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# Genome analysis of *Cryphonectria hypovirus 4*, the most common hypovirus species in North America

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**Abstract**

Many different viruses that reduce virulence and alter the phenotype to varying extents have been identified in the chestnut blight fungus *Cryphonectria parasitica*. Most viruses identified in this fungus fall within the *Hypoviridae* family of positive-sense RNA viruses, which contains one genus and four species. Different species predominate in different geographic locations in chestnut-growing areas around the world. In this paper, we describe the genome organization and some variants of *Cryphonectria hypovirus 4* (CHV-4), the species most commonly found in eastern North America. CHV-4 is distinguished from other hypoviruses by having little effect on fungal virulence and colony morphology. The 9.1-kb genome of strain CHV-4/SR2 is the smallest of any member of the family characterized to date. Like the recently characterized species CHV-3, a single ORF was predicted from deduced translations of CHV-4/SR2. Sequence analysis revealed the presence of a putative glucosyltransferase domain in both CHV-4 and in CHV-3, but no such homolog was detected in the more thoroughly examined CHV-1 or in CHV-2. Alignments with 8 other CHV-4 isolates from different regions of eastern North America revealed sequence diversity within the species and the likelihood that RNA recombination has led to this diversity.


*Keywords:* Chestnut blight; *Cryphonectria parasitica*; Hypovirus; Hypovirulence; Fungal virus; Glucosyltransferase; RNA recombination

## Introduction

*Cryphonectria parasitica*, the filamentous fungus that causes chestnut blight, originated from East Asia and leads to relatively mild infection of native chestnuts. The fungus established in North America early in the 20th century where it found highly susceptible chestnut hosts and spread very rapidly; the pathogen became established in Europe 30–40 years later. A number of different viruses have been identified in *C. parasitica* in those three continents. Most of the viruses belong to the family *Hypoviridae* and are generically called hypoviruses (Hillman et al., 2000b).

When and where hypoviruses arrived in the latter two regions are unknown, but the resulting virus populations and their effects on the chestnut blight epidemics were markedly different. In Europe, a single hypovirus species (CHV-1) spread relatively quickly in the fungal population, likely slowing the destruction of the chestnut stands (Heiniger and Rigling, 1994). On the contrary, the fungus decimated the American chestnut, and virus presence was apparently not a factor in slowing the epidemic (Milgroom and Cortesi, 2004).

The overall incidence of hypoviruses in the *Cryphonectria* population of North America is higher (28%) than that observed in China (2%) or in Japan (6%) (Peever et al., 1997, 1998). Three major hypovirus groups were originally identified in North America through hybridization tests: CHV-2 in New Jersey (Hillman et al., 1994), CHV-3 found predominantly in Michigan (Paul and Fulbright, 1988; Smart et al., 1999; Yuan and Hillman, 2001) and in Ontario (Melzer and Boland, 1999), and the “SR2-type” dsRNA

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widespread throughout eastern North America (Enebak et al., 1994; Peever et al., 1997). The few CHV-3 isolates found outside of Michigan (in West Virginia and Kentucky) were likely the result of an early release for biological control (Peever et al., 1997). CHV-1 is predominant in Europe (Heiniger and Rigling, 1994) and in Asia (Peever et al., 1998) but largely absent in North America in spite of several deliberate releases.

Virulence levels among different hypoviruses vary considerably. CHV-1 infection dramatically reduces pigmentation, sporulation, and virulence, and results in female sterility of the fungus (Hillman et al., 1990), but variation within the species has been observed (Chen et al., 1996). CHV-2 is the most debilitating hypovirus in terms of reducing fungal virulence, and also interferes with fungal development and fecundity (Hillman et al., 1992, 1994). CHV-3 has less impact on fungal colony morphology than CHV-1 or 2, but it too reduces the virulence of *C. parasitica* substantially (Fulbright, 1984; Smart et al., 1999).

The three previously characterized hypovirus species differ in genome size and organization (Fig. 1). The CHV-1 genome is 12.7 kb and contains two open reading frames, ORF A and ORF B, divided by a translational stop–start (Shapira et al., 1991). ORF A encodes a papain-like protease that cleaves its two products, p29 and p40 (Choi et al., 1991). The N-terminal product of the ORF B polyprotein is a paralog of the ORF A protease and is followed by a region with RNA-dependent RNA polymerase (RdRp) and helicase (Hel) motifs (Shapira and Nuss, 1991; Shapira et al., 1991). The 12.5-kb CHV-2 genome also contains two ORFs. ORF A encodes a 50-kDa protein whose N-terminal region is similar to the N-terminal region of CHV-1 p29 and whose C-terminal region is similar to CHV-1 p40. ORF B of CHV-2 is organized similarly to ORF B of CHV-1, with a few differences of unknown significance (Hillman et al., 1994). CHV-3 has a genomic RNA of 9.8 kb that contains a single ORF encoding a large polyprotein with a cis-acting papain-like protease at the N-terminus and conserved RdRp and Hel domains downstream (Hillman et al., 2000a; Smart et al., 1999; Yuan and Hillman, 2001). Details of possible proteolytic processing of the C-terminal two-thirds of the large polyprotein have not been reported for any hypovirus.

