# Revised Estimates of the Molecular Weights of dsRNA Segments in Hypovirulent Strains of *Endothia parasitica*

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I am grateful to R. F. Bozarth (Department of Life Sciences, Indiana State University, Terre Haute, 47809) and J. Van Etten (Department of Plant Pathology, University of Nebraska, Lincoln 68583) for gifts of Hm9 VLP ds-RNA and $\phi 6$ bacteriophage dsRNA, respectively. The technical assistance of Margaret Finkbeiner, Michael Tiffany, and Nancy DePalma is gratefully acknowledged.
Accepted for publication 9 June 1980.

## ABSTRACT

DODDS, J. A. 1980. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica*. Phytopathology 70:1217-1220.

Multisegment dsRNAs were detected by polyacrylamide gel electrophoresis of preparations purified by CF-11 cellulose chromatography from twelve hypovirulent strains of *Endothia parasitica* isolated from France, Italy, and North America. Ethidium bromide-stained dsRNA segments were identified by incubating individual gels with ribonuclease, first in 0.3 NaCl and then in water. The molecular weights of the dsRNA segments were between 4.0 and $7.0 \times 10^6$. The types of dsRNA in European strains (type 1 in one strain, type 2 in seven strains) were distinct from each other and from the dsRNAs in North American strains (type 3 in four strains). Levels of pathogenicity or growth rate were not correlated with types of dsRNA. The concentration of dsRNA was lower in North American strains than in European strains.

In a previous study (6) all hypovirulent strains of *Endothia parasitica* tested were found to contain double-stranded RNA (dsRNA). The quality of dsRNA was variable, but one common major dsRNA segment (MW = $3.3 \times 10^6$) was reported for all strains. The molecular weights were previously estimated assuming a linear relationship between the logarithm of molecular weight and mobility of dsRNA on polyacrylamide gel electrophoresis. This assumption has since been shown to be inadequate (3). Improvements in subsequent analyses of dsRNA from selected European and North American hypovirulent strains have included longer electrophoresis times, a different staining technique, a postelectrophoresis ribonuclease test, standards of more similar molecular weight, and electron microscopy. These approaches, which have produced data that give a clearer picture of the size, variability, identity, quality, and quantity of dsRNA in *E. parasitica,* are the subject of this report.

## MATERIALS AND METHODS

Hypovirulent strains of *E. parasitica* isolated from Europe and North America were selected from the culture collection at The Connecticut Agricultural Experiment Station; the identities and origins of the 12 chosen strains are shown in Table 1. They were grown in liquid culture as previously described (6) and the dsRNA they contained was purified by the method of Morris and Dodds (12) with minor modifications. Mycelium was extracted at a ratio of 1 g (fresh weight) to 2 ml of double-strength STE buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.0) which contained bentonite (1 mg/ml). The extract was adjusted to 1% SDS and emulsified with an equal volume of water-saturated phenol. The emulsion was broken by centrifugation and ethanol was added to the aqueous phase to a final concentration of 15% (v/v). The dsRNA was purified by chromatography on small columns of CF-11 cellulose (6,12).

