Double-Stranded RNA Molecules from Michigan Hypovirulent Isolates of *Endothia parasitica* Vary in Size and Sequence Homology

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ABSTRACT

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The sizes and homology relationships among double-stranded RNA (dsRNA) molecules from American and European hypovirulent isolates of *Endothia parasitica* were compared. The number, size, and relative intensity of dsRNA bands in gels varied among isolates. The sizes of dsRNA segments from each isolate ranged from a molecular weight of 0.56×10^6 to 6.2×10^6. No single size of dsRNA molecule was common to all isolates. The dsRNA from isolate GH2 hybridized to dsRNA from all other Michigan isolates tested, with the exception of dsRNA from isolate RC1. RC1 dsRNA did not hybridize to dsRNA from any other Michigan isolates tested. Neither GH2 nor RC1 dsRNA shared homology with dsRNA from other states or from Europe.

Additional keywords: chestnut blight, *Cryphonectria parasitica.*

Virulent *Endothia parasitica* (Murr.) Anderson (*Cryphonectria parasitica* (Murr.) Barr) is responsible for the demise of American and European chestnut trees (*Castanea dentata* (Marshall) Borkh. and *Castanea sativa* Miller). Hypovirulent *E. parasitica* isolates have been found in Europe (2) and in the United States (12,14,18) where chestnut trees have survived infection. Hypovirulence was found to be transmitted cytoplasmically through hyphal anastomosis (31) and is correlated with the presence of cytoplasmic double-stranded RNA (dsRNA) (7). Many of these isolates also exhibit altered culture morphology. When the dsRNA is removed, the hypovirulent phenotype is eliminated (13). For the purpose of this paper, hypovirulent isolates are defined as those that exhibit reduced virulence, harbor dsRNA, and can transmit these characteristics through hyphal anastomosis.

Although hypovirulent isolates harbor dsRNA, the number, size, and relative amounts of the dsRNA segments vary among isolates (9,14). The dsRNA segments within an isolate have been found to share sequence homology and common termini (17,28). However, isolates may carry dsRNA segments that do not share homology (28). Knowledge of the similarities and differences among dsRNA molecules found in different isolates may provide clues to the origin of the dsRNA and its ability to spread geographically. It may also provide insights into the mechanism by which dsRNA results in the hypovirulent phenotype. Toward these ends, comparisons were made among the dsRNAs from a selection of hypovirulent isolates of *E. parasitica*. The number and sizes of dsRNA segments from selected Michigan hypovirulent isolates were examined in polyacrylamide gels. The dsRNA from Michigan hypovirulent isolates, hypovirulent isolates from other states, and hypovirulent isolates from Europe were tested for sequence homology using Northern blot hybridization. Portions of this work have been reported elsewhere (16,23,24).

MATERIALS AND METHODS

Cultures. Virulent and hypovirulent isolates used in this study are described in Table 1, and the locations from which Michigan isolates were recovered are shown in Figure 1. Strains 780 and 422 are derived from American virulent isolates into which dsRNA of Italian (11) and French origin, respectively, has been transferred through hyphal anastomosis.

All cultures were maintained on potato-dextrose agar (Difco, Detroit, MI) at 20 C under fluorescent lights with a 16-hr photoperiod (15). For dsRNA isolation, cultures were grown in stationary liquid culture in *Endothia*-complete medium (25) modified by the omission of glucose. Cultures were usually grown for 10–14 days, although older cultures were occasionally used.

dsRNA isolation. The dsRNA used in size comparisons in polyacrylamide gels was isolated as described by Morris and Dodds (22) and Fulbright et al (14). Mycelia were washed with 1× STE (0.05 M Tris, pH 6.8, 0.1 M sodium chloride, 0.001 M ethylenediamine tetraacetic acid [EDTA]) and pressed dry. Samples were then ground in a mortar and pestle using glass beads (0.12–0.18