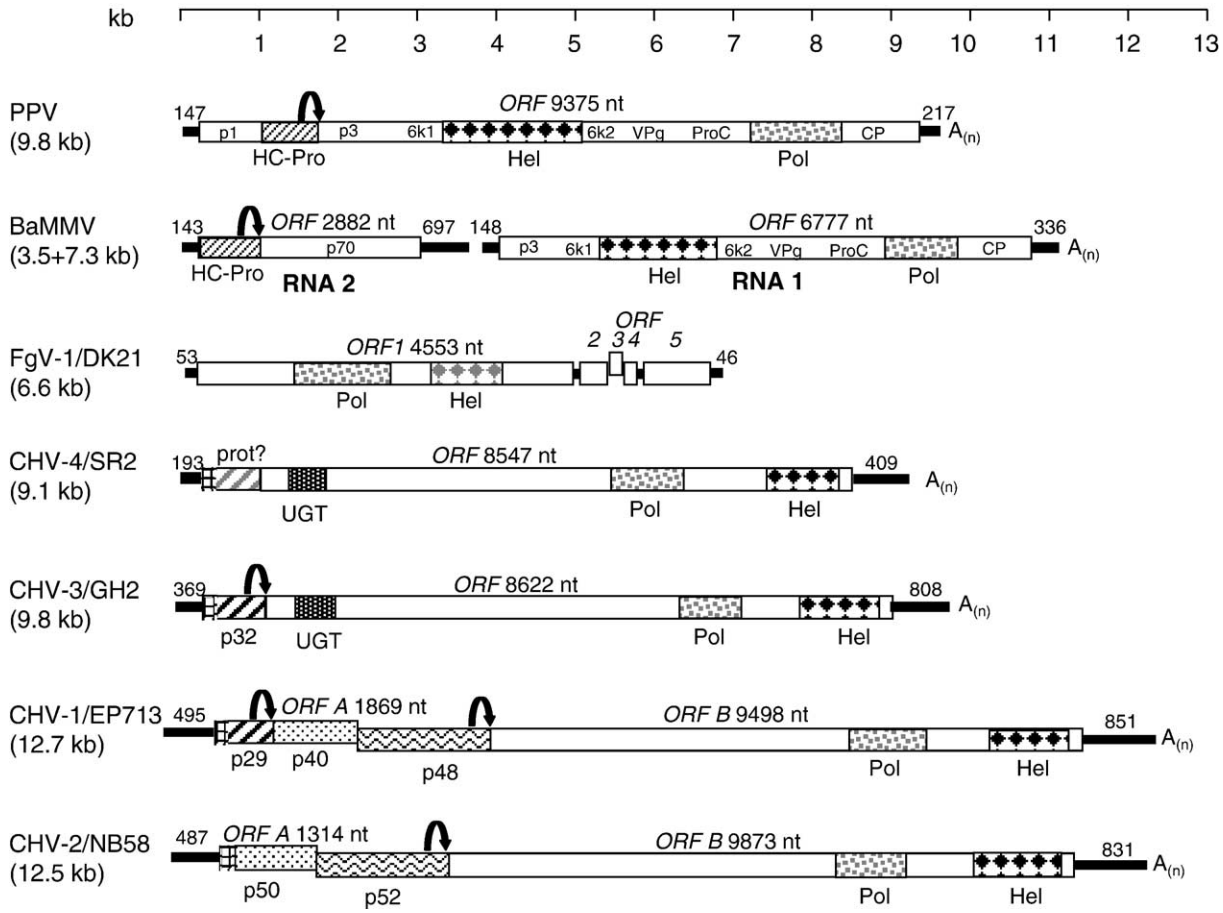


Fig. 1. Graphic representation of the genome organization of CHV-4/SR2 (accession no. AY307099) compared with CHV-3/GH2 (AF188515), CHV-1/EP713 (M57938), and CHV-2/NB58 (L29010), *Fusarium graminearum* virus 1/DK21 (FgV-1/DK21; AAT07067), *Barley mild mosaic virus* (BaMMV; P90245), and *Plum pox virus* (PPV; 628428). Hatched patterns in the ORF represent homologous sequences; thick black lines indicate the 5′- and 3′-end UTR. Arrows indicate the cleavage sites of cis-acting proteinases; the question mark indicates the potential proteinase in CHV-4/SR2, which has not yet been confirmed. Abbreviations indicate the following: UGT glucosyltransferase, Pol RNA-dependent RNA polymerase (also RdRp in text), Hel helicase domain. A poly(A)-tail at the 3′-end of the (+) strand of each virus is indicated as $A_{(n)}$.

The genome of CHV-4/SR2, the subject of this study, was previously identified as a single dsRNA of ~9 kb present at very low titer that does not cross-hybridize with CHV-1, -2, or -3 (Enebak et al., 1994). In a large study covering the entire natural range of American chestnut, dsRNA hybridizing to CHV-4/SR2 was recovered from 26% of the *C. parasitica* isolates examined (Peever et al., 1997). Unlike CHV-1, -2, or -3, most strains of CHV-4 do not substantially debilitate the host. Virulence of infected isolates is generally high and significant damage or death to infected American chestnut trees results (Enebak et al., 1994). In this regard, CHV-4/SR2 is similar to many other fungal viruses, which tend to be asymptomatic (Buck, 1986; Ghabrial, 1994).

The purpose of this study was to examine the genome organization of CHV-4/SR2 in comparison to other hypoviruses, and to begin to examine variability among these viruses in North America. Two observations lead to the conclusion that the divergence of the CHV-1 and -2 lineage from the CHV-3 and -4 lineage was not recent. The first was the surprising finding of a glucosyltransferase domain in CHV-3 and -4 but absent in CHV-1 and -2; the second was the identification of a closer relationship between the CHV-3 and 4 RdRp domains and the RdRp of a recently characterized virus from *Fusarium graminicola* than to the RdRps of CHV-1 and -2. Partial sequence analysis of a few related viruses from different regions of the United States provides a preliminary view of the diversity within the species and shows that recombination has likely occurred in at least two instances.

## Results and discussion

### *Nucleotide sequence and genome organization*

The 9149-nt CHV-4/SR2 dsRNA contains a 5′-untranslated region (UTR) of 193 nt followed by a single putative open reading frame (ORF) of 8547 nt and a 3′-terminal UTR of 409 nt (Fig. 1). A 333-nt ORF in an alternative frame at nt 6111–6443 is unlikely to be expressed because of its position on the genome and the lack of evidence for subgenomic RNA in this and other hypoviruses. CHV-4/SR2 has a poly(A)+ tail at the 3′ terminus of the positive strand, as do other hypoviruses. The average number of adenosine residues in the poly(A)+ tail has not been determined.

### *5′-UTR*

The 193-nt 5′-UTR is the shortest among hypoviruses characterized to date. Like other hypoviruses, the CHV-4/SR2 5′-UTR contains multiple mini-ORFs, with 4 AUG codons preceding the large ORF. The first 100 nt of the 5′-UTR include 21 nt at positions 75–95 that correspond to a conserved region in the genomic, defective, and satellite dsRNA elements of CHV-3/GH2 (Smart et al., 1999) (Fig. 2A), and an overall identity level of 63%. The rest of the 5′-UTR shows negligible sequence identity.

