduction of the fungus into North America has long been attributed to the importation into the Bronx Zoo of oriental nursery-stock chestnut species (2). A more recent detailed historical account, however, has suggested that the importation of diseased trees into the Bronx Zoo probably followed the earlier establishment of the epidemic, perhaps from Japanese trees grafted before 1900 (3).

The original discovery of hypovirulence in *C. parasitica* was in Europe (14). Researchers at the Connecticut Agricultural Experiment Station subsequently demonstrated with European and North American isolates that hypovirulence resulted from double-stranded (ds)RNA (7). The movement of these dsRNAs, which we now recognize as the genomes of a novel family of nonencapsidated viruses (17), from an infected fungal isolate to a noninfected one depends on the vegetative compatibility of the two isolates (24).

Besides reducing fungal virulence, *C. parasitica* viruses or dsRNAs affect other fungal attributes, including sporulation and pigmentation (19), laccase and cutinase expression (32,33,36), and other proteins and messenger RNAs normally produced (30,31). Virus infection or dsRNA presence often affects culture morphology, sometimes resulting in slow growth, lobate margins, or reduced aerial mycelium (24). Variations in size, number, concentration, and hybridization properties of dsRNAs have been observed between various fungal isolates from the same or different locations (5,8,9,22).

Hypovirulent strain NB58 was isolated in the central eastern part of New Jersey in 1988. Colonies are orange-brown on potato-dextrose agar (PDA), grow slowly, and produce little aerial mycelium compared to their virulent, dsRNA-free counterparts. The virus of NB58 (Cryphonectria hypovirus 2-NB58 [CHV2-NB58] [17]) contains only one dsRNA with a size of 12,507 base pairs, excluding the poly(A) tail at the 3′ end of the plus strand (18,20). CHV2-NB58 dsRNA is organizationally similar to CHV1-EP713, previously designated hypovirulence-associated virus (HAV) (34), the first hypovirulence-associated dsRNA sequenced, and the two share approximately 60% nucleotide sequence identity (18,20). Because different regions of the CHV2-NB58 genome share different degrees of nucleotide sequence identity with CHV1-EP713, predictions about the usefulness of oligonucleotides for amplification of conserved or nonconserved regions can be made based on two-way alignments of these sequences.

Several fairly recent innovations in nucleic acid technology have permitted the detailed analysis of viral genomes. The polymerase chain reaction (PCR) amplification of complementary (c)DNA products of reverse transcription reactions allows, in effect, the amplification of RNA segments as DNA copies. When combined with sequencing reactions, rapid primary characterization of large numbers of isolates in a population is possible. The objective of this research was to examine the cultural, morphological, and virulence characteristics of dsRNA-containing isolates from within a specific geographic area in New Jersey and to relate these to the genetic diversity of their dsRNAs.

MATERIALS AND METHODS

Isolates and maintenance. Isolates of *C. parasitica* used in these studies are listed in Table 1. Eleven isolates were selected from among New Jersey isolates containing dsRNAs to examine diversity among viruses within the same canker, from different cankers on the same tree, among cankers collected from trees within a defined site, and among cankers collected from geographically

separated sites. The geographic areas of Five Points Road (Howell Township) and Navesink, NJ, and specific host-tree locations in the Five Points area are described in Table 1 and Figure 1. Hypovirulent isolate Bennet 23-X1 from Rhode Island and EP713 (ATCC 52571) were provided by S. Anagnostakis (Connecticut Agricultural Experiment Station, New Haven). In several experiments, virulent dsRNA-free isolates from New Jersey (SC58-19, ATCC 76221; isogenic to NB58) and Connecticut (EP155, ATCC 38755; isogenic to EP713) also were included. Isolates were routinely grown on Difco PDA amended with methionine (100 mg/l) and biotin (1 mg/l) (PDAmb) as described by Anagnostakis and Day (4), in 15- × 100-mm petri dishes with 25 ml of medium per dish.

Culture phenotype and growth rates. Observations on culture morphology and growth rates for 15 isolates were made on PDAmb and half-strength PDAmb (PDAmb/2). Plates were inoculated with 6-mm plugs of mycelia cut from the margins of 7-day-old cultures with a cork borer. Two colony diameters at right angles were measured 5 and 7 days after inoculation, with five replicates per isolate. Analysis of variance was used to examine the effects of isolates and media on colony size. Tukey's method of multiple comparisons was used to identify significant differences among isolates.

Virulence assays. Virulence was assessed by inoculation of Granny Smith apple fruits using the methods of Elliston (9) and Fulbright (11) with minor modifications. Each apple was inoculated with EP155, and two additional isolates were chosen at random. Apples were incubated in small storage boxes with saturated paper blotters at 25 C. Nine and 16 days after inoculation the diameter of each wound was measured along two axes. Radial growth (10 replications per isolate) was expressed as a percentage of the radial growth of EP155. Statistical analysis was performed as described above.