The purified dsRNA from 0.5–2.0 g of mycelium was analyzed by electrophoresis on 5% polyacrylamide gels in 0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.8 for 10 hr at 6 mA/gel. The absorbance profile at 280 nm of gels in quartz tubes was recorded after electrophoresis with an ISCO Model 1310 gel scanner and Model UA-5 absorbance monitor (ISCO, Lincoln, NE 68505). A preferred wavelength of 254 nm was not used because the baseline absorbance profile of blank gels was unacceptable. Gels were stained with ethidium bromide (5 $\mu$g/ml) in 0.3 M NaCl for 4 hr and destained in 0.3 M NaCl for 4 hr. Ribonuclease (50 $\mu$g/ml) was added for the final 3 hr. Gels were photographed and then incubated for a further 3 hr in the presence of ribonuclease in water and re-examined. Any fluorescence resistant to the first treatment and digested by the second treatment was assumed to be associated with dsRNA (12). The dsRNAs from a VLP (Hm9) of *Helminthosporium maydis* (MW = $5.7 \times 10^6$) and the bacteriophage $\phi 6$ (MW = 4.3, 2.6, and $1.8 \times 10^6$) were used as molecular weight standards. Molecular weights were calculated by the graphical method of Bozarth and Harley (3). The values for the standards differ from those used previously which were calculated from molecule lengths by using a factor of $2.39 \times 10^6$ per $\mu$meter (3). They have been reduced by a factor of 2.15/2.39 because $2.15 \times 10^6$ (11) rather than $2.39 \times 10^6$ was used to convert molecule lengths to molecular weights in this study. This correction was made to avoid over-estimating the molecular weight of the large dsRNAs in *E. parasitica.*

Purified dsRNA molecules were spread and shadowed by the method of Davis et at (4) and photographed at ×4,950. Magnification was calculated from a grating replica ( E. F. Fullam, Inc., Schenectady, NY 12301) photographed at the same time. Projected images were traced and their lengths were measured with a map measurer. Molecular weights were estimated using a factor of $2.15 \times 10^6$ per $\mu$meter (11).

## RESULTS

The dsRNA patterns (types 1,2, and 3) observed, the standard dsRNAs and the effects of ribonuclease are illustrated in Fig. 1. Fluorescence before (not shown) and after (Figs. 1 A–F) ribonuclease treatment of gels in 0.3 M NaCl was the same. Fluorescence associated with bands began to disappear when the stained gels were transferred to water and ribonuclease (Figs. 1G, H). Clearing began at the surface and progressed to the core of the gel, and within 3–4 hr no fluorescent bands remained. The resistance of standard dsRNAs to RNAse in 0.3 M NaCl is shown in Figs. 1 E,F. The standards were completely digested by RNAse in water (not illustrated). The fluorescent components illustrated in Fig. 1 were therefore dsRNA.

Type 1 dsRNA, observed in only EP 113 (French) was resolved as four major segments (Figs. 1A, D) in a background of minor segments. The two fastest-migrating major segments had mobililties within the range of the standards (Figs. 1 D, E, and F) and their molecular weights were calculated to be 4.6 and $5.0 \times 10^6$. The other two segments had slower mobilities than Hm9 VLP dsRNA, the highest molecular weight standard available (3);

0031-949X/80/12121704/$03.00/0

therefore, their molecular weights had to be calculated by extrapolation and were estimated to be 5.9 and $6.2 \times 10^6$. These are to be considered minimum values, since small differences in mobility correspond to large differences in molecular weight for dsRNA segments of this size (3).

Type 2 dsRNA was detected in three French strains and four Italian strains (Fig. 1B). One major (MW = $6.0 \times 10^6$), one intermediate (MW = $5.5 \times 10^6$), and four minor (MW = 4.5, 4.7, 4.9, and $5.2 \times 10^6$) segments characterized these strains. The minor segments are not clear though they were present in the gel illustrated in Fig. 1B. They also were consistently detected in all seven strains designated as containing type 2 dsRNA, suggesting that they are not likely to be in vitro degradation products of the major segment.

Type 3 dsRNA was detected in four North American strains. They contained one major segment (MW = $5.5 \times 10^6$). Strain EP 60 (Fig. 1C) contained sufficient dsRNA to distinguish two minor dsRNA segments (MW = $4.8 \times 10^6$ and $4.3 \times 10^6$). The other three North American strains did not. The minor segments are resolved in Fig. 2 which illustrates separate and co-electrophoresis of type 1

