Effect of dsRNA Associated with Isolates of *Cryphonectria parasitica* from the Central Appalachians and Their Relatedness to Other dsRNAs from North America and Europe

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ABSTRACT

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A 12-kb segment of double-stranded (ds) RNA was associated with 25% of the isolates of *Cryphonectria parasitica* recovered from actively growing cankers in the central Appalachians. The relative virulence of these dsRNA-containing isolates ranged from a level comparable to that of the dsRNA-containing hypovirulent control isolate GH-2 to a level significantly greater than that of the dsRNA-free virulent control isolate Ep-155. When dsRNA-containing isolates and their dsRNA-free progeny were inoculated into Golden Delicious apples, excised dormant chestnut stems, or American chestnut sprouts, none of the lesions produced by the dsRNA-free progeny was significantly larger than those produced by the dsRNA-containing isolate from which they were derived. No differences in cultural morphology were noted when dsRNA-free and dsRNA-containing isogenic isolates were compared. These studies indicate that the single 12-kb segment of dsRNA neither alters morphology nor confers hypovirulence to the pathogen, and therefore it has little potential for biological control of chestnut blight. The relationships of the dsRNAs found in these isolates to dsRNAs associated with hypovirulent isolates from Europe and North America were examined by using recombinant cDNA libraries from three different dsRNAs. The first type, designated SR-2, contains one segment of dsRNA (12 kb); the second type, designated D2, contains two segments of dsRNA (5 and 12 kb); and the third type, designated C-18, contains 11 segments of dsRNA (1–5 kb). Clones from isolates SR-2 (Maryland), D2 (Pennsylvania), and C-18 (West Virginia) did not cross-hybridize, indicating that these dsRNAs have neither close affinities to one another nor to the other dsRNAs tested from New Jersey (NB58) and Europe (Ep-713 and Ep-747). However, clones from isolate SR-2 cross-hybridized with the single 12-kb segment of dsRNA from 26 other isolates, indicating that dsRNAs of the SR-2 type are closely related.

Additional keyword: *Endothia*.

The ability of chestnut trees in Europe and many sites in Michigan to recover from chestnut blight has been associated with the presence of double-stranded (ds) RNA in the chestnut blight fungus *Cryphonectria* (*Endothia*) *parasitica* (Murr.) Barr (2,3, 18–20). When dsRNA is present in the cytoplasm, the fungus typically is altered in cultural morphology and shows decreases in both virulence and sporulation when compared with dsRNA-free isolates (11). As more isolates of *C. parasitica* have been examined for biocontrol properties, many different types of dsRNAs have been recovered. One means to determine whether dsRNA has any influence on *C. parasitica* is to eliminate dsRNA from the cytoplasm of hypovirulent isolates. This can be accomplished either by single sporing of conidia or by treatment with cycloheximide. The morphology and virulence of isogenic dsRNA-containing and dsRNA-free isolates are then compared (5,11,17).

When dsRNAs associated with the pathogen are examined closely, the amount, size, and number of segments often differ among isolates (4,23,26). A few of the dsRNAs associated with hypovirulence in North America and Europe have been examined for nucleotide sequence relatedness at the molecular level by DNA hybridization techniques. These studies have shown that a number of dsRNAs recovered from Michigan share common sequences with each other (26) as well as with one dsRNA from Virginia (23). However, in these same studies, one dsRNA from Michigan did not cross-hybridize with dsRNAs from Michigan nor with other dsRNAs recovered from West Virginia or Tennessee (23). Until recently, no cross-hybridization had been found between dsRNAs associated with isolates from North America and those associated with European isolates (23,26); however, a dsRNA from a hypovirulent isolate (NB58) recovered from New Jersey did cross-hybridize with two European dsRNAs (22). While this may indicate a common origin for hypovirulence across continents, there are records of numerous releases of European dsRNA-containing isolates into the area for the control of chestnut blight, which may explain the cross-hybridization of these isolates (22).

One of the first indications that dsRNA was present within the *Cryphonectria* population of West Virginia was in a survey completed by Double et al (6), who examined over 1,000 isolates from the field and found nine that contained dsRNA. More recently, Likins (24) and Sillick and MacDonald (28) collected 360 isolates from virulent-appearing cankers at sites in Maryland, Virginia, and West Virginia. Among these isolates, 25% were found to contain a single segment of dsRNA approximately 12 kb in size. Because these dsRNA-containing isolates were recovered at random, little was known about their usefulness for biological control or their relatedness to other known hypovirulence-inducing dsRNAs. These studies were to examine the effect of this 12-kb segment on cultural morphology and virulence as well as its genetic relatedness to other dsRNAs associated with European and North American isolates of *C. parasitica*. Preliminary reports of this work have been published (14–16).