Phenol oxidase assay. Phenol oxidase activity was examined by growing the isolates on malt and tannic acid agar (MTAA; 15 g of Difco malt extract, 5 g of tannic acid, and 20 g of Difco Bacto agar per liter of water [Difco Laboratories, Detroit]) (35). Five replicates per isolate were inoculated onto individual petri dishes and incubated at 25 C in the dark. Nine days after inoculation, individual plates were inverted and backlit from below with a fluorescent lightbox. An image of the underside of each plate was produced by scanning it with an Eikonix digital camera (Eikonix, Inc., Bedford, MA) operated with ERDAS (Earth Resources Data Analysis System, Eardas, Inc., Atlanta, GA) on an MS-DOS-based computer. A 20- × 200-pixel transect across the center of the image, which represented a 150 mm^2 area of the culture, was sampled, and the average relative brightness was determined. Statistical analysis was performed as above.

DsRNA isolation. DsRNA was isolated from 7- to 10-day-old cultures essentially as described by Morris and Dodds (27). Quality and quantity of dsRNA from each isolate was assessed by agarose gel electrophoresis as described previously (19).

Complementary DNA reactions and PCR. First strand cDNA was synthesized from 1 μg of dsRNA primed with random hexadeoxyribonucleotides essentially as described previously (20). Reaction products were diluted in distilled water; typically a 1:10 to 1:100 dilution was used for PCR.

Three sets of specific oligonucleotides from different sites of the CHV2-NB58 genome were used as primers to amplify cDNA fragments from the first strand cDNA reactions (Fig. 2). Based on alignments with CHV1-EP713, these were predicted to be the least (primer set 1), intermediate (primer set 2), or most conserved (primer set 3), respectively. Reaction mixtures (100 μl) consisted of 10 μl of the cDNA dilution, 10 μl of 10× PCR buffer (500 mM KCl, 100mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, and 0.01% [w/v] gelatin), 20 μl of oligonucleotide primers (10 ng/μl), 10 μl of 2 mM dNTP, and 0.5 μl of Ampli*Taq* polymerase (5 units/μl) (Perkin-Elmer Cetus, Norwalk, CT). Amplification was accomplished by 35 cycles with the following parameters: denaturation at 92.5 C for 1.5 min, annealing at 52.5 C for 1.5 min, and extension at 72 C for 1 min. Reaction products were analyzed by electrophoresis through 1% agarose gels.

Nucleotide sequence analysis. PCR products were purified by agarose gel electrophoresis and removed from the gel by binding and elution from powdered glass using Geneclean (Bio 101, Inc., La Jolla, CA). Sequencing reactions of PCR products primed with oligonucleotides NB58-50 and NB58-52 were performed with reagents and protocols of the CircumVent thermal cycle dideoxy DNA sequencing system (New England Biolabs, Beverly, MA) and [^{35}S]dATP (Dupont Company, Wilmington, DE). Analysis of reaction products was completed by electrophoresis on 5% Long Ranger (J. T. Baker Inc., Phillipsburg, NJ) sequencing gels, which were dried and exposed to XAR film (Kodak) for 24–48 h.

Sequence analysis was aided by the software programs CLUSTAL V (15) and PHYLIP (10). Sequences determined in this study were compared to CHV1-EP713 and CHV2-NB58 (18,34).

RESULTS

Growth and culture morphology. Culture morphologies of *C. parasitica* isolates are described in Table 1. None of the hypovirulent isolates from New Jersey exhibited the white phenotype

TABLE 1. Descriptions of *Cryphonectria parasitica* isolates[a]

Isolate	Site[b]	Year	Tree	HV or V	Color	Aerial mycelium[c]	Lobed margin[c]
NB58	Five Points	1988	18	HV	Brown	−	−
SC58-19	Five Points	Lab isolate	18	V	Orange	+	±
NB548	Five Points	1989	12	HV	Brown	−	−
NB599	Five Points	1989	28	HV	Borwn	±	+
NB601	Five Points	1989	28	HV	Brown	±	+
NB621	Five Points	1989	42	HV	Brown	−	−
NB724	Five Points	1992	18	HV	Brown	±	+
NB725	Five Points	1992	25	HV	Brown	−	+
23-4C	Five Points	1989	23	HV	Brown	−	+
NB459	Navesink[d]	1989	RB14	HV	Brown	±	+
NB468	Navesink[d]	1989	RB14	HV	Brown	±	±
NB691	Navesink[e]	1992	HC3	HV	Brown	−	+
EP155	Connecticut			V	Orange	+	−
EP713	French-derived			HV	White	+	±
Bennet 23-X1	Rhode Island			HV	Brown	−	±