TABLE 1. *Endothia parasitica* isolates used in this study

Strain	Description	Origin
CL1	Virulent, bark isolate	Crystal Lake, MI
CL1-16	Virulent, single spore isolate of CL1	Crystal Lake, MI
GH2	Hypovirulent,[a] bark isolate	Grand Haven, MI
GHU4	Hypovirulent, bark isolate	Grand Haven, MI
RF	Hypovirulent, bark isolate	Rockford, MI
RC1	Hypovirulent, bark isolate	Roscommon, MI
CoLi 5′	Hypovirulent, bark isolate	County Line site, MI
MSU 12#1	Hypovirulent, bark isolate	County Line site, MI
FF	Hypovirulent, bark isolate	Frankfort, MI
BF5	Hypovirulent	West Virginia
9-B-2-1	Hypovirulent	West Virginia
905	Hypovirulent	Tennessee
780	Hypovirulent, ATCC no. 38755	Italian dsRNA in American background
422	Hypovirulent, ATCC no. 48537	French dsRNA in American background

[a] Hypovirulent isolates are reduced in virulence as determined by rate of growth in apple and they contain dsRNA. They are able to transmit these characteristics through hyphal anastomosis.

mm) and liquid nitrogen. The ground mycelia were suspended in 10 ml of 2× STE per 2–3-g sample. One-tenth volume of 100 mg of bentonite per milliliter, 0.15 volume of 10% sodium dodecyl sulfate (SDS) (w/v), and 1.5 volumes of STE-saturated phenol were added. The mixture was shaken on ice for 30 min and then centrifuged to separate the phases. The aqueous phase was adjusted to 15% ethanol and subjected to CF-11 chromatography. Ten-milliliter CF-11 columns (Whatman, Clifton, NJ) were washed with 80 ml of STE:15% ethanol before the elution of the dsRNA with 14 ml of 1× STE. Column chromatography was repeated and the dsRNA was concentrated by ethanol precipitation.

The dsRNA used in hybridization experiments was isolated as described, except that the STE used in the initial stages of the isolation contained 5 mM ethylene-glycol-bis (β-aminoethyl ether) N, N′ tetraacetic acid, 5 mM N-ethylmaleimide, 100 μg of heparin per milliliter, 0.5% 2-mercaptoethanol (v/v), and 50 μg of spermine per milliliter (4). The aqueous phase was ethanol precipitated after phenol extraction, and nucleic acids were resuspended in 1× STE without additives. CF-11 chromatography was performed as described. The concentration of dsRNA was determined spectrophotometrically. Equivalent amounts of fungal mycelia from the virulent isolates, CL1 and CL1-16, which do not contain dsRNA, were carried through the extraction procedure. The resulting solution was handled in the same way as the dsRNA isolated from hypovirulent isolates.

Polyacrylamide gels. The dsRNA was separated in 5% polyacrylamide gels run for 12 hr at 40 milliamperes (mA) in 0.04 M Tris, pH 7.8, 0.02 M sodium acetate, 0.001 M EDTA. Gels were stained in 0.25 μg of ethidium bromide per milliliter for 1 hr, followed by destaining in distilled water for 15 min before photography. Reovirus serotype 3 dsRNA, phage ϕ6 dsRNA, and dsRNA from viruses of *Cochliobolus heterostrophus*, *Penicillium stoloniferum*, and *P. chrysogenum* were included on the gels as molecular weight markers (5,6,26,27). Molecular weights were estimated by the method described by Bozarth and Harley (5).

Agarose gels. Two micrograms of dsRNA was loaded onto 1% agarose gels. Electrophoresis was performed for 1–1.5 hr at 60 V in 0.04 M Tris, pH 7.8, 0.02 M sodium acetate, and 0.001 M EDTA. Gels were stained in 0.5 μg of ethidium bromide per milliliter for 10 min, followed by brief destaining in distilled water before photography.

Fig. 1. Location of sites from which Michigan isolates of *Endothia parasitica* were collected. GH = Grand Haven; RF = Rockford; FF = Frankfort; CoLi = County Line; CL = Crystal Lake; RC = Roscommon.