MATERIAL AND METHODS

Morphology of dsRNA-containing isolates. Surveys completed in 1988 identified 89 isolates of *C. parasitica* that contained a single 12-kb segment of dsRNA (24,28). Isolates were transferred from culture tubes to petri plates containing 25 ml of Difco potato-dextrose agar amended with methionine (100 mg/L) and biotin (5 mg/L). The plates were incubated for 2 wk at 20 C with a 16-h photoperiod. After incubation, all 89 plates were examined for morphologic characters, including diurnal zonation, unusual pigmentation, flat or aerial hyphal growth, and growth rate, that have been reported for other hypovirulent isolates (10,18,19). Virulent dsRNA-free isolates Ep-155 and Ep-523 from Connecticut were used for comparisons.

Relative virulence of dsRNA-containing isolates. Excised dormant American chestnut (*Castanea dentata* (Marsh.) Borkh.) stems were used to determine the relative virulence of the dsRNA-containing isolates as previously described by Elliston (10). The isolates chosen for the study were replicated eight times in a completely randomized design. Chestnut stem pieces (6–12 cm in diameter and 45 cm long) were wounded four times with a #11 leather punch (8 mm). Each wound received an agar and mycelial plug from the margin of an actively growing culture. Canker length and width were measured 3 wk after inoculation. The relative virulence of each dsRNA-containing isolate was determined by comparing the mean lesion size of each isolate to the dsRNA-positive (Euro-7, Italy; GH-2, Michigan; and 26-2-1, Virginia) and dsRNA-negative (Ep-523, Ep-155, and 5-9-1-B, West Virginia) controls.

Influence of dsRNA on cultural morphology. Six of the dsRNA-containing isolates were chosen for further study because they displayed low virulence (WD-1 and SR-2), moderate virulence (SH-4 and HM-3), or high virulence (BS-2 and NM-2) in excised dormant American chestnut stems (Fig. 1). Another central Appalachian isolate, D2, was included because it contained 12- and 5-kb segments of dsRNA and showed reduced virulence in field studies (W. L. MacDonald, *unpublished data*). Attempts to free these seven isolates of their dsRNA were made by isolating single conidia as previously reported by Dodds and Elliston (5). Sixty to 100 single-spore colonies from each parent isolate were examined for morphological differences that may have been due to the presence of dsRNA. Extraction and analysis of dsRNA from putative isogenic cultures were done essentially as described by Morris and Dodds (25) and modified by Likins (24).

dsRNA-free single-conidial isolates were paired with their dsRNA-containing parent isolates to determine whether the 12-kb segment was transmitted via hyphal anastomosis (1). After incubation, the pairings were examined for an alteration of colony morphology, which may indicate that dsRNA had been transferred from the dsRNA-containing isolate to the dsRNA-free isolate. The transmission of dsRNA from parent to progeny isolates was confirmed by extraction and analysis as described above.

Influence of dsRNA on virulence. The effect of dsRNA on the relative virulence of *C. parasitica* was determined by comparing lesions produced on three different substrates after inoculation with the parent isolates and their progeny isolates. The first of three tests used Golden Delicious apples as described by Elliston (11) and Fulbright (17). dsRNA-containing parent isolates (SR-2, BS-2, NM-2, HM-3, and SH-4) and their dsRNA-free single-conidial progeny (SR2-6, BS2-25, NM2-5, HM3-18, HM3-24, and SH4-31) were included. Isolates GH-2 and Ep-155 were hypovirulent and virulent controls, respectively. Isolates were replicated eight times (three wounds per apple) in a completely randomized design. Lesion size was measured 3 wk after inoculation.

A second study was performed on excised dormant American chestnut stems as described above. dsRNA-containing parent isolates used in the study were SR-2, HM-3, and SH-4 along with their dsRNA-free (SR2-6, HM3-18, HM3-24, HM3-30, SH4-1, and SH4-31) and dsRNA-containing (SR2-19, SR2-27, HM3-6, HM3-16, HM3-25, SH4-19, and SH4-25) single-conidial progeny. Inoculations were replicated eight times in a completely randomized design. Canker size was measured 4 wk after inoculation.

A third study was conducted in the field with American chestnut sprouts. The field plot was established in June 1990 near Parsons, West Virginia, in a 6-yr-old clear-cut that contained chestnut sprouts 1–20 cm in diameter. dsRNA-containing parent isolates used in the study were SR-2, HM-3, and SH-4 along with their dsRNA-free (SR2-6, HM3-18, HM3-24, HM3-30, SH4-1, and SH4-31) and dsRNA-containing (SR2-1, SR2-19, SR2-27, HM3-6, HM3-16, HM3-25, SH4-6, SH4-19, and SH4-25) single-conidial progeny. The parental isolate and its dsRNA-containing and dsRNA-free progeny were inoculated into the same stem. A randomized complete block design was used with 10 replicates for each isolate. The formula for the area of an ellipse was used to determine canker size 12 mo after inoculation.