### *Coding sequence*

At the N-terminal portion of the deduced translation product, sequence similarity with the corresponding region of other hypovirus polyproteins is low: only 12% amino acid identity was found between CHV-4 and its closest relative CHV-3 (Fig. 3). In most parts of this region, these two sequences could not be aligned, and alignments of CHV-4 with CHV-1 or -2 in this region were also not informative. All other members of the *Hypoviridae* family encode at least one papain-like cysteine protease (Fig. 1) that is characterized by a cysteine-rich amino terminal domain, two key residues (Cys and His) required for the autoproteolytic activity, and a cleavage site located not far downstream from the active residues (Hillman et al., 1994; Koonin et al., 1991; Smart et al., 1999). The putative

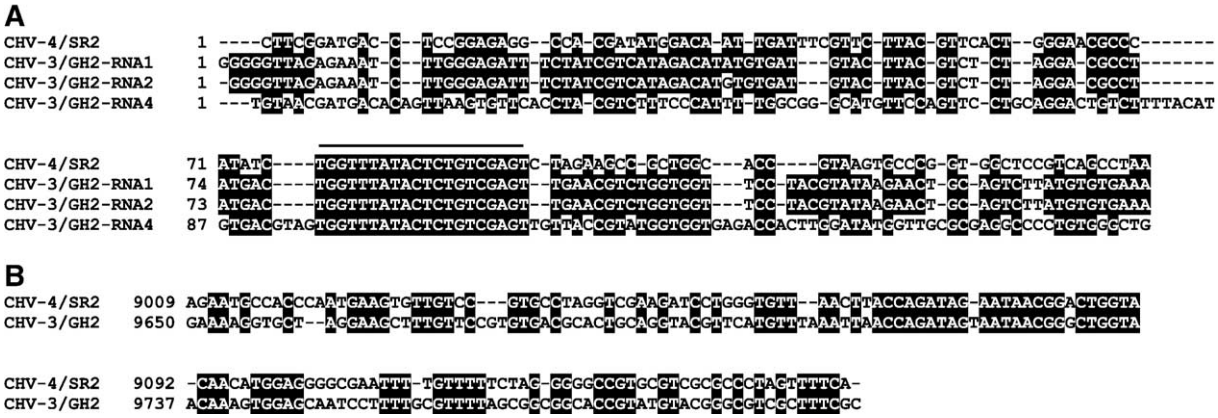


Fig. 2. Alignment of the 5′-UTR (A) and 3′-UTR (B) of CHV-4/SR2 with CHV-3/GH2 genomic (1), defective (2), and satellite (4) RNAs (Hillman et al., 2000a; Smart et al., 1999) obtained with ClustalW (Thompson et al., 1994) and corrected by hand. Blackened nucleotides are identical. The overlined region indicates the conserved region among all the CHV-3/GH3 segments and CHV-4/SR2.

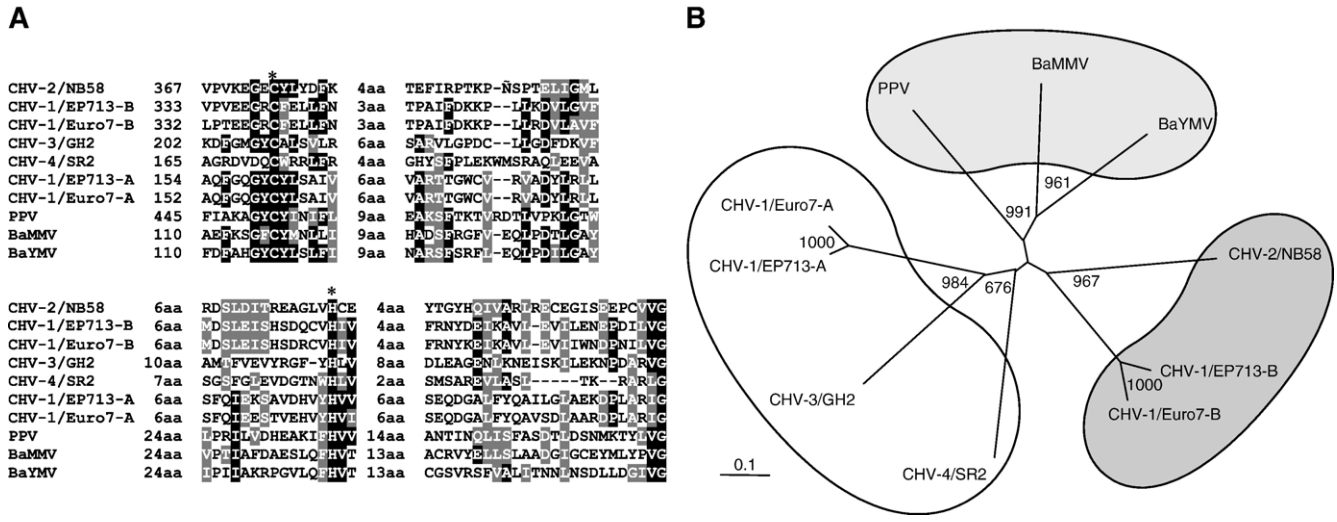


Fig. 3. Alignment (A) and phylogenetic tree (B) of the putative protease region of CHV-4/SR2 compared those demonstrated for other hypoviruses and potyviruses. Sequences were aligned with ClustalX (Thompson et al., 1997). Identical amino acids in the alignment are shaded in black and functionally similar amino acids in gray, with the number of amino acid residues between the conserved region shown. The asterisk (*) indicates the catalytic Cys and His residues demonstrated for CHV-1, CHV-2, and CHV-3. Accession numbers are as in Fig. 1, with the addition of CHV-1/Euro7 (AF082191) and *Barley yellow mosaic virus* (BaYMV; 4210318). The unrooted, bootstrapped phylogram (1000 repetitions) was drawn with TreeView (Page, 1996).

protease of CHV-4/SR2 contains several Cys and His residues that could potentially represent the catalytic site of a papain-like proteinase. $His^{223}$ of CHV-4/SR2 most closely aligns with the putative catalytic $His^{262}$ of CHV-3/GH2; $Cys^{173}$ would be the predicted catalytic Cys residue of CHV-4/SR2 based on its position approximately 50 residues upstream of $His^{223}$ (Fig. 3A). Proteolytic activity of this region has not yet been examined.

Downstream from the putative protease, CHV-4/SR2 contains a region that is homologous to UDP-glucose/sterol glucosyltransferase (UGT) (Fig. 4), a protein commonly found in eukaryotes. A similar UGT homolog was also identified in the genome of CHV-3/GH2 (Fig. 4). The highly conserved C-terminal domain, representing the signature sequence of the protein superfamily of UDP-glucosyltransferase (Warnecke et al., 1999), is present in both CHV-3 and -4, as are domains 1, 2, and 3 identified by Warnecke et al. (1999). Surprisingly, there is no evidence for such a motif in either CHV-1 or -2. In CHV-1, the same region (nt 3575–5310) corresponds to an area affecting the virulence of the fungus on chestnut trees and acts in conjunction with the N-terminal protease of the ORF B, p48 (Chen et al., 2000). In this region, CHV-1 shares 73% amino acid identity with CHV-2, but overall only 14–15% identity with CHV-3 and CHV-4/SR2.