Denaturing gels and transfer to nitrocellulose. Four micrograms of dsRNA was denatured by heating at 65 C for 5 min in 50% formamide (v/v), 6% formaldehyde (v/v), 20 mM sodium borate, pH 8.3, 0.2 mM EDTA. The samples were then adjusted to 0.03% bromophenol blue (w/v) and 10% glycerol (v/v) and loaded onto denaturing gels. The 1.2% agarose, 3% formaldehyde gels were then run for approximately 17 hr at 40 mA in 20 mM sodium borate, pH 8.3, 0.2 mM EDTA, 3% formaldehyde buffer (8). Lanes containing size standards were cut off of the gel and stained with ethidium bromide (21).

The dsRNA was transferred from the gel to nitrocellulose filters by blotting in 10× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) (8,21). Nitrocellulose filters were baked under a vacuum at 80 C for 2 hr.

End-labeling of dsRNA. T4 polynucleotide kinase was used to end-label dsRNA by the procedure of Jordan and Dodds (19). Two to ten micrograms of dsRNA was subjected to alkaline hydrolysis in 25 mM glycine, 5 mM magnesium chloride, pH 9.0, for 30–60 min at 60 C. The dsRNA was ethanol precipitated and resuspended in distilled water. Reaction mixtures contained 70 mM Tris-HCl, pH 7.0, 100 mM potassium chloride, 10 mM magnesium chloride, 5 mM dithiothreitol, 7–10 units T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD), and 25–60 μCi of 5′-(γ-^{32}P) ATP (3,000–5,000 Ci/mmol; Amersham, Arlington Heights, IL). Reactions were allowed to proceed overnight at 37 C. Four-tenths volume of sonicated calf thymus DNA was added, followed by one volume of 4 M ammonium acetate. The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was subjected to Sephadex G-50 chromatography, and fractions were monitored for radioactivity by absorption onto DE-81 filters (Whatman, Clifton, NJ), which were then counted in a liquid scintillation counter (21). Fractions showing the highest levels of radioactivity were ethanol precipitated. Specific activities of 10^5–10^7 cpm/μg were obtained.

Hybridization. Nitrocellulose filters were prehybridized in 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 6.5, 250 μg of sonicated denatured calf thymus DNA per milliliter, 0.02% bovine serum albumin (w/v), 0.02% Ficoll (MW ~ 400,000) (w/v), 0.02% polyvinylpyrrolidone (MW = 40,000) (w/v) for a minimum of 2 hr. ^{32}P-labeled dsRNA was denatured by heating at 100 C for 5–10 min. Filters were hybridized to the ^{32}P-dsRNA in the above hybridization solution containing 10^4–10^6 cpm/ml labeled RNA for 24 hr at 42 C (29). The filters were washed four times with 2× SSC, 0.1% SDS for 5 min at room temperature and then twice in 0.1× SSC, 0.1% SDS at 50 C for 15 min. Kodak XAR-5 film was exposed to the filter at −75 C using a Dupont Cronex Lightning Plus intensifying screen. Sizes of hybridized segments were determined by comparison with size markers in lanes cut from the gel before transfer of dsRNA to nitrocellulose.

Selected isolate combinations were examined under lower stringency conditions. GH2 dsRNA was hybridized to dsRNA from isolates RC1, 780, 422, and BF5 as described, with the omission of the two final washes at 50 C. GH2 dsRNA was also hybridized to dsRNA from isolates RC1, 780, 422, and BF5 for 30 hr, during which the temperature was allowed to drop from 80 C to 32 C. The filter was then washed as usual.

RESULTS

A variety of dsRNA banding patterns from Michigan isolates of *E. parasitica* were observed in polyacrylamide gels. The patterns varied in size, number, and relative intensity of dsRNA bands. Six isolates with representative dsRNA banding patterns were chosen, and the sizes of their brightest and most consistent dsRNA bands were estimated by comparing their migration in polyacrylamide gels with that of dsRNA molecular weight markers (Fig. 2). Most isolates consistently yielded one to three brightly stained dsRNA