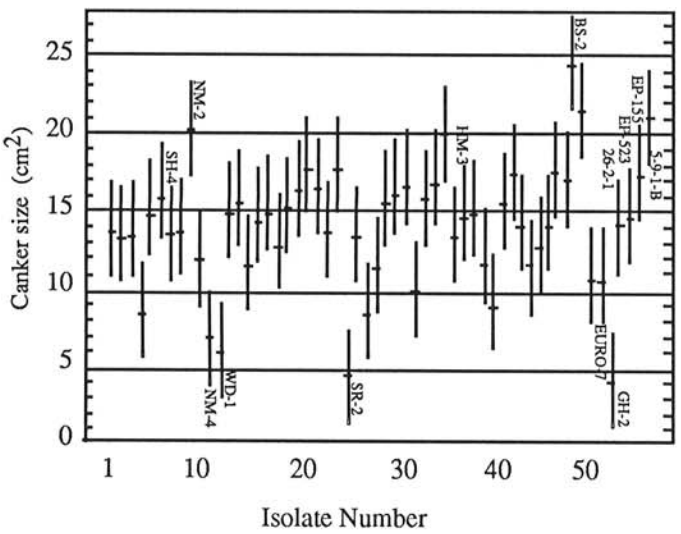


Fig. 1. Relative virulence as measured by mean canker size and least significant difference analyses for 51 double-stranded (ds) RNA-containing isolates and six control isolates of *Cryphonectria parasitica* 3 wk after inoculation on excised dormant American chestnut stems. dsRNA-positive (Euro-7, GH-2, and 26-2-1) and -negative (Ep-523, Ep-155, and 5-9-1-B) controls are isolate numbers 52–57, respectively.

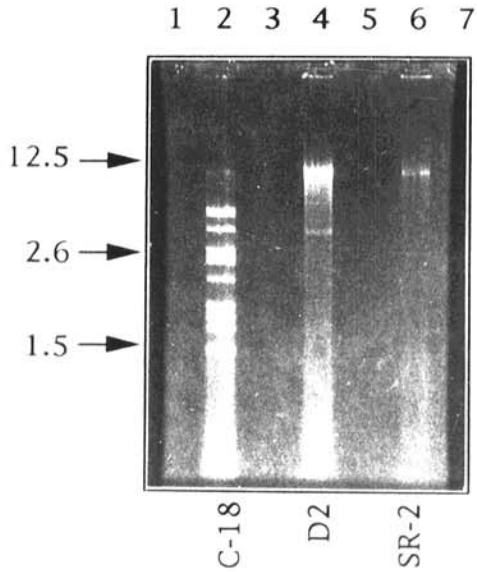


Fig. 2. Double-stranded (ds) RNA banding patterns and sizes (kb) of three *Cryphonectria parasitica* isolates. Molecular weights were determined from the comigration of dsRNA from NB58, wound tumor virus, and C-18. Lane 2 contains dsRNA from isolate C-18; lane 4 contains dsRNA from isolate D2; and lane 6 contains dsRNA from isolate SR-2.

The effect of dsRNA on the virulence of *C. parasitica* in all three studies was determined by comparing the mean lesion size for each dsRNA-free progeny to that of the dsRNA-containing progeny and that of the parent isolate from which they were derived. Significant differences ($P = 0.05$) among isolates in all virulence studies were determined by analysis of variance (STATISTIX, NH Analytical Software, St. Paul, MN) and Fisher's least significant difference (7).

Construction and analysis of the cDNA libraries. dsRNA from three *C. parasitica* isolates was used to construct cDNA libraries. Approximately 1 μg of DNase-treated dsRNA from isolates SR-2 (Maryland), D2 (Pennsylvania), and C-18 (West Virginia) was used for cloning (Fig. 2). The synthesis and analysis of the cDNA libraries were conducted as described by Hillman et al (22).

Spot hybridization. The genetic relationships among dsRNAs were examined in two separate spot hybridization experiments. In the first, dsRNA from isolates C-18 (West Virginia), D2 (Pennsylvania), NB58 (New Jersey), and Ep-747 and Ep-713 (Europe) was used; pUC9 and H_2O were included as positive and negative controls, respectively. dsRNA was quantified by gel electrophoresis prior to spot hybridization, and approximately 5 ng of each dsRNA was spotted onto two separate nylon membranes. Randomly selected plasmids from each cDNA library of isolates D2 (pD2-7, 1.1 k) and C-18 (pC18-3, 1.4 k) were labeled with ^{32}P-dCTP with a random primer labeling kit from Bethesda Research Laboratories (Gaithersburg, MD) to a specific activity of $>10^8$ cpm/μg.

In the second hybridization, the single 12-kb segment of dsRNA associated with 28 isolates recovered from Maryland, Virginia, and West Virginia was examined. Plasmid DNA from pUC9 and dsRNA from isolate C-18 were included on the membrane as positive and negative controls, respectively. Approximately 1 μg of each dsRNA sample was blotted onto the nylon membrane as well. One plasmid from the cDNA library of SR-2 (pSR2-13, 1.2 k) was labeled with ^{32}P as described above. After spotting, the membranes were prehybridized, hybridized, and washed as described by Hillman et al (22) and Rae et al (27). Membranes were wrapped in plastic wrap and exposed to X-ray film with an intensifying screen at −80 C.