[a]Tree numbers correspond to Figure 1. HV or V = hypovirulent or virulent. Color of colonies, amount of aerial mycelium, and whether margins were lobate in culture were not quantified but represent a gross visual description of these properties.
[b]Five Points Road area or Navesink area of central-eastern New Jersey.
[c]Aerial mycelium and lobed margin: − = not prominent; + = prominent; ± = intermediate.
[d]Sutter's Woods.
[e]Huber Conservation Area.

typical of European-derived isolate EP713. Although they were somewhat variable in colony morphology, all hypovirulent isolates were brown to orange-brown in color and darker than virulent isolates examined in this study. DsRNA-containing isolates were easily distinguished from dsRNA-free isolates by their colony morphologies. This parallels results for NB58 and its isogenic, dsRNA-free counterpart, SC58-19. The reduction of aerial mycelium apparent in strain NB58 also was evident in other New Jersey hypovirulent isolates.

Significant effects ($P < 0.001$) of isolate and media on radial growth were identified by analysis of variance (ANOVA). The interaction of isolate and media also was significant ($P < 0.05$), so separate analyses were done for each medium. Mean radial growth of isolates on PDAmb ranged from 30.9 mm for EP155 to 16.0 for NB725 (Fig. 3A). In contrast, analysis of radial growth experiments for a subset of six isolates on PDAmb and PDAmb/2 identified a significant isolate effect ($P < 0.001$) but not a significant media effect ($P < 0.05$).

Virulence assay. Average untransformed lesion size on Granny Smith apples, expressed as a percentage of the radial growth of EP155, ranged from 1.3% for NB599 to 90.0% for SC58-19 (Fig. 3B). Data were transformed by taking the natural logarithm of the percent radial growth to correct for heterogeneity of sample variances. ANOVA identified a significant isolate effect ($P < 0.05$) on the size of lesions produced. Virulent isolate SC58-19 produced lesions that were significantly larger ($P < 0.05$) than the 11 hypovirulent isolates. Hypovirulent isolate 23-4C produced significantly larger lesions ($P < 0.05$) than the other hypovirulent isolates with single-dsRNA species.

Phenol oxidase assay. ANOVA identified a significant isolate effect ($P < 0.05$) on the relative brightness of *C. parasitica* on MTAA, transformed by natural logarithms to correct for heterogeneity (Fig. 3C). Relative brightness values ranged from 0 to 250: 0 was black, and 250 was white. Average untransformed values ranged from 27.5 for SC58-19 to 53.8 for NB621, respectively. The two virulent isolates (SC58-19 and EP155) were significantly darker ($P < 0.05$) than the hypovirulent isolates. Significant differences ($P < 0.05$) among hypovirulent isolates also were observed.

DsRNA analysis. By gel electrophoresis, dsRNAs from all New Jersey isolates in this study were indistinguishable from CHV2-NB58. Representatives are shown in Figure 4. With the exception of CHV1-EP713, all dsRNAs examined had only one segment of similar mobility. In fact, all dsRNA-containing New Jersey isolates examined to date contain a single dsRNA segment. DsRNA from one isolate, NB631 from tree 72, was phylogenetically unrelated to all other New Jersey isolates (J. J. Polashock, P. J. Bedker, and B. I. Hillman, *unpublished data*) and was not included in this study. Excluding multiple isolates from a single canker, 19 of 94 (20.2%) of New Jersey *C. parasitica* isolates collected in intensive sampling from the Five Points, Navesink, and nearby areas during 1990 contained dsRNA.