segments. Many dsRNA segments that stained less intensely were not consistently detected during repeated assays. A continuum of bands was present below the largest segment in gels from many extractions. The most intensely stained segments and the continuum of bands were resistant to 5 mg of RNase A per milliliter in 0.3 M sodium chloride, but were degraded by RNase A in distilled water, confirming their identity as dsRNA (22) (results not shown). The molecular weights of the dsRNA segments in the hypovirulent isolates varied from 0.56 to 6.2×10^6. No one size segment was common to all isolates, but five of the six isolates analyzed consistently contained high molecular weight segments ($4.4–6.2 \times 10^6$). Isolates GH2, GHU4, and RF also consistently exhibited smaller segments with molecular weights between 0.56×10^6 and 2.4×10^6. The RC1 isolate was an exception; the only segment consistently present had a molecular weight of 1.1×10^6. Segments with molecular weights of 1.9×10^6 and 0.92×10^6 were often, but not always, detected. Size comparison studies were performed several times.

Because of the lack of a single-size dsRNA segment correlated with the hypovirulent phenotype, hybridization experiments were conducted to determine whether any of the various segments were related by sequence homology. The ability of dsRNA from two Michigan isolates, GH2 and RC1, to hybridize to dsRNA from other Michigan isolates, as well as isolates from Tennessee, West Virginia, France, and Italy, was examined. Resolution of dsRNA segments in polyacrylamide is improved relative to resolution in agarose (Figs. 2 and 3). The continuum of bands below the largest band is sometimes visible in agarose as well as in polyacrylamide gels (Fig. 3, lane 3). Northern blots used in this study were from agarose gels. Banding patterns might look different than those shown in polyacrylamide (Fig. 2), so banding patterns in agarose are shown (Fig. 3). A wide variety of banding patterns was found among these isolates (Fig. 3). The isolates from West Virginia (Fig. 3, lanes 9 and 10) exhibited many bands compared to the other isolates shown. All gels were not run concurrently, so direct size comparisons cannot be made in Figure 3.

Double-stranded RNA from GH2 hybridized to all dsRNAs of Michigan origin (GHU4, RF, FF, CoLi 5′, MSU 12#1), except to dsRNA from isolate RC1 (Fig. 4A and B, Table 2). Hybridization to the 0.58×10^6 MW GHU4 segment was observed in some autoradiograms (data not shown). GH2 dsRNA did not hybridize to dsRNA from other states or from Europe (905, BF5, 9-B-2-1, 422, 780). RC1 dsRNA, when used as a probe, did not share homology with dsRNA from any of the isolates tested except itself (Fig. 4C, Table 2). The background of less intensely staining dsRNA bands seen on ethidium bromide stained gels was also often visible in autoradiograms; this is probably responsible for the smear observed in some lanes (Fig. 4). Reducing the stringency of hybridization did not result in hybridization of GH2 dsRNA to RC1 dsRNA or to dsRNA from outside of Michigan (results not shown). No hybridization was ever observed in lanes containing extracts from CL1-16, a virulent isolate lacking detectable dsRNA.