RESULTS

Morphology of dsRNA-containing isolates. The most common cultural characteristic associated with the 89 dsRNA-containing isolates, compared with virulent isolates Ep-155 and Ep-523, was a lobate growth pattern. All 89 isolates exhibited diurnal patterns of growth, produced aerial mycelia, and were yellow orange in pigmentation, similar to virulent control isolates Ep-155 and Ep-523. The intent of this evaluation was to identify isolates with unusual morphology that may be hypovirulent. Because the lobate margins were a feature of the 89 colonies examined, the isolates were grouped by area of collection, and three or four isolates from each site were chosen that had the most lobate growth patterns and the slowest rates of growth. These 51 isolates were randomly assigned numbers and used to determine the relative virulence on excised dormant American chestnut stems.

Relative virulence of dsRNA-containing isolates. The ability of the 51 dsRNA-containing isolates to produce cankers in excised dormant American chestnut stems varied significantly (Fig. 1). Cankers ranged in size from as large as those produced by the dsRNA-free virulent controls Ep-523, Ep-155, and 5-9-1-B to as small as those produced by the dsRNA-containing hypovirulent control isolate GH-2. Only one isolate, BS-2, produced lesions that were significantly larger than cankers produced by the virulent control isolate Ep-155. Most of the isolates, however, produced cankers that were comparable in size to those of the dsRNA-free control isolates (15 cm^2), and only three isolates (NM-4, WD-1, and SR-2) produced cankers as small as those of the dsRNA-containing control isolate GH-2. The cankers produced by these isolates were approximately 6 cm^2.

Influence of dsRNA on cultural morphology. When the cultural morphologies of single-spore isolates from the seven parental isolates were examined, there was no indication whether dsRNA was present or absent. Because no morphologic alteration was observed with any of the seven parent isolates, the single-spore isolates were analyzed to determine whether the 12-kb segment of dsRNA was present. The transmission rate of dsRNA into conidia varied from 100% for isolates D2 and WD-1 to 10% for isolate BS-2. dsRNA-free progeny were never obtained from isolates D2 and WD-1; therefore, the effect of dsRNA on the morphology of these two isolates is unknown. However, the morphology of the dsRNA-containing single-conidial progeny did not appear unusual when compared with the other isolates containing the 12-kb segment. Isolates D2 and WD-1 were excluded from further virulence studies because of the inability to obtain dsRNA-free progeny from them.

When the dsRNA-containing parental isolates (BS-2, NM-2, SR-2, HM-3, and SH-4) were each paired with one of their dsRNA-free progeny (BS2-25, NM2-5, SR2-6, HM3-24, and SH4-31, respectively), no morphological alteration of colony growth was observed. However, dsRNA analysis of agar plugs taken from the advancing margins (dsRNA-free progeny side) of the pairings yielded a 12-kb segment of dsRNA, indicating that transmission of dsRNA by hyphal anastomosis had occurred without an obvious corresponding alteration of cultural morphology.

Influence of dsRNA on virulence. dsRNA-free progeny from isolates SR-2, BS-2, NM-2, HM-3, and SH-4 did not produce significantly larger lesions when compared with the dsRNA-containing parent isolates from which they were derived. All dsRNA-containing isolates and their dsRNA-free progeny produced lesions that were significantly larger than those of the dsRNA-containing hypovirulent control isolate GH-2, and all lesions were as large or larger than those of the dsRNA-free virulent control isolate Ep-155 (Fig. 3).

None of the dsRNA-free progeny produced cankers on dormant stems that were significantly larger than those of their dsRNA-containing sister isolates or of the dsRNA-containing parental isolate from which they were derived. The mean sizes of the cankers for the isolates examined are given in Figure 4A–C and represent data collected 4 wk after inoculation.

The dsRNA-free isolates inoculated into chestnut sprouts in the field produced cankers that were similar in size to lesions produced by the dsRNA-containing isolates from which they were