As a general class, glycosyltransferases are usually responsible for the synthesis of sterol glycosides, which are normal membrane-bound lipids, and act by catalyzing the transfer of sugars to lipophilic compounds. Several different types of glycosyltransferases have been identified in dsDNA viruses of eukaryotes (Markine-Goriaynoff et al., 2004 for review), including the phycodnavirus *Paramecium bursaria Chlorella virus-1* (PBCV-1) isolated from *Chlorella*-like green algae (Graves et al., 2001) and baculoviruses and nucleopolyedroviruses from insects (Manzan et al., 2002; O'Reilly and Miller, 1989). The phycodnavirus

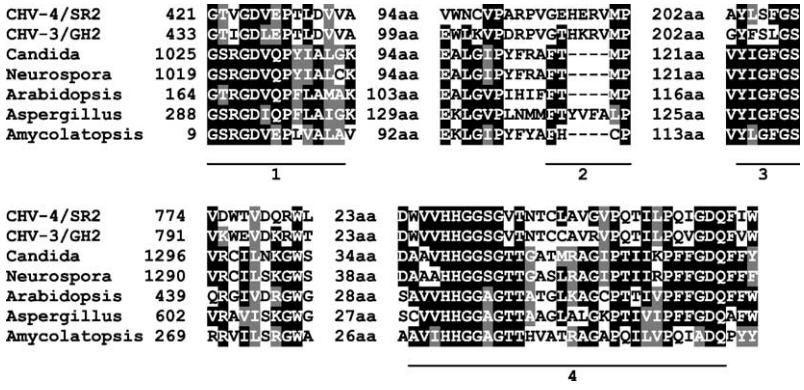


Fig. 4. Alignment of the putative glucosyltransferase homologs in CHV-4/SR2, CHV-3/GH2 (accession no. AF188515), *Candida albicans* (46440140), *Neurospora crassa* (28919811), *Aspergillus nidulans* (40745571), *Arabidopsis thaliana* (5080759), *Amycolatopsis orientalis* (13591785). Alignments and shading were as done in Fig. 3. The lines indicate the signature sequences 1–4 of the UDP-glucosyltransferases superfamily (Warnecke et al., 1999).

glycosyltransferase glycosylates a viral protein p54 (Graves et al., 2001), while the insect virus enzymes inactivate molting hormones by sugar conjugation (O'Reilly et al., 1998). Such inactivation extends the larval stadium, facilitating the propagation of the virus in the developmentally retarded host. No other glycosyltransferases have yet been reported in viruses more closely related to hypoviruses.

UGTs have been characterized from many organisms and their sequences are available in GenBank. It would be reasonable to postulate that the origin of the homolog found in CHV-4/SR2 may be fungal. Comparison with sequences of cellular glucosyltransferases, however (Fig. 4), shows closer relationship with those from yeasts such as *Candida* (e value: 4e-07) and *Pichia* (7e-05) and with plants such as *Arabidopsis* (1e-05) and *Oryza* (2e-05) than with filamentous fungi such as *Neurospora* or *Magnaporthe*, which are more closely related to *Cryphonectria* (Dawe et al., 2003). These relationships could be explained by early acquisition and independent evolution by the common ancestor of CHV-3 and -4, as nothing is currently known about the origin of hypoviruses. Acquisition of useful coding sequences by non-homologous recombination between viruses and host RNAs is well documented. For example, the mammal-infecting *Bovine viral diarrhea virus* contains several ubiquitin sequences inserted into non-structural protein coding regions (Meyers et al., 1991), the plant-infecting *Beet yellows virus* contains heat-shock protein homologs (Agranovsky et al., 1991), and the fungal virus M2 from *Rhizoctonia solani* contains a homolog of the host cellular protein QUTR, which acts as a repressor of the quinic acid pathway and thus affects expression of the shikimic acid pathway and amino acid biosynthesis (Lakshman et al., 1998; Liu et al., 2003a).

It is particularly interesting that neither CHV-1 nor CHV-2 appears to contain a UGT domain. Possible explanations for this difference in the genomes are (1) that the UGT domain in CHV-3 and -4 is a relic and not enzymatically active; (2) that the UGT domain is present but unrecognizable in CHV-1 and -2; (3) that the same function is carried out in CHV-1 and -2 by a fungal UGT; or (4) that something in the CHV-1 and -2 genome substitutes for the UGT domain. There is considerable amino acid similarity and similar spacing of all four domains of known functional UGTs compared to the putative homologs of CHV-3 and -4, but no such similarity in the CHV-1 and -2 sequences, so the first two possible explanations seem unlikely.

The function of the UGT domain in CHV-3 and 4 is unknown. Among the possibilities are that the viral UGT interferes with function of a cellular UGT and somehow gives advantage to the virus, or that the viral UGT is more directly involved in virus replication and/or movement. The association of cellular membranes and vesicles with replication of positive-sense RNA viruses is well documented (Salonen et al., 2005 for review). Indeed, the complexity and variability of membrane structures depending on viral replicase properties are now becoming apparent (Schwartz et al., 2004). UDP-glucosyltransferase is usually active inside the ER and synthesizes glycolipids for the membrane (Warnecke et al., 1999). The viral homolog could be involved in membrane-associated RNA replication or packaging of the dsRNA/protein complex in vesicles. If this is the case, it seems surprising that there would be a different mechanism for such a critical function in CHV-1 and -2. Several studies have examined the composition and trafficking of dsRNA-containing vesicles in hypovirulent isolates of *C. parasitica* (Dodds, 1980; Hansen et al., 1985; Newhouse and MacDonald, 1991; Newhouse et al., 1983; 1990). Most of these studies have been done using only CHV-1-infected isolates. Although no thorough comparative studies have been reported, Newhouse (1988, 1990) noted that dense clusters of dsRNA-containing vesicles were observed in CHV-1-infected isolates, but that similar vesicles were observed only sparsely in North American isolates infected with CHV-3.