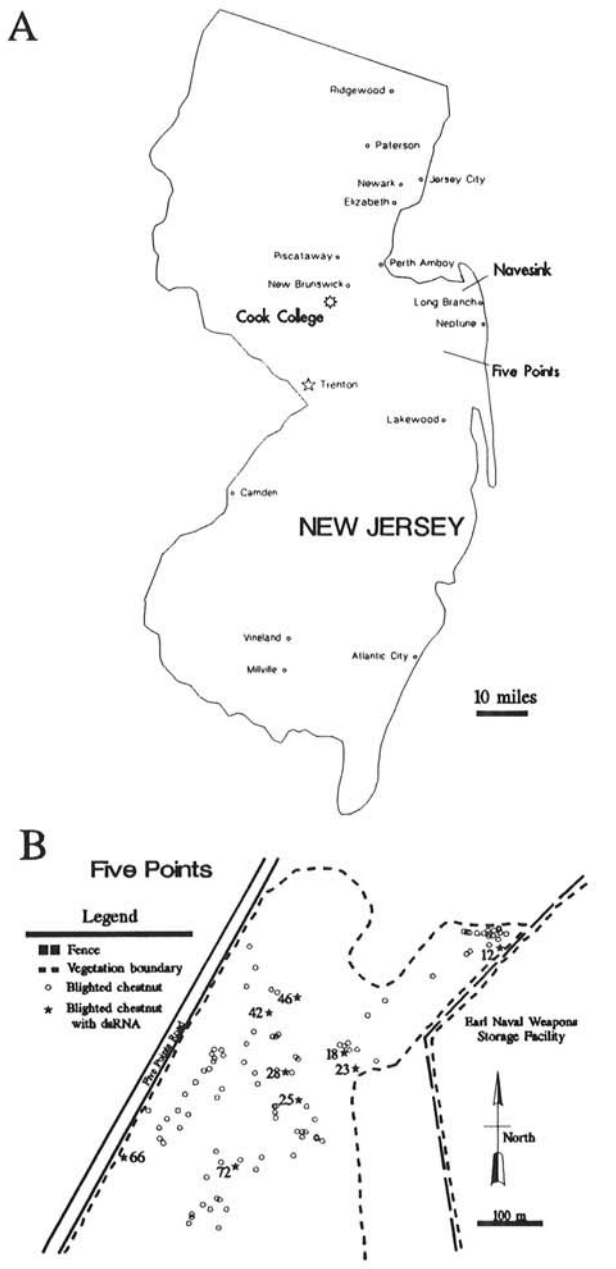

Fig. 1. Sites of *Cryphonectria parasitica* isolate collection. **A,** The Huber Conservation Area and Sutter's Woods, contiguous sites near Navesink, and the Five Points Road site in Howell Township are approximately 10 miles apart in central-eastern New Jersey. **B,** Most samples were collected from the Five Points Road area. Trees from which the double-stranded RNA-containing isolates referred to in the text and Table 1 were collected are numbered; the trees from which double-stranded RNA-free isolates were collected are indicated with circles. Isolates from trees 46, 66, and 72 were not used in this study.

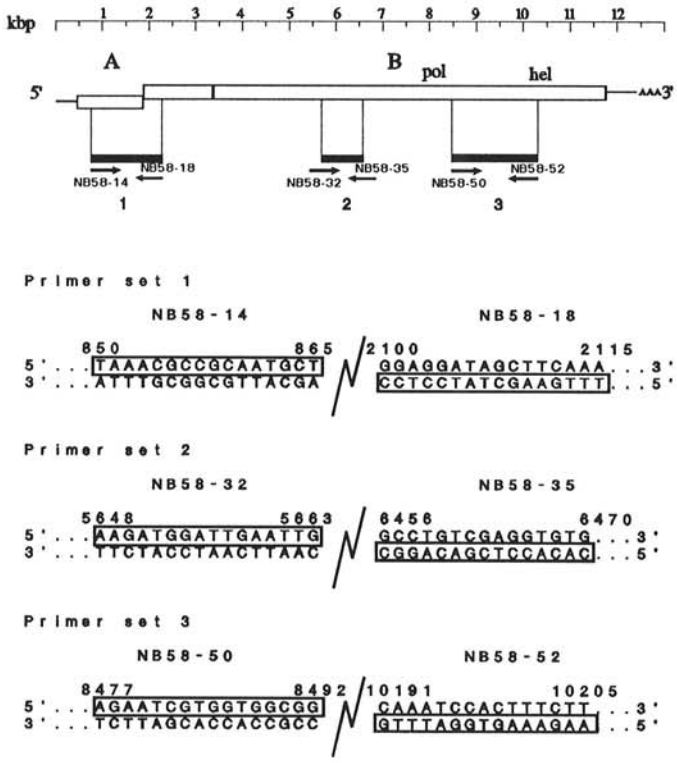

Fig. 2. Positions and sequences of primer sets used for polymerase chain reaction amplification. The approximate sizes of expected amplification products are shown below the genomic map. Sequences of plus strand (5′→3′) and minus strand (3′→5′) are presented.

PCR analysis. Results of PCR amplifications are presented in Table 2. The only dsRNA from New Jersey that did not amplify was NB691. In the case of dsRNAs that failed to amplify, PCR experiments were repeated at least three times with independent dsRNA preparations. Oligonucleotides NB58-50 and NB58-52 primed synthesis of the predicted 1.7-kb products for 10 of the 11 dsRNAs. Oligonucleotides NB58-32 and NB58-35 amplified 0.8-kb products from eight isolates, whereas NB58-14 and NB58-18 amplified 1.3-kb products from five of the dsRNAs.

Nucleotide sequence analysis. DsRNAs that were successfully amplified with oligonucleotides NB58-50 and NB58-52 were sequenced on the upper strand with oligonucleotide 50 and the lower strand with oligonucleotide 52. At least 200 residues were readable from each of the reaction sets. Sequences were aligned with the homologous region of CHV2-NB58 and CHV1-EP713 with the program CLUSTAL V (15) (Fig. 5). Sequences were combined, phylogenetic trees were inferred with the program DNAPARS, and phenograms were drawn with the program DRAWGRAM, both part of the PHYLIP package (10) (Fig. 6). DsRNAs of NB58, NB621, and 23-4C clustered together, as did NB601 and NB599.