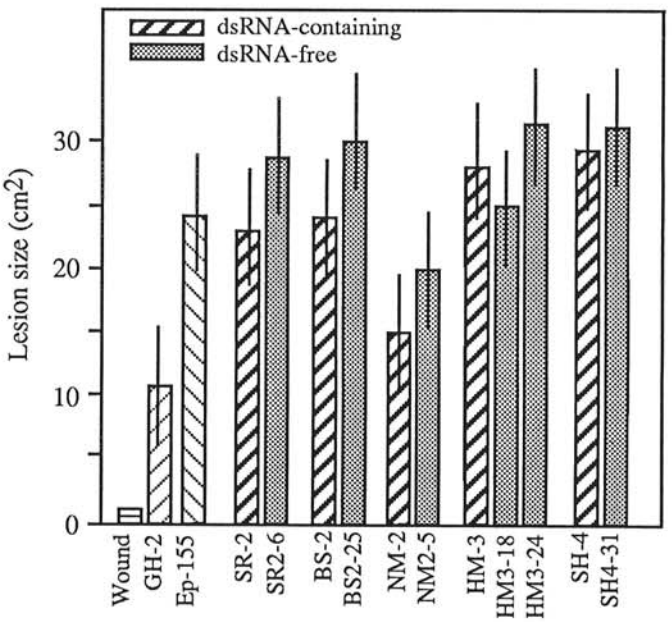


Fig. 3. Effect of cytoplasmic double-stranded (ds) RNA on the size of lesions on Golden Delicious apples 3 wk after inoculation with dsRNA-containing parent and dsRNA-free progeny isolates of *Cryphonectria parasitica*. Bars represent Fisher's least significant difference.

derived (Fig. 5A and B). The dsRNA-free isolate SR2-6 produced significantly smaller lesions than did its dsRNA-containing parent SR-2 (Fig. 5C).

Construction and analysis of the cDNA libraries. The construction of the cDNA libraries and plating of transformed *Escherichia coli* cells yielded in excess of 300 white colonies for isolates D2 and C-18 and 32 white colonies for SR-2. Plasmid preparations from transformed bacteria from the D2, C-18, and SR-2 libraries yielded inserts ranging in size from 500 to 3,000 bp.

Spot hybridization. The first spot hybridization experiment was performed with four labeled clones from the D2 and C-18 libraries. These clones were used to probe dsRNA from isolates C-18, D2, and NB58 (North America), and Ep-713 and Ep-747 (Europe). In each blot, the recombinant plasmid used as a probe hybridized only to the positive control (pUC9) and to its own template dsRNA, indicating that dsRNA from D2 and C-18 do not have close affinities to each other or to the other dsRNAs tested (Ep-713, Ep-747, and NB58). One representative autoradiogram of each hybridization is shown in Figure 6A and B.

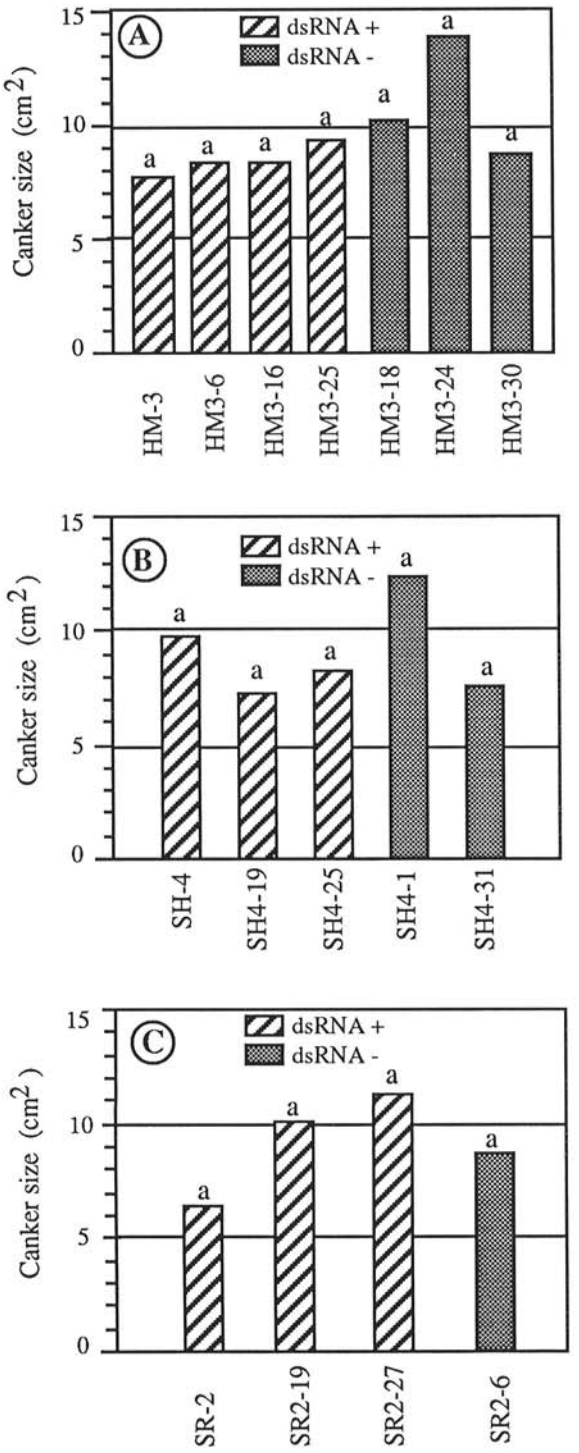


Fig. 4. Effect of cytoplasmic double-stranded RNA on the size of cankers induced on excised dormant American chestnut stems by *Cryphonectria parasitica* 4 wk after inoculation with single-conidial isolates from strains **A,** HM-3, **B,** SH-4, and **C,** SR-2. Bars with the same letter are not significantly different according to Fisher's least significant difference ($P = 0.05$).