Downstream from the putative UGT domain, the CHV-4/SR2 polyprotein contains RdRp and Hel domains, conserved among the supergroup I of positive-strand ssRNA viruses and in the *Hypoviridae* (Koonin et al., 1991). Both the polymerase and the helicase contain the conserved motifs identified in CHV-1, -2, and -3 (Hillman et al., 1994; Shapira et al., 1991; Smart et al., 1999) (data not shown). Of particular interest, the CHV-4/SR2 RdRp domain is more closely related to the RdRps of CHV-3 and the recently characterized virus referred to here as FgV-1/DK21 from the filamentous ascomycete *F. graminicola* (Chu et al., 2004; accession no. AAT07067) than to the other *Cryphonectria* hypovirus species, CHV-1 and CHV-2 (Fig. 5). Relationships inferred from Hel domain alignments were similar to those based on the RdRps, with one notable exception: the hypovirus Hel domains were much more closely related to each other and even to plant potyvirus Hel domains than to the FgV-1/DK21 Hel domain (Fig. 6). Since the hypovirus and potyvirus Hel domains were previously found to derive from a common ancestor (Koonin et al., 1991), this implies that the FgV-1/DK21 RdRp and Hel domains were likely acquired from two different sources. The genome organization of FgV-1/DK21 is substantially different from any of the four hypovirus species: the genome is much smaller and contains four other deduced ORFs that are 3′-proximal to the Hel domain in addition to the large ORF that contains the RdRp and Hel domains. We found no evidence in the FgV-1/DK21 sequence for a glycosyltransferase or papain-like proteinase domain.

### *3′-UTR*

The 3′-UTR of CHV-4/SR2 is only 409 nt long, the shortest among hypoviruses characterized to date (Fig. 1). Its 3′-terminal 200 nt share a relatively high level of identity with CHV-3 (53%), particularly in a stretch of 34 nt located at positions 9068–9101 (Fig. 2B). The 34 nt stretch, conserved in the 3′-UTR of CHV-3 and -4 but absent in

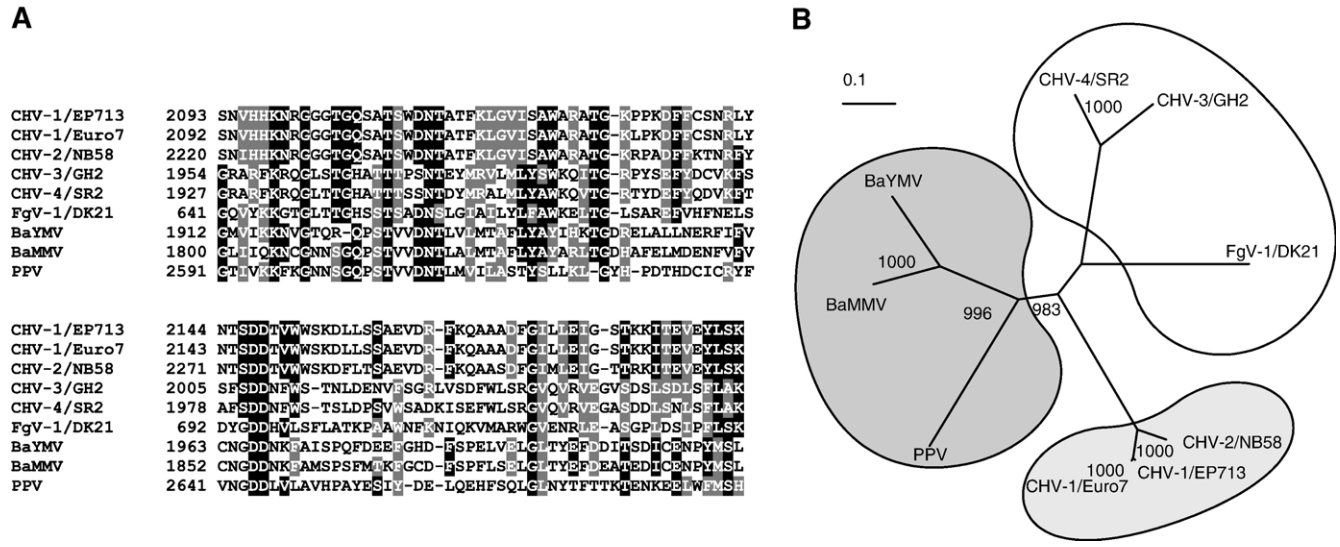


Fig. 5. Alignment (A) and phylogenetic tree (B) of the RNA-dependent RNA polymerase domain of CHV-4/SR2 with the homologs in other related viruses. The core 100 amino acid residues that aligned substantially without the introduction of gaps were chosen for analysis, with alignments performed and trees drawn as in Fig. 3. Accession numbers are as in Figs. 1 and 3.

CHV-1 and -2, and the short sequence conserved within the 5′-UTR likely play a role in polymerase recognition and/or replication, but their specific roles are unknown. No obvious conserved structures were identified in the predicted folded structures of the RNAs (data not shown), but this has not yet been thoroughly investigated.

*Variability within the CHV-4 species*

To begin to examine diversity of CHV-4 sequences, several isolates from different locations in the United States were selected. Five regions of the genome were amplified and their sequences used for the comparative analysis. As expected, the nucleotide sequence was more informative than the amino acid sequence, since most of the substitutions were located in the third position of the codon (Table 1).

From the phylogenetic analysis of the nucleotide sequence, three main groups were identified: the majority (BRU, EP, MI, MD, and SR2), the NJ isolates (119, 120, 129), and the KY isolate (Fig. 7). Within the clades, the identity level was very high (98%), but between clades it reached only 89%. In the R1 region, located within the possible protease, the NJ sequences diverge from the rest of the samples including KY. In the R2 region, located upstream from the polymerase domain, a high level of identity was observed among all the dsRNAs, with no isolate segregating from the majority. The R3 region, covering the C-terminal of the polymerase domain and the downstream region, must be subdivided into two separate subregions. The first 350 nt reveals homogeneity similar to that observed for the R2 region. In the following 310 nt, however, the KY dsRNA diverges clearly from the rest of