Besides NB724 (see below), none of the sequences from different isolates were identical nor were they identical to the original CHV2-NB58 sequence. To examine the variation among the sequences from these different dsRNAs, deduced translation products were determined, and amino acid variation was compared using CHV2-NB58 as the base isolate (Table 3). Most of the changes noted were in the third position of each codon and were nucleotide transitions (A→G, G→A, C→T, or T→C,) that caused no amino acid differences.

Stability of dsRNA sequences within a fungal isolate or from different isolates from the same canker at different times were examined by extracting dsRNA from strain NB58 after maintenance by subculture for 4 yr and from a 1992 isolate (NB724) from the original canker. These sequences were identical, indicating that there was no change in CHV2-NB58–related sequences

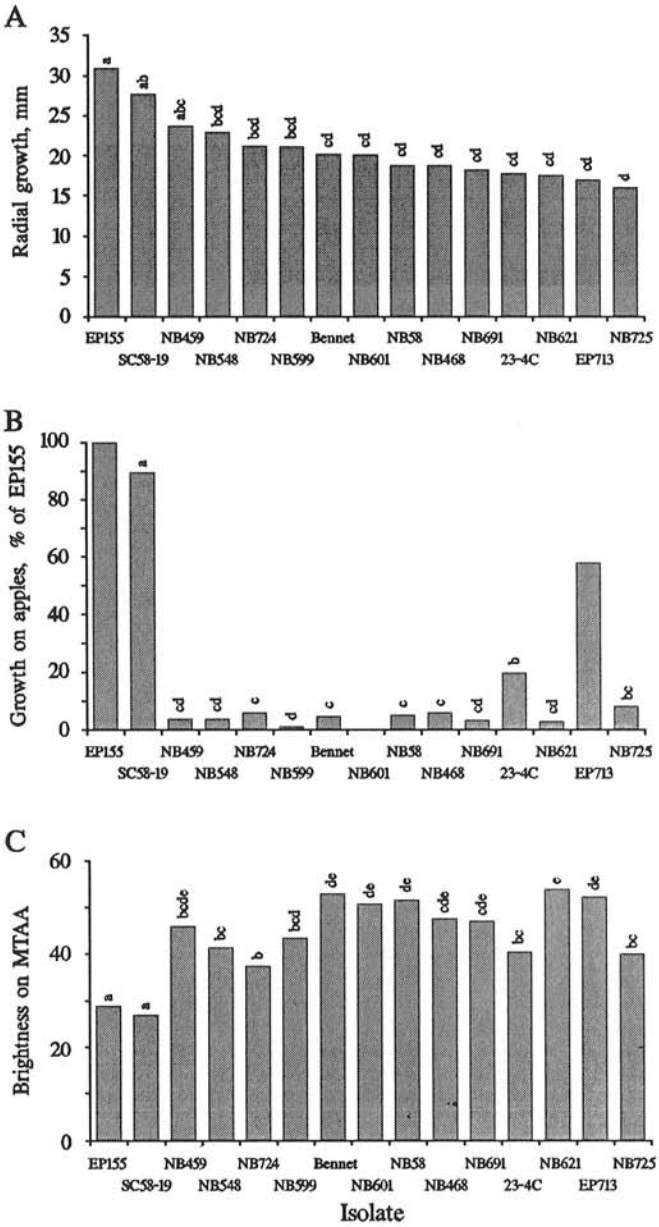

Fig. 3. Properties of *Cryphonectria parasitica* isolates. Significant differences ($P < 0.05$) among isolates were identified by Tukey's studentized range test and are indicated by different letters above the bars. **A,** Radial growth of *C. parasitica* isolates on potato-dextrose agar amended with methionine and biotin. Mean radial growth was measured 7 days postinoculation. **B,** Average untransformed size of *C. parasitica* lesions on Granny Smith apples, 16 days postinoculation, expressed as a percentage of the radial growth of virulent strain EP155. The average radial growth of EP155 was 19.6 mm ($s = 3.7$, $n = 69$). Isolate NB601 was not included in this study. The result for EP713 is from a separate experiment and is included here for reference only. **C,** Relative laccase activity of *C. parasitica* isolates. Relative brightness values of isolates grown on malt and tannic acid agar were measured as described in the text.

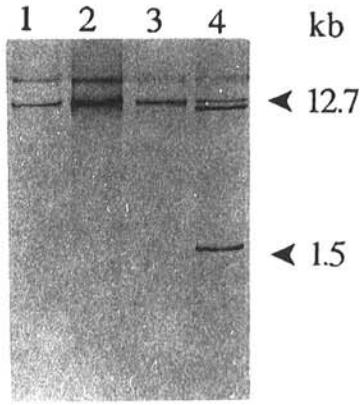

Fig. 4. Silver-stained polyacrylamide gel of four double-stranded (ds)RNA isolates used in this study. DsRNAs were isolated, as described in the text, from the following fungal isolates: lane 1: NB467; lane 2: NB58 (overloaded to show the absence of low molecular weight dsRNAs); lane 3: Bennet 23-X1 from Rhode Island; and lane 4: EP713. Stained material in the upper portion of the gel is caught at the interface of the 5% stacking gel and the 8% resolving gel.