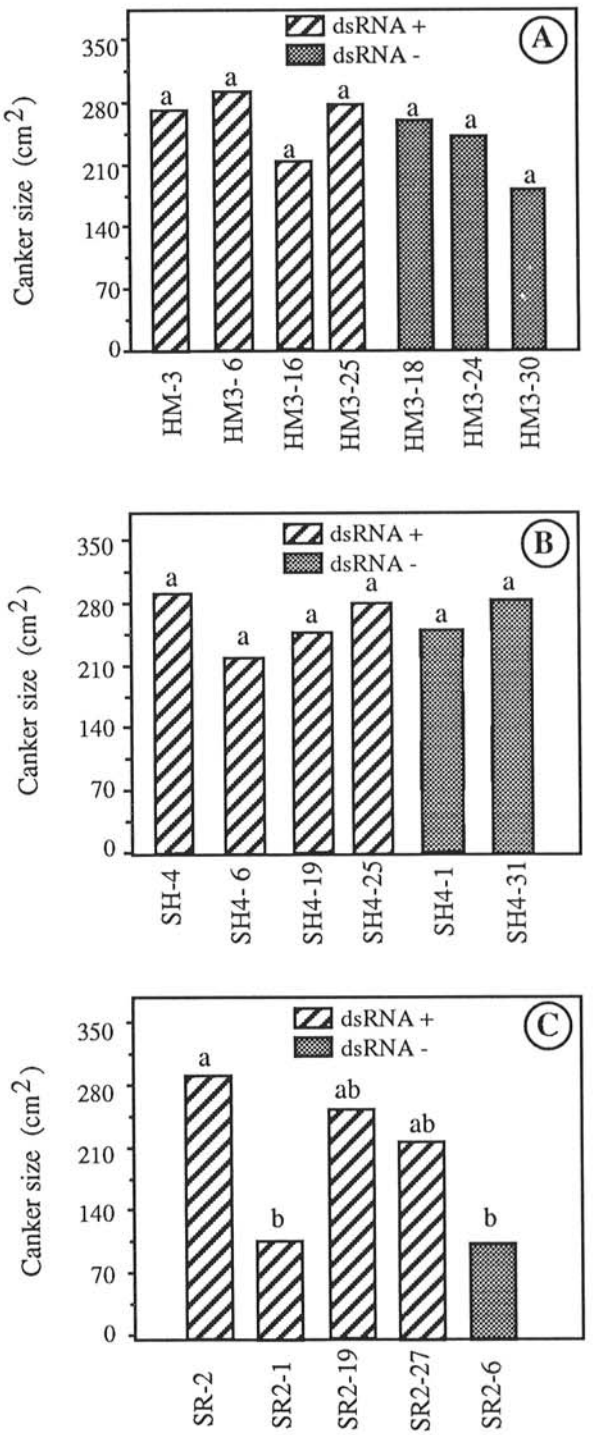


Fig. 5. Effect of cytoplasmic double-stranded RNA on the size of cankers induced on American chestnut sprouts by *Cryphonectria parasitica* 1 yr after inoculation with single-conidial isolates from strains **A,** HM-3, **B,** SH-4, and **C,** SR-2. Bars with the same letter are not significantly different according to Fisher's least significant difference ($P = 0.05$).

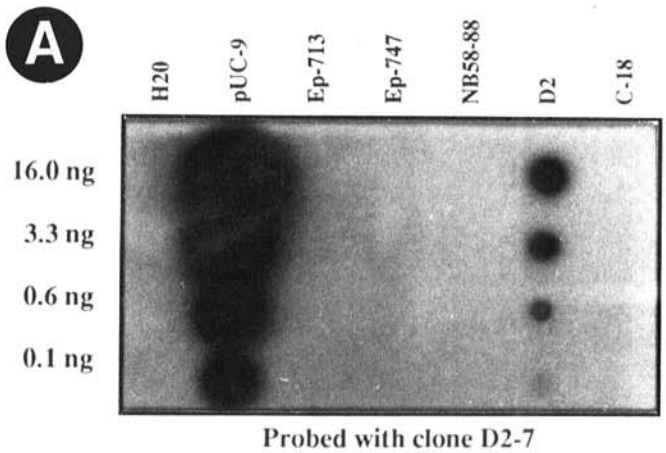
In the second spot hybridization, one clone from the library constructed from SR-2 dsRNA was used to probe 28 dsRNAs associated with *Cryphonectria* recovered from the central Appalachians. The recombinant plasmid hybridized to the positive control pUC9 (row 1), to its own template dsRNA SR-2 (row 3), and to 26 (rows 4–8) of the 28 single segmented dsRNAs tested from the central Appalachians. It did not hybridize to the negative control dsRNA from isolate C-18 (row 2) or to dsRNA from isolates WD-2 and BM-2 (Fig. 7).