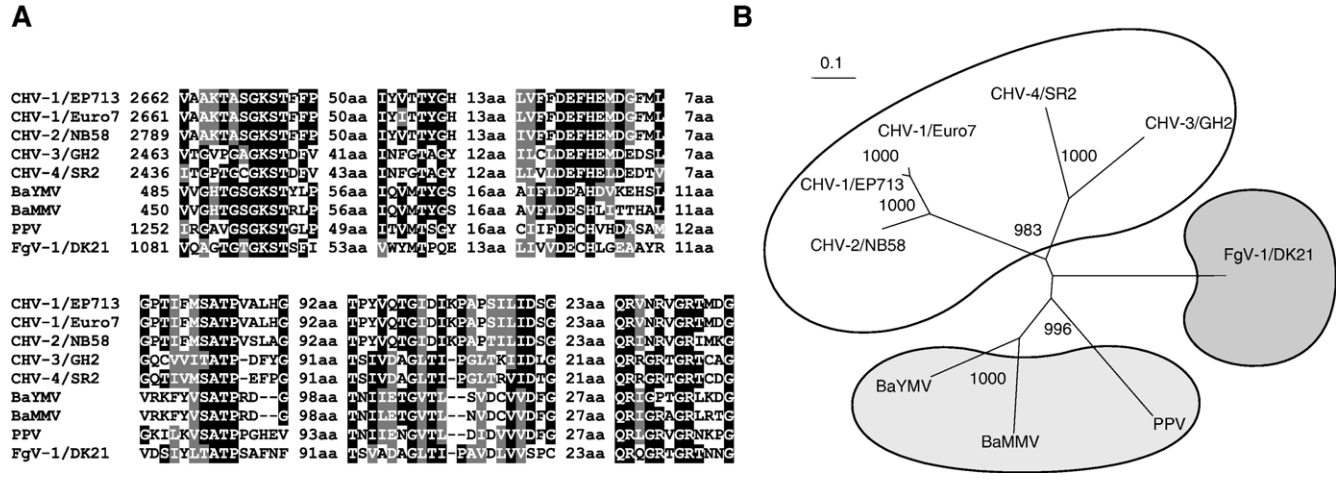


Fig. 6. Alignment (A) and phylogenetic tree (B) of most conserved regions of the helicase domain of CHV-4/SR2 with the homologs in other related viruses. Methodology and isolates were as in Fig. 5.

Table 1
Summary of the nucleotide substitutions in the nine CHV-4 dsRNAs analyzed

| | | R 1 | R 2 | R 3 A | R 3 B | R 4 | R 5 |
|---|---|---|---|---|---|---|---|
| Location on CHV-4/SR2 | | 724–1341 | 4319–4743 | 5994–6343 | 6344–6653 | 7682–8476 | 8597–9033 |
| nt analyzed | | 618 | 425 | 350 | 310 | 795 | 437 |
| Total substitutions | | 68 | 9 | 5 | 20 | 129 | 69 |
| Coding | | 68 | 9 | 5 | 20 | 129 | 15 |
| 1st position | | 16 | 1 | 0 | 6 | 16 | 3 |
| 2nd position | | 6 | 2 | 1 | 1 | 4 | 1 |
| 3rd position | | 46 | 6 | 4 | 13 | 109 | 11 |
| Noncoding | | 0 | 0 | 0 | 0 | 0 | 54 |
| Transitions | | 47/68 | 8/9 | 4/5 | 15/20 | 101/129 | 53/69 |
| Transversions | | 21/68 | 1/9 | 1/5 | 5/20 | 28/129 | 16/69 |
| Deletions (in 3′UTR) | | 0 | 0 | 0 | 0 | 0 | 4 |
| Total amino acids | | 208 | 142 | 116 | 103 | 259 | 47 |
| aa substitutions | | 16 | 4 | 1 | 1 | 13 | 2 |
| Same functional group | | 4 | 0 | 1 | 1 | 5 | 0 |
| Different group | | 12 | 4 | 0 | 0 | 6 | 2 |
| *Substitutions in specific isolates* | | | | | | | |
| MD64 | nt | 1 | 2 | 1 | 1 | 2 | 3 |
| | % | 0.2 | 0.3 | 0.3 | 0.3 | 0.2 | 0.7 |
| NJ119-3 | nt | 63 | 0 | 0 | 1 | 74 | 34 |
| | % | 10 | 0 | 0 | 0.3 | 9 | 8 |
| KY14-c1 | nt | 0 | 1 | 1 | 15 | 78 | 39 |
| | % | 0 | 0.2 | 0.3 | 5 | 10 | 9 |

Region 1 is located in the putative protease domain of the CHV-4/SR2 dsRNA, Region 2 upstream from the polymerase domain, and Region 3 in and downstream from the polymerase domain, R4 in the helicase domain, and R5 in the 3′-UTR.

the samples. In regions R4 and R5, located in the helicase and in the 3′-UTR, respectively, two independent clades diverge from the majority: one with the three NJ isolates, and the other with the KY isolate.

These results suggest that the KY virus could have originated from the recombination between two strains: its 5′-end is almost identical to the majority but the genome sequence diverges substantially after the polymerase

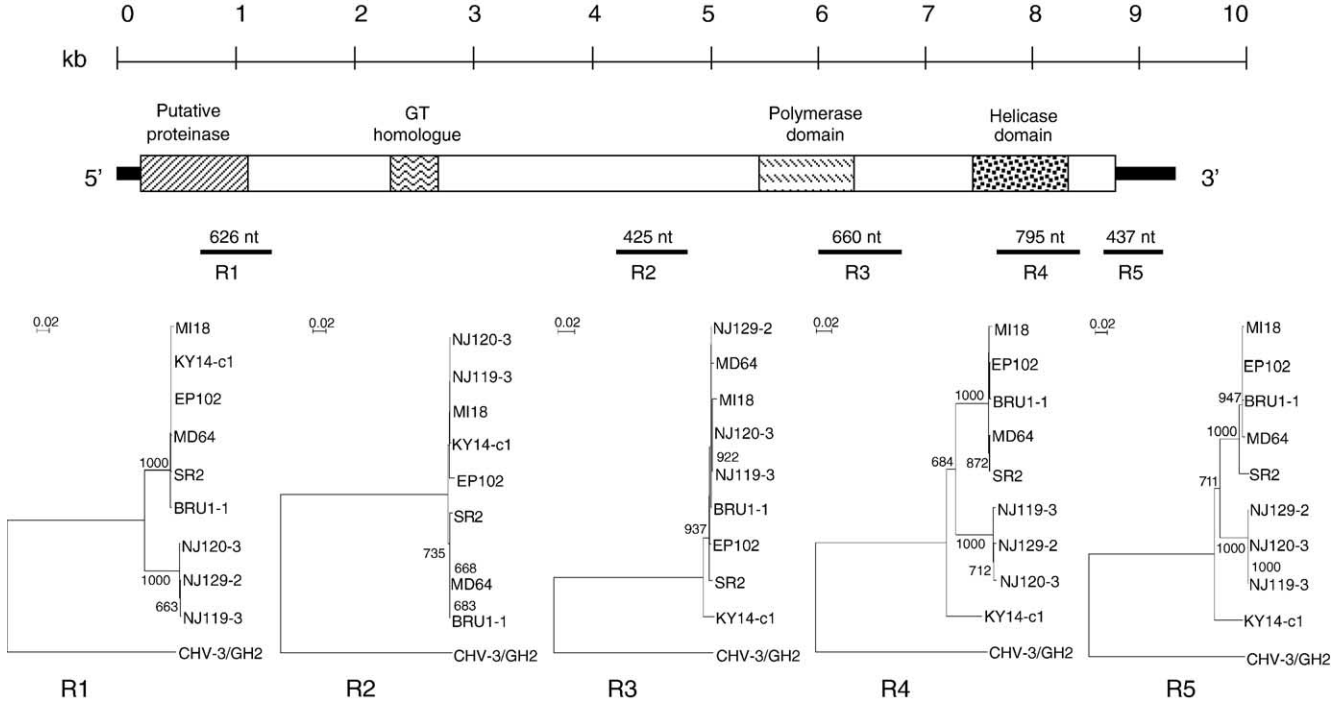


Fig. 7. Map of the genomic location of the five regions analyzed in the CHV-4 isolates in Table 2 and phylograms of the aligned nucleotide sequences. Alignments were performed and trees drawn as in Fig. 5.