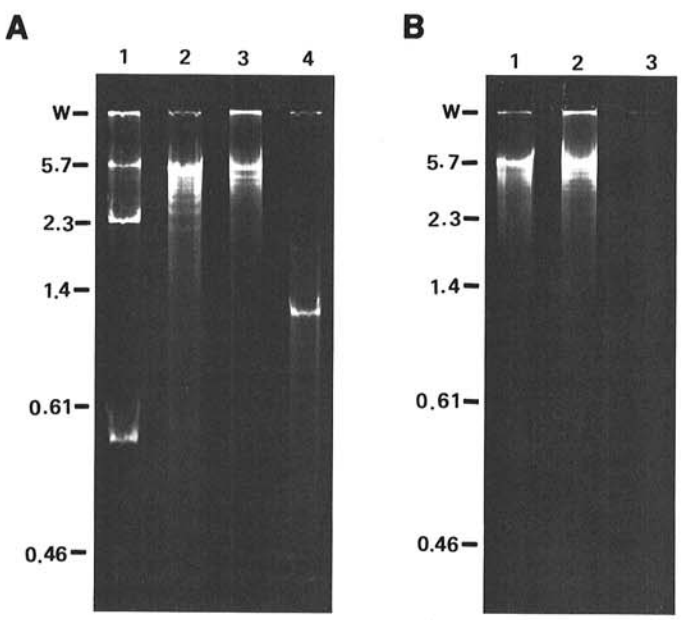

Fig. 2. Banding patterns of dsRNA from representative Michigan isolates of *E. parasitica* in 5% polyacrylamide gels run for 12 hr and then stained with ethidium bromide. The position of the wells is indicated (w). Lanes: **(A)** 1, GH2; 2, GHU4; 3, RF; 4, RC1; **(B)** 1, CoLi 5′; 2, FF; 3, CL1. CL1 represents a virulent isolate that does not contain dsRNA. The molecular weights ($\times 10^6$) of selected size markers that were run in adjacent lanes are indicated.

DISCUSSION

Hypovirulence is exhibited by Michigan isolates of *E. parasitica* with varying numbers, sizes, and relative amounts of dsRNA segments. No one size dsRNA segment was found to correlate with hypovirulence. As we have reported (14), many dsRNA banding

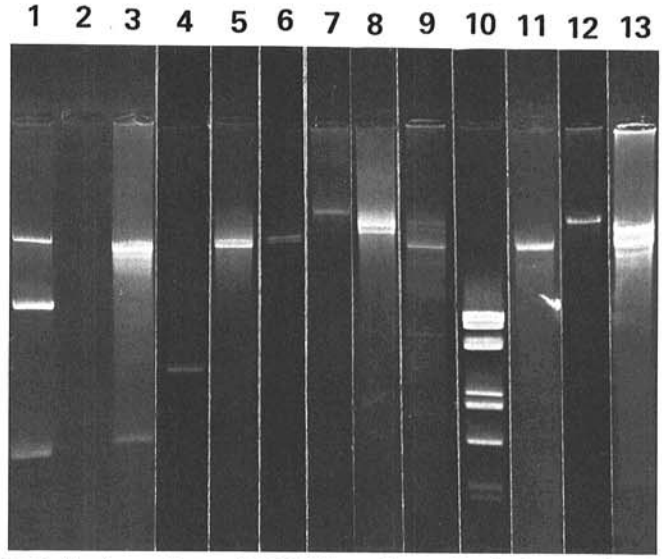

Fig. 3. Banding patterns of dsRNA from *E. parasitica* isolates run in 1% agarose gels and stained with ethidium bromide. Lanes: 1, GH2; 2, CL1-16; 3, GHU4; 4, RC1; 5, FF; 6, RF; 7, CoLi 5′; 8, MSU 12#1; 9, BF5; 10, 9-B-2-1; 11, 905; 12, 780; 13, 422. CL1-16 (lane 2) represents a virulent isolate that does not contain dsRNA. All isolates were not run concurrently; therefore, direct size comparisons cannot be made.

TABLE 2. Summary of hybridization results between dsRNA from isolates GH2 and RC1, and dsRNA from other isolates from Michigan, other states, and Europe

Source of dsRNA used in hybridizations	Source of probe dsRNA	
	GH2	RC1
Michigan		
GH2	+	−
RC1	−	+
GHU4	+	−
RF	+	−
FF	+	−
CoLi 5′	+	−
MSU 12#1	+	−
CL1-16[a]	−	−
Other states		
905	−	−
BF5	−	−
9-B-2-1	−	−
Europe		
422	−	−
780	−	−

[a]CL1-16 is a virulent isolate that does not carry dsRNA.

patterns in gels are found in Michigan other than the three reported by Dodds (9). Two of the banding patterns described by Dodds were found in European isolates and the other in North American isolates. Molecular weights of the dsRNA segments reported by Dodds ranged from 4.3–6.2 $\times 10^6$. He also found minor bands, with molecular weights of less than 4×10^6. These bands were very faint and required overloading of the gel and long photographic exposures to be visualized (9). All but one of the Michigan hypovirulent isolates included in this study harbored dsRNA corresponding to the large molecular weight segments described by Dodds. The smaller segments found in isolates GH2, GHU4, and RF were easily detected, unlike the low molecular weight segments found by Dodds. One isolate, RC1, was exceptional in that it did not carry detectable large molecular weight segments, but carried three segments of molecular weight between 0.92 and 1.9×10^6. The molecular weights assigned to these segments should be regarded as estimates (5,9), but are useful for comparative purposes.