DISCUSSION

The lack of discernible alteration in cultural morphology when the 12-kb segment is present in these Appalachian isolates is in sharp contrast to what occurs when isolates are infected with dsRNAs associated with hypovirulent isolates from Europe and North America. The presence of dsRNAs from other hypovirulent isolates usually results in pigmentation change so that isogenic dsRNA-containing and dsRNA-free isolates can be identified in culture (2,10,17,19,20). Because progeny of these isolates were indistinguishable, it was impossible to visually screen for dsRNA-positive and -negative cultures. Consequently, dsRNA-free progeny had to be identified via dsRNA-extraction. In the case of isolates D2 and WD-1, dsRNA-free progeny were never identified, and the effect of dsRNA on cultural morphology and virulence could not be determined.

Like other hypovirulent isolates from Michigan and Europe, isolate D2 was recovered from a canker that was swollen and superficial. dsRNA analysis yielded two segments of dsRNA, approximately 12 and 5 kb in size (Fig. 2), and isolate D2 has been found to be hypovirulent (W. L. MacDonald, *unpublished data*). Because D2 was associated with a swollen canker, had reduced virulence, and contained a 12-kb segment of dsRNA, we attempted to further examine the effects of this dsRNA on morphology and virulence as well as its relationship to other dsRNAs. The larger 12-kb segment associated with D2 is similar in size to European (Ep-713 and Ep-747) and North American dsRNAs (SR-2). However, clones derived from D2 did not hybridize to any of the North American or European dsRNAs examined in dot blot hybridization (Fig. 6), indicating that the relationship of D2 to other dsRNAs included in this study is too distant to be detected by the hybridization methods used here.

A common dsRNA-associated trait in *C. parasitica* is a reduction in fungal virulence. To quantify the role of the SR2-related 12-kb dsRNA on virulence, a number of dsRNA-containing isolates and their dsRNA-free progeny were inoculated into Golden Delicious apples, excised dormant chestnut stems,



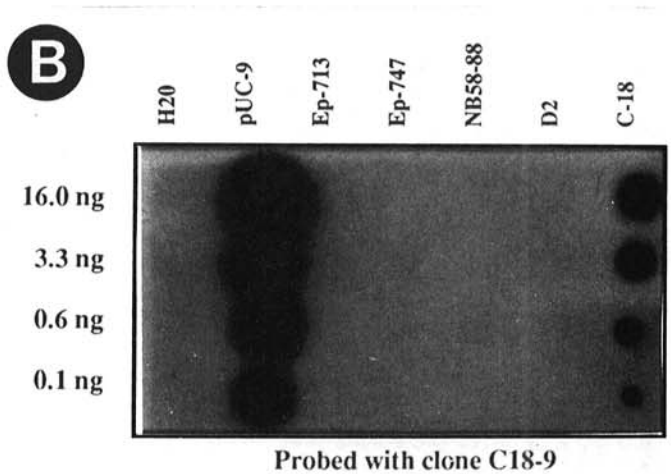


Fig. 6. Dot blot hybridization of double-stranded (ds) RNA preparations from North American and European isolates of *Cryphonectria parasitica* probed with a cDNA clone of North American dsRNA from isolates **A,** D2 and **B,** C-18. Approximate amounts of dsRNA spotted onto nylon membranes are listed at the left of each membrane; dsRNA preparations and the clones used as probes are listed at the top and bottom of each membrane, respectively. Lanes 1 and 2 are H_2O and pUC9, negative and positive controls, respectively; lanes 3–7 are dsRNA preparations from isolates Ep-713 and Ep-747 (Europe), NB58 (New Jersey), D2 (Pennsylvania), and C-18 (West Virginia), respectively.