domain, throughout the 3′-end (Fig. 7). This conclusion was supported by analysis using PlotSimilarity (Devereux et al., 1984), which plots the average similarity in sliding windows around specific nucleotide residues (Fig. 8). The substitution rate is less than 1% in the 5′-proximal region, but rises to 5–10% in the 3′-proximal region (Table 1). Such unbalanced substitution rates strongly suggest that a recombination event resulted in exchanging the 3′-end of the genome. Since no other sample from KY was available, no inference is possible about the frequency of this genotype.

The NJ isolates diverge from the main population in the protease and helicase domains as well as in the 3′-UTR, where the substitution rate reaches 10% (Fig. 8; Table 1). The central region, however, is highly conserved with only 0–0.5% total variation. Such a high conservation level could be related to the importance of this region in virus replication, but may also signal a recombination event exchanging the 5′- and the 3′-ends. Coinfection and recombination would be not be surprising in New Jersey, as variability of strains has previously been observed in this region (Chung et al., 1994; Enebak et al., 1994; Peever et al., 1997).

Homologous and non-homologous recombinations have been described in RNA viruses, and most RNA viruses that have been studied in detail at the population level have been shown to undergo recombination (Worobey and Holmes, 1999 for review). Mixed virus infections represent the basis for intergenomic recombination, an important source of genetic variation in viruses (Domingo and Holland, 1997; Ramirez et al., 1995). Mixed hypovirus infections are common and may result in symptoms in the infected fungus that differ from those resulting from either virus alone (Elliston, 1985a, 1985b; Peever et al., 1997; Smart and

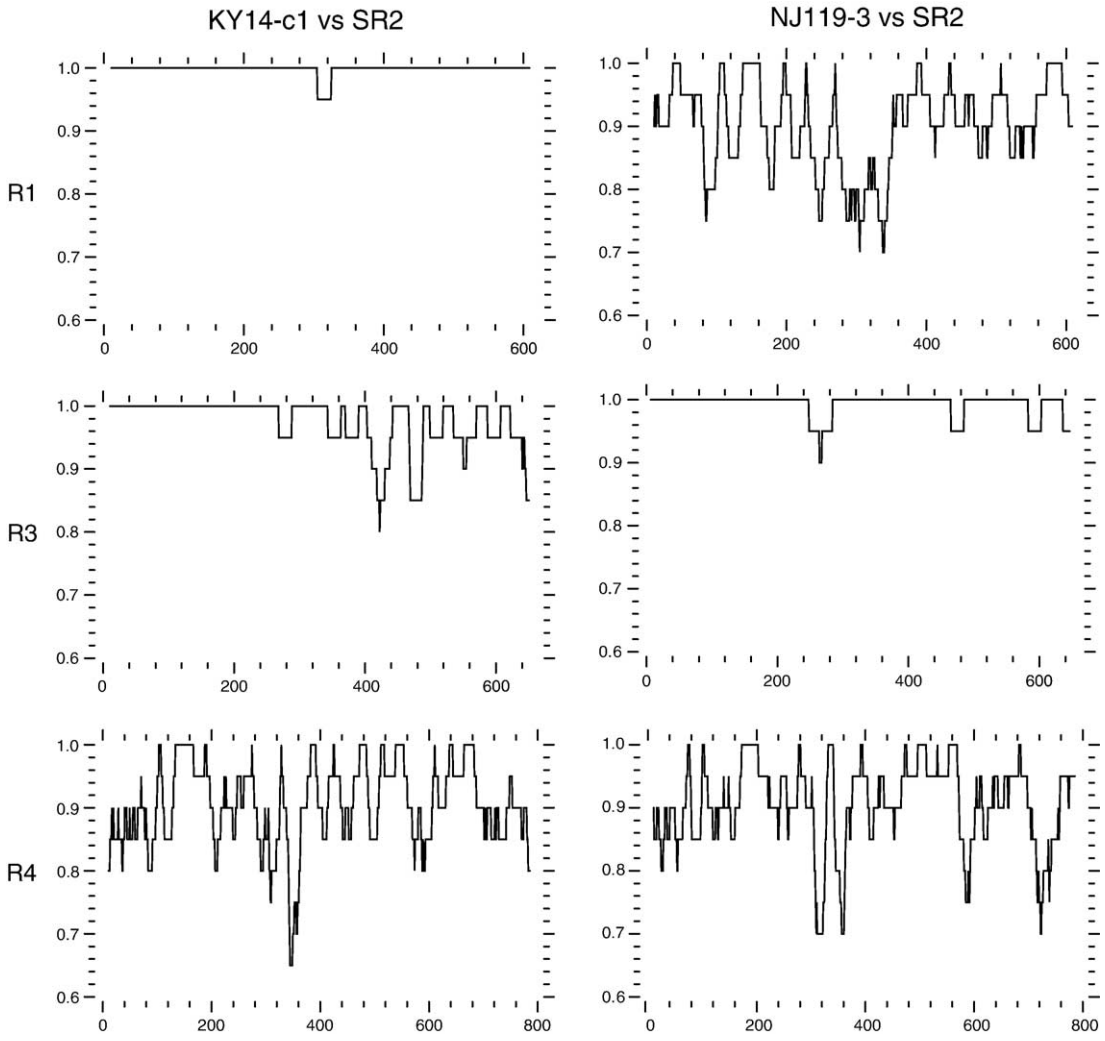


Fig. 8. Nucleotide identity profile of two CHV-4 strains (KY14-c1 and NJ119-3) exhibiting putative recombination events. The diagrams were constructed with the program PlotSimilarity from the GCG package (Devereux et al., 1984). Each diagram depicts the similarity between homologous regions of the two sequences named at the top, as calculated within a sliding window of 20 nt around the nucleotide position shown on the *x*-axis. Identity at all residues within the window equals a value of 1.0 on the *y*-axis. The regions considered were located as follows: R1 nt 724–1341, R3 nt 5994–6653, and R4 nt 7682–8476.