TABLE 2. Summary of polymerase chain reaction (PCR) amplification results for complementary DNA of double-stranded (ds)RNAs from 13 *Cryphonectria parasitica* isolates primed with three pairs of primers[a]

	Primer pairs		
Isolate	NB58-14,18	NB58-32,35	NB58-50,52
NB58	+	+	+
NB724	+	+	+
NB621	+	+	+
23-4C	+	+	+
NB459	+	+	+
NB548	−	+	+
NB599	−	+	+
NB601	−	+	+
NB468	−	−	+
NB725	−	−	+
NB691	−	−	−
EP713	−	−	−
Bennet 23-X1	−	−	−

[a] Primer pairs and conditions are described in the text and in Figure 2. A positive result (+) indicates a PCR product of the predicted size based on NB58 results.

in this region of the genome, in the forest or in culture, since the original isolation.

DISCUSSION

This study represents the first molecular examination of sequence divergence among members of a population of interrelated *C. parasitica* isolates from a given geographic area. Meaningful comparisons can be made with results from other less-detailed studies focusing on *C. parasitica* or with other studies focusing on genetic drift among viruses in a population.

Hypovirulent isolates of *C. parasitica* from New Jersey showed differences in colony morphology, virulence, and phenol oxidase activity. Although some of the differences noted in colony morphology were consistent and reproducible, most were minor and somewhat inconsistent. Therefore, colony morphology on PDA cannot be used as a reliable indicator to differentiate New Jersey hypovirulent isolates. Variation in virulence among hypovirulent isolates was relatively small. The most notable variation was in 23-4C, which was significantly more virulent than the other dsRNA-containing isolates examined.

Although its role in the biology of *C. parasitica* is unclear, laccase activity is usually reduced in dsRNA-containing isolates, and therefore, it has been presented as one of the hypovirulence-associated traits of the fungus (19,32). We quantified colors of colonies growing on MTAA as an indicator for activity of the laccase enzyme. Numbers obtained were consistent with our visual approximations of these plates. Although laccase activity was significantly lower in hypovirulent than virulent isolates, there was no correlation between virulence and laccase activity among hypovirulent isolates, supporting observations by Choi and Nuss that these dsRNA-suppressed traits are not directly connected (6).

Elliston (9) noted that hypovirulence-associated phenotypes exhibited by *C. parasitica* isolates are most dependent on dsRNAs rather than host genotype. Our examination of more than 600 isolates from New Jersey (including multiple isolates from the same canker; P. J. Bedker and B. I. Hillman, *unpublished data*) confirms these observations; however, we have noted consistent differences in morphology, virulence, and laccase activity among virulent, dsRNA-free strains. Differences in morphology, growth rate, virulence, and laccase activity among hypovirulent isolates in this study may suggest that individual dsRNAs modulate host gene expression to different extents, but these variations also could be due to the genomic diversity among fungal isolates.

RNA-RNA hybridization studies by Paul and Fulbright (29) indicate that hypovirulence-associated dsRNAs from Michigan are somewhat variable both in their genomic structures and phenotypic effects on their host fungi. Studies by L'Hostis et al (22)