The dsRNA banding patterns in *E. parasitica* have been shown to change when transferred to another genetic background or upon repeated subculturing (1,3,7,10,13,15,30). Because of their occasional instability and the fact that no one size of band is common to the hypovirulent isolates, banding patterns cannot be relied on to categorize isolates (30). The middle segment in isolate GH2 may be the result of an internal deletion of the large segment, because they share sequence homology and common termini (28). Internal deletion has also been suggested as an explanation for the presence of smaller segments in a European isolate (17). However, the small segment of the GH2 isolate does not share extensive sequence homology with the two larger GH2 segments (28), suggesting an independent origin for this segment. A 3′-poly(adenylic acid):5′-poly(uridylic acid) sequence is found at one terminus in all segments of both the American and the European isolate examined, indicating dsRNAs from *E. parasitica* share common features that may be important for dsRNA replication or gene expression. Defective dsRNA segments retaining the necessary structural features would be maintained in the fungal isolate resulting in the complex and changing banding patterns observed.

Our results indicate that the dsRNA in populations of *E. parasitica* in North America is varied, with at least three types of dsRNA based on sequence homology. Even within a single state, dsRNA from all isolates did not share common sequences at a detectable level (Table 2). Although different isolates were used, the lack of homology found between American and European dsRNA is in agreement with the results of L'Hostis et al (20). Thus, the European dsRNA constitutes a fourth type of dsRNA. Our results differ from those of L'Hostis et al in that we found dsRNAs of American origin that do not share homology. Thus, homology relationships among dsRNAs from different geographical areas are more complex than previously reported. The lack of homology suggests different origins for the dsRNA. More than one independent origin for dsRNA in *E. parasitica* may have occurred even within the state of Michigan, as well as on two continents.

The Michigan isolates used in this study were collected from six different stands of trees in the western half of Michigan's lower peninsula (Fig. 1). Hypovirulent isolates were found at all sites, except for Crystal Lake. The Crystal Lake isolates, represented here by CL1-16, did not contain detectable dsRNA. Different dsRNA banding patterns were found within the same recovering stand of trees, as shown by the GH2 and GHU4 banding patterns (Fig. 2), whereas similar banding patterns were found at the County Line and Frankfort sites (data not shown). These two sites are located approximately 10 miles apart.

The RC1 isolate is unique among the Michigan hypovirulent isolates examined. RC1 dsRNA did not hybridize to the dsRNA from any of the Michigan isolates tested and also has the most distinct banding pattern (13). Additionally, the RC1 isolate exhibits a unique culture morphology, differing from other hypovirulent strains isolated from Michigan in that it has a fibrous appearance, smooth colony margins, and lacks aerial hyphae. This isolate was collected from a site that is farther east in Michigan than any of the other sites: approximately 70 miles from County