Fulbright, 1995). Recently, the role of RNA recombination in hypoviruses has become apparent, as recombinant CHV-1 molecules have been identified in isolates from Italy (Carbone et al., 2004) and from Japan (M. Milgroom, personal communication).

Horizontal transfer among fungi occurs during hyphal anastomosis, a process that requires vegetative compatibility (vc). The *Cryphonectria* population in North America is characterized by an elevated number of vc-groups that renders anastomosis between isolates very rare (Cortesi et al., 2001; Milgroom and Cortesi, 1999). The repeated failure of introducing CHV-1 into the United States for biological control may be related to vc type diversity (Milgroom and Cortesi, 2004). CHV-4/SR2 likely spread especially effectively very early in the North American chestnut blight epidemic when only few vc-groups were present, and expanded with the epidemic front, as postulated for spread of viruses of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier, 1988; 1990).

The ability of a virus to spread in populations depends on the capacity of the virus for horizontal and vertical transmission. This in turn is affected by factors such as the replicative competence or aggressiveness of the virus, its effects on the host, its ability to move into reproductive structures, and the size and structure of the population (Milgroom, 1999; Taylor et al., 1998). Classically, these factors cause a cycling between highly virulent strains that spread rapidly and less virulent strains that are more stable in individual hosts (Fenner, 1995; Garcia-Arenal et al., 2003). CHV-4 does not substantially alter the fitness and the reproductive capability of the host, and it is transmitted through conidia at a rate of almost 100% (Enebak, 1992). These factors would be predicted to lead to virus spread and stability. Although the migration of CHV-4 across eastern North America occurred 6–10 decades ago, our results indicate that the sequences among the different CHV-4 isolates examined from different regions are largely conserved. Coupled with previous findings that more than a quarter of all *C. parasitica* isolates from North America contain CHV-4 (Enebak et al., 1994; Peever et al., 1997), this suggests that the virus is well adapted to this geographic region.

The origin of CHV-4 remains unknown. No dsRNA hybridizing with CHV-4 has been discovered in Asia (Liu et al., 2003b; Peever et al., 1998; M. Milgroom, personal communication), although *C. parasitica* was probably introduced into North America from Japan (Milgroom et al., 1996). It has been hypothesized that the *Cryphonectria* hypoviruses evolved in an undetermined progressive fashion following the introduction of a progenitor virus into *C. parasitica* (Smart et al., 1999). The differences in genome organization, presence or absence of a UDP-glucosyltransferase domain, relationships among conserved replication-associated motifs, and the detection of a closer relationship between the RdRp domains of CHV-3 and -4 to FgV-1/DK21 than to CHV-1 or -2 suggest an early split between the clades containing CHV-1 and -2 from those of CHV-3 and -4, possibly followed by intergenomic recombination.

## Materials and methods

### *Fungal isolates and culture*

The isolates of *C. parasitica* used in this study are listed on Table 2. The dsRNA from isolate SR2 from Savage River, MD (Enebak et al., 1994), was used for complete genome sequencing. Isolates were maintained on potato dextrose agar (PDA) as previously described (Hillman et al., 1990), or in long-term storage as dried, frozen cultures. Since the dsRNA titer in strain SR2 was very low (Enebak et al., 1994), the mycelium was grown in large-scale cultures of $4 \times 500$ ml in potato dextrose broth (PDB), incubated for 1 week at RT, harvested, washed, air-dried, and stored at 4 °C.

### *DsRNA extraction, cDNA cloning, and screening*

DsRNA was extracted and purified by the column method described by Morris and Dodds (1979). Synthesis

Table 2
DsRNA-containing fungal isolates used in this study, and GenBank accession numbers of sequences analyzed in Fig. 7

| Name | Origin | GenBank accession nos. | | | | |
|---|---|---|---|---|---|---|
| | | R1 nt 724–1341 | R2 nt 4319–4743 | R3 nt 5994–6653 | R4 nt 7682–8476 | R5 nt 8597–9033 |
| SR2 | Maryland | AY307099 (complete genome) | | | | |
| BRU1-1 | W. Virginia | AY501435 | AY501443 | AY501451 | AY501459 | AY501467 |
| EP102 | Virginia | AY501436 | AY501444 | AY501452 | AY501460 | AY501468 |
| KY14-c1 | Kentucky | AY501437 | AY501445 | AY501453 | AY501461 | AY501469 |
| MD64 | Maryland | AY501438 | AY501446 | AY501454 | AY501462 | AY501470 |
| MI18 | Michigan | AY501439 | AY501447 | AY501455 | AY501463 | AY501471 |
| NJ119-3 | New Jersey | AY501440 | AY501448 | AY501456 | AY501464 | AY501472 |
| NJ120-3 | New Jersey | AY501441 | AY501449 | AY501457 | AY501465 | AY501473 |
| NJ129-2 | New Jersey | AY501442 | AY501450 | AY501458 | AY501466 | AY501474 |

The isolates were previously described by Enebak et al. (1994) and Peever et al. (1997).

of a cDNA library representing the SR2 dsRNA was prepared as described previously (Hillman et al., 2004). Colonies were screened first by minipreps and later by Southern blot hybridization using random-primed cDNA from the SR2 dsRNA as probe. The regions lying between the clones were amplified by PCR with primers designed from the available sequence. 5′-RACE reactions for the determination of the terminal dsRNA sequences were performed as described previously (Smart et al., 1999).

*Sequencing, analysis, and alignments*

Plasmids and PCR products were sequenced with an ABI 373A automatic sequencer following the protocol of the reagents' manufacturer (Perkin-Elmer, Boston, MA). Data were analyzed with the DNAstar computer software and the sequences aligned using the ClustalX program (Thompson et al., 1994, 1997). Neighbor-joining trees were drawn using NJ Plot software (Saitou and Nei, 1987) or TreeView (Page, 1996) and bootstrap values obtained with 1000 replicates.

Five regions of the viral genome were chosen for intraspecies sequence comparison: R1 in the possible protease domain (nt 724–1341), R2 upstream from the polymerase domain (nt 4319–4743), R3 in and downstream from the polymerase domain (nt 5994–6653), R4 in the helicase domain (nt 7682–8476), and R5 in the 3′-untranslated region (nt 8597–9033).

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