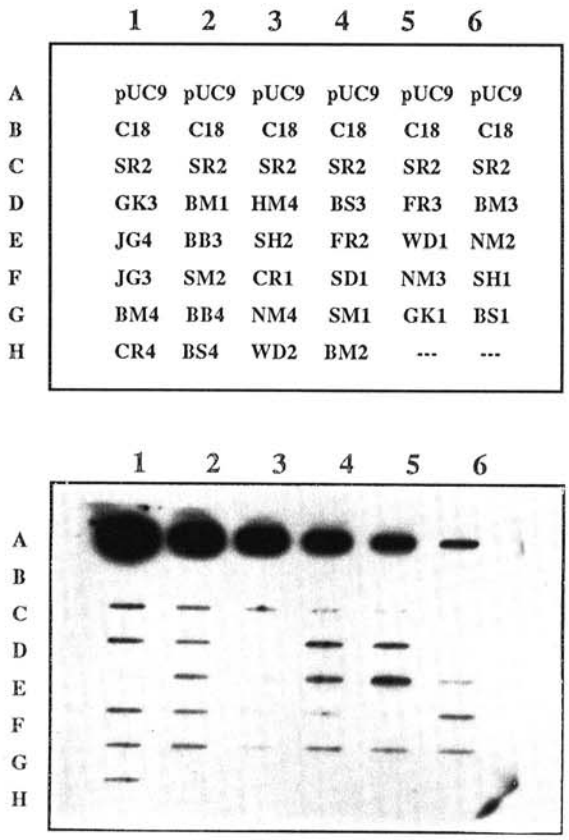


Fig. 7. Slot blot hybridization of double-stranded (ds) RNA preparations from 28 isolates of *Cryphonectria parasitica* probed with a cDNA clone from isolate SR-2. Rows 1 and 2 are plasmid preparations and dsRNA from pUC9 and isolate C-18, positive and negative controls, respectively. Row 3 is dsRNA from isolate SR-2; rows 4–8 are dsRNA preparations from Maryland, Virginia, and West Virginia. Approximately 1 μg of dsRNA was spotted for each sample with pUC9, C18, and SR-2 serially diluted (1:9 μl) six times prior to blotting. BB = Blue Bend, WV; BM = Brushy Mt., WV; BS = boy scout camp, WV; C-18 = Hopkins Tower, WV; CR = Clover Run, WV; FR = Frost, WV; GK = Grindstone Knob, WV; HM = Hankey Mt., VA; JG = Judy Gap, WV; NM = Nathanial Mt., WV; SH = Stillhouse, WV; SD = Shenandoah, VA; SM = Snowy Mt., WV; SR = Savage River, MD; and WD = Waldrup, WV.

and chestnut sprouts. Results of the three experiments revealed that the SR2-related 12-kb dsRNA common to these isolates does not significantly affect virulence (Figs. 3–5). These results support other evidence that this dsRNA has no discernible effect on the fungus, including the lack of altered morphology in culture as described above and the lack of association with swollen cankers that are typical of hypovirulent strains (24,28).

These studies indicate that the presence of dsRNA within the cytoplasm is not enough to elicit the response necessary for hypovirulence in the chestnut blight fungus. The dsRNA described here is similar in size (12 kb) to Ep-713 from Europe, NB58 from New Jersey, and GH-2 from Michigan, yet it does not cross-hybridize with any of these. The situation with Michigan dsRNA is similar: whereas dsRNA from strain GH-2 cross-hybridizes with many other dsRNAs from Michigan hypovirulent strains, it does not cross-hybridize with dsRNAs from outside Michigan (26). However, nucleotide sequence analysis of GH-2 dsRNA through genomic regions encoding the conserved putative polymerase and helicase domains reveals that GH-2 dsRNA is phylogenetically related to Ep-713 (8,9). Thus, dsRNAs in the Michigan population appear to have diverged to the point where they do not cross-hybridize with their more distant relatives. A similar situation may apply to the dsRNAs from the central Appalachians. While they may have progenitors in common with Michigan, New Jersey, and European dsRNAs, a divergent population that does not cross-hybridize with other populations may have become established in this region. Information about the molecular structure and coding potential of these dsRNAs, which is out of the purview of this study, would be required to address those questions.

The reason for the apparent avirulence of the dsRNAs associated with these isolates (i.e., their failure to induce hypovirulence in the fungus) is a matter of speculation. Since all chestnut blight dsRNAs examined to date have the potential to encode their own putative replicase molecules, the same is assumed to be true of these dsRNAs. In addition to RNA-dependent RNA polymerase, however, other chestnut blight dsRNAs examined to date encode additional polypeptides that affect symptom expression in the fungus and may be quite variable (2; M. P. Brown and B. I. Hillman, *unpublished data*). Thus, a specific coding region may be responsible for the hypovirulence phenotype. It is also possible that the level of accumulation or expression of the dsRNAs is critical, because these dsRNAs accumulate at much lower levels than the 10–20 μg/g of tissue associated with European dsRNAs such as Ep-713 (21). However, the level of dsRNA accumulation is not directly correlated with hypovirulence in other systems. For example, NB58 dsRNA, which accumulates to only 10–20% of the level of Ep-713 dsRNA, causes a great reduction in fungal virulence (22).

In epidemiological terms, a more important question may be whether the avirulent SR2-related dsRNAs described here prevent other dsRNAs from entering or replicating in the same fungal thallus. Although several studies indicate that at least some chestnut blight dsRNAs are capable of replicating in a common mycelium (12,13), it is not clear that this is commonly the case. In surveys of *C. parasitica* in West Virginia, other types of dsRNA have been identified that are indeed hypovirulent and have potential for the biological control of chestnut blight (6,15,26). These are found less frequently than is the 12-kb segment, and perhaps their spread is suppressed by the more common SR2-related 12-kb segment. Studies that involve the double infection of *Cryphonectria* and include the SR2-related 12-kb segment and other dsRNAs associated with hypovirulence need to be undertaken to determine whether this is the situation. If multiple infections are not possible, then natural hypovirulence in the central Appalachians has yet another obstacle to overcome.

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