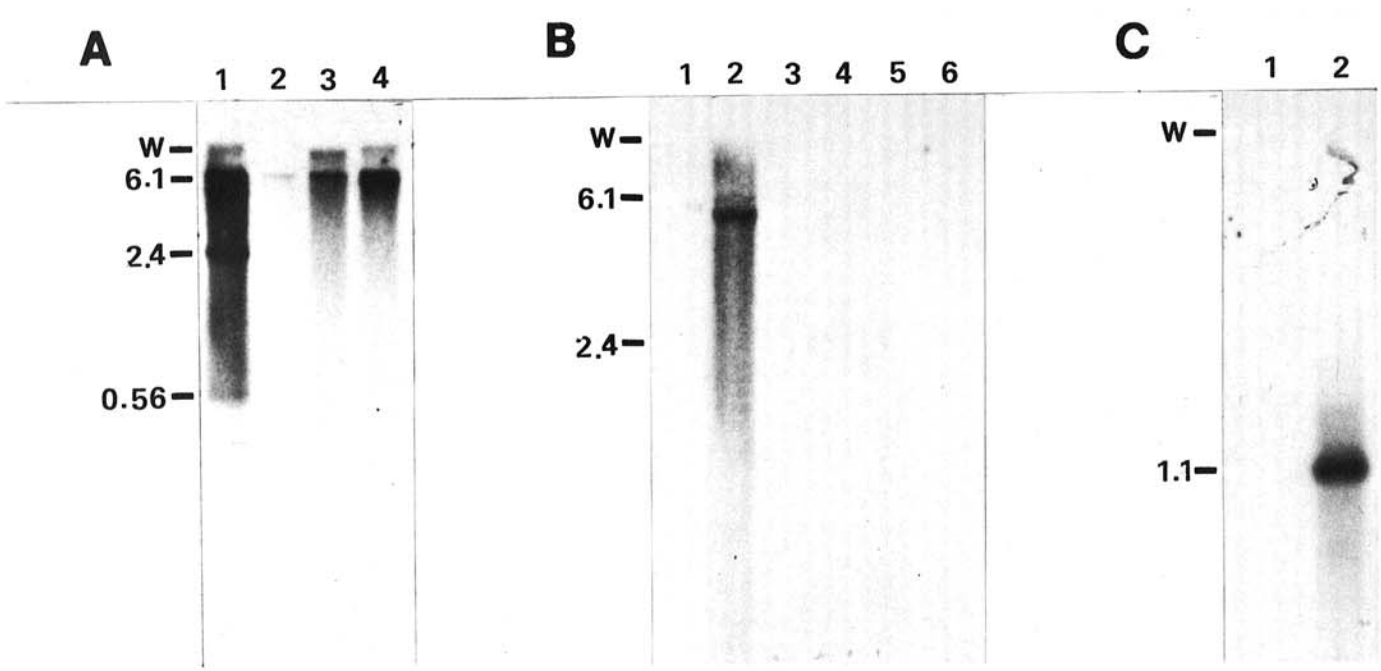

Fig. 4. Hybridization of GH2 and RC1 dsRNA to dsRNA from other *E. parasitica* isolates. The dsRNA from various isolates was denatured and run in 3% formaldehyde, 1.2% agarose denaturing gels and then blotted onto nitrocellulose filters. Probe dsRNA was end-labeled with ^{32}P using T4 polynucleotide kinase and then hybridized to the dsRNA on the filter. X-ray film was exposed to the filter using an intensifying screen. Sizes of segments were determined by comparison with reovirus dsRNA run in the same gel. Lanes containing reovirus dsRNA were cut off of the gel and stained with ethidium bromide (21). Molecular weights ($\times 10^6$) and the position of the wells (w) are indicated in each figure. A, B, and C were run separately, and the time of the runs varied. **(A),** dsRNA from selected isolates probed with GH2 dsRNA. Lanes: 1, GH2; 2, RF; 3, FF; 4, MSU 12#1. Some material remained in the wells of this gel. Lane 2 contained 2 ng of dsRNA, whereas all other lanes contained four μg of dsRNA. Results with isolate CoLi 5′ were similar to lane 3. **(B),** dsRNA from selected isolates probed with GH2 dsRNA. Lanes: 1, CL1-16; 2, GHU4; 3, RC1; 4, 780; 5, 422; 6, 9-B-2-1. Results with isolates BF5 and 905 were identical to lanes 4–6. **(C),** dsRNA from selected isolates probed with RC1 dsRNA. Lanes: 1, GHU4; 2, RC1. Results with all other isolates tested were identical to lane 1.

Line and 125 miles from Grand Haven.

The presence of isolates within Michigan carrying dsRNAs that share homology suggests that the natural spread of dsRNA within the *E. parasitica* population has occurred. The sequence of the dsRNA has apparently been more highly conserved than the size of the segments. Isolates with dsRNA sharing homology with GH2 dsRNA were collected up to 105 miles from the Grand Haven site. Within a localized area, dsRNA sequences are related, indicating that the dsRNA may have spread from one original source.

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