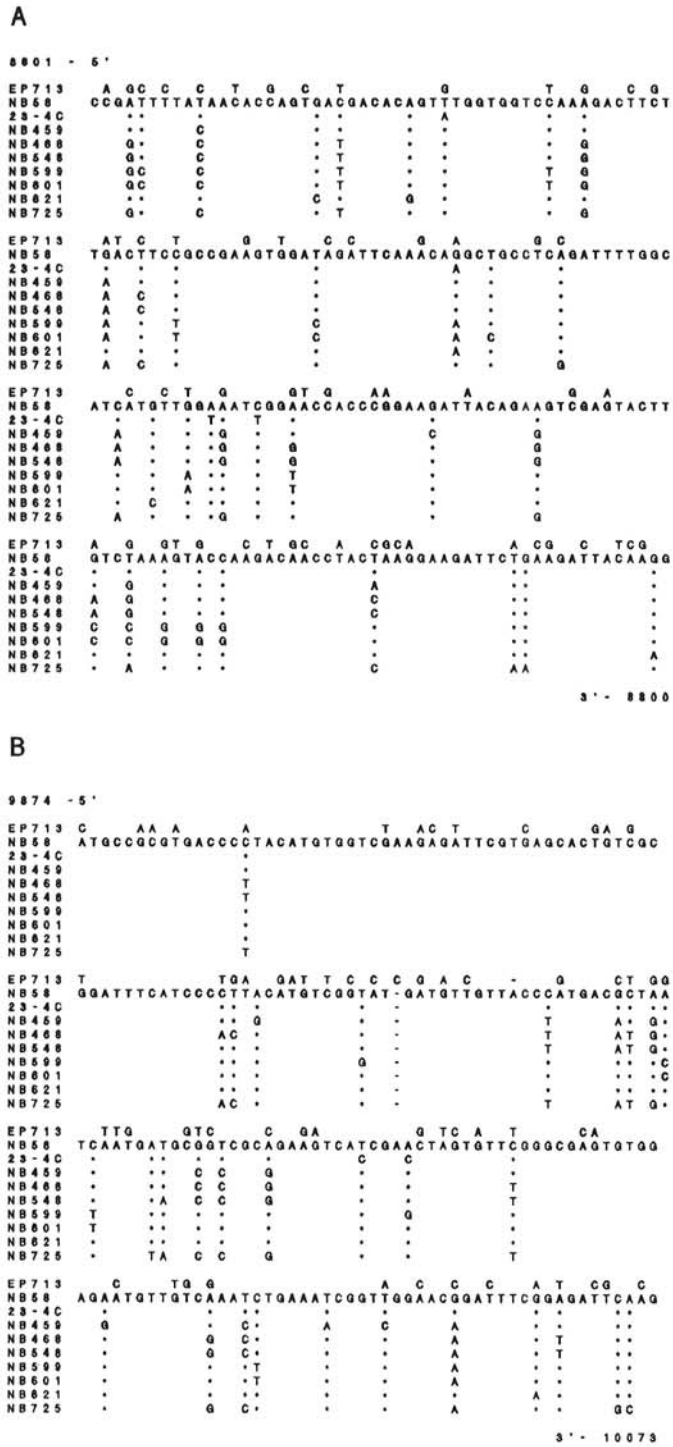

Fig. 5. Alignments of nucleotide sequences of eight double-stranded RNAs from the New Jersey population primed with **A,** oligonucleotide NB58-50 and **B,** oligonucleotide NB58-52. Alignments are relative to the CHV2-NB58 sequence (GenBank Accession L29010), shown in full. The CHV1-EP713 sequences in the same regions (34) are shown above the CHV2-NB58 sequence. The origins of the isolates are described in Table 1.

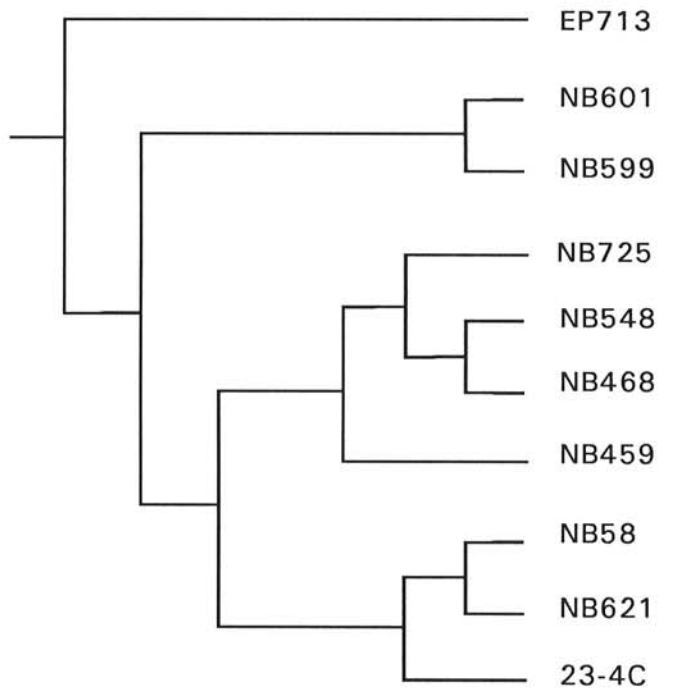

Fig. 6. Dendrogram drawn from alignments of nucleotide sequences presented in Figure 5. The phenogram was drawn with the DRAWGRAM program of PHYLIP, version 3.4 (10). This was one of three possible trees. The other two were identical in their grouping of sequences from EP713, NB601, NB599, NB58, NB621, and 23-4C. They varied slightly in groupings of NB725, NB548, NB468, and NB459.

TABLE 3. Sequence differences within combined 400-nucleotide regions (Fig. 5) and predicted amino acid sequences for New Jersey double-stranded (ds)RNAs primed with oligonucleotides NB58-50 and 52[a]

	New Jersey isolates							
	23-4C	NB459	NB468	NB548	NB599	NB601	NB621	NB725
Nucleotide differences	6	20	28	27	23	22	6	32
Differences in codon position								
1	0	1	2	1	3	3	1	3
2	2	1	1	1	1	1	0	3
3	4	18	25	24	19	18	4	26
Transition	3	14	23	22	18	18	4	23
Transversion	3	6	5	5	5	4	2	9
Nucleotide differences resulting in								
No amino acid change	3	17	27	25	19	18	3	27
Amino acid changes	3	3	1	2	4	4	3	5
Conservative amino acid substitution	1	2	1	2	1	1	1	2
Nonconservative amino acid differences	2	1	0	0	3	3	2	2

[a]Amino acid results for oligonucleotide 52 are from a sequence converted to the positive-sense.

demonstrate the similarity of European isolates to each other and the differences from North American isolates used in the study. New Jersey isolates showed more homogeneity in dsRNA structure and similarity in their effect on the phenotype of the host fungus.

Only one New Jersey dsRNA was not amplified with oligonucleotides NB58-50 and NB58-52. Of the seven dsRNAs from the Five Points Road area, only four (including NB724) could be amplified by all three primer sets. The remaining three dsRNA isolates amplified with one or two primer sets. Diversity was found even in dsRNAs isolated from different cankers on the same tree. For example, NB459 and NB468 were both isolated from Navesink tree RB14 in 1989 and were successfully amplified by primers NB58-50 and NB58-52, but only NB459 dsRNA could be amplified by the two primer sets NB58-14 and NB58-18 and NB58-32 and NB58-35. These two isolates were not vegetatively compatible and were probably the result of independent infections. Isolates NB599 and NB601 also came from different cankers. In this case, PCR amplification and nucleotide sequence analysis revealed that these dsRNAs were almost identical, with only a single different nucleotide in the sequenced regions. NB599 and NB601 were vegetatively compatible, so it is reasonable to predict that one represents a secondary infection from the other or that they are independent infections from another hypovirulent strain.

Isolate NB691 from the Huber Conservation Area, Navesink, was the only dsRNA from New Jersey that could not be amplified in this study. Two hypovirulence-associated dsRNA isolates from outside New Jersey, CHV1-EP713 and the dsRNA of Bennet 23-X1, also could not be amplified from any set of primers. Although the dsRNA of CHV1-EP713 hybridized with CHV2-NB58 cDNA clones corresponding to the region amplified by oligonucleotides NB58-50 and NB58-52 (20), similar hybridization information was not available for Bennet 23-X1. We therefore do not know how closely related the isolate Bennet 23-X1 is to other isolates in this study.

NB724 was isolated from the same tree as NB58 but was collected 4 yr later. DsRNA from NB724 was successfully amplified by all three primer pairs, and the sequence derived from oligonucleotides NB58-50 and NB58-52 proved to be identical to that of CHV2-NB58. Thus, regardless of whether the CHV2-NB58-infected fungus survived in the original canker or was maintained in culture in the laboratory, no viral sequence drift was observed. This strongly suggests that NB724 carries the same cytoplasmic dsRNA (CHV2-NB58) as fungal strain NB58. The observation that strain NB724 was able to pass its dsRNA to strain SC58-19 provides evidence that the fungal strain is the same as well, although stronger confirmatory experiments, such as DNA fingerprint analysis (25), have not been performed. The minor differences between NB58 and NB724 in hypovirulence-associated traits and in colony morphology, therefore, may be explained by a mutation in the fungus that did not affect the vegetative compatibility system, by the presence of an undetected element besides the large dsRNA, or by variation in dsRNA sequences outside those analyzed.

Hypovirulence-associated dsRNAs, such as those described here, are transmitted vertically only through conidia, not ascospores (1,12). Therefore, dispersal by conidia may be important for the spread of the dsRNAs. Results of spatial pattern analysis (26) suggest that isolates representing a single vegetative compatibility group are often found on a given tree, consistent with previous observations (4,23,28). Furthermore, Garrod et al (13) suggested that conidia commonly infected wounds on the same tree from which they originated but found only limited evidence for conidia dispersal from one tree to another. The large number of vegetative compatibility groups in North American forests would be predicted to restrict long-distance movement of a particular dsRNA, although this has not been formally demonstrated.

Variation among sequences examined in this study was high but was within the range of other RNA viruses in similar studies. Although sequence drift is often high in RNA viruses (21), this is not always the case. Some plant viruses are extremely stable genetically through many passages in a particular host (16). It is unknown how the lack of a protein coat might influence the evolutionary rate of these dsRNAs, but the stability noted in the CHV2-NB58 sequence would suggest that this is not a major factor. We had no evidence in the current study for greater sequence variation between the relatively close geographic sites of Five Points and Navesink than within those sites, but the small sample sizes preclude drawing any conclusions based on the data.

Although the taxonomy of the Hypoviridae family is now firmly established, important details will be added as more information accumulates. As the genomic structures of more species and strains of the genus *Hypovirus* are examined from within and across populations, more expansive phylogenetic trees can be drawn and a better understanding of the viral evolutionary processes will be gained. This will be particularly interesting when connected to the genotype of the fungal host.

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