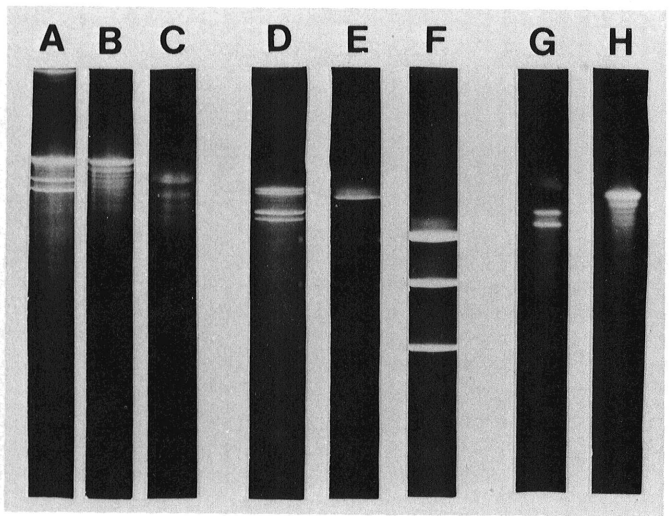


**Fig. 1.** Types of dsRNA associated with hypovirulence in *Endothia parasitica* compared with the dsRNAs from Hm9 VLP (MW = $5.7 \times 10^6$) and $\phi$6 bacteriophage (MW = 4.3, 2.6 and $1.8 \times 10^6$). **A,** Type 1 dsRNA (EP 113). **B,** Type 2 dsRNA (EP4, 47, 49, 50, 51, 166, and 167). **C,** Type 3 dsRNA (EP 60, 90, 102, and 103). **D,** Type 1 dsRNA. **E,** Hm9 VLP dsRNA. **F,** $\phi$6 bacteriophage dsRNA. **G and H,** Partial in situ ribonuclease digestion of types 1 and 2 dsRNA. Electrophoresis was through 5% polyacrylamide gels at 6mA/gel for 10 hr in 0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.8. Gels A–F were stained with ethidium bromide for 4 hr in 0.3 M NaCl, and destained in 0.3 M NaCl for 4 hr and treated with ribonuclease (50 $\mu$g/ml) at the same time. Gels G and H were subsequently incubated in water and ribonuclease for 2 hr. Gels A–C, D–F, and G–H were electrophoresed at different times.

TABLE 1. Growth rate, pathogenicity, and dsRNA content of strains of *Endothia parasitica* grouped by the types of dsRNA

| Type of dsRNA | Strain[a] number | Country of origin | dsRNA[b] content ($\mu$g) | Growth[c] rate | Pathogenicity[d] |
|---|---|---|---|---|---|
| 1 | 113 | France | 40 | ++ | + |
| 2 | 167 | France | 24 | + | – |
| | 4 | France | 16 | + | – |
| | 51 | Italy | 12 | ++ | ++ |
| | 47 | Italy | 8 | +++ | – |
| | 49 | Italy | 6 | ++ | ++ |
| | 166 | France | 6 | + | – |
| | 50 | Italy | 2 | +++ | ++ |
| 3 | 60 | USA | 4 | ++ | – |
| | 90 | USA | 1 | ++ | ++ |
| | 102 | USA | 1 | + | ++ |
| | 103 | USA | 1 | ++ | +++ |
| None | 6 | USA | 0 | ++ | +++ |

[a] Numbers refer to entries in the culture collection of The Connecticut Agricultural Experiment Station.

[b] Estimated from absorbance profiles (280 nm) of polyacrylamide gels through which the dsRNA from 2.0g (fresh weight) of mycelium had been electrophoresed.

[c] Expressed as fresh weight of mycelium after 7 days of growth in 100 ml of medium. + = 0.4–0.6 g, ++ = 1.0–1.9 g, +++ = 2.4 g.

[d] – = no canker developed on living stems of *Castanea dentata* + = canker ceased to grow within 3 wk of inoculation, ++ = canker ceased to grow after first winter, +++ = canker continued to grow in second growing season. The first three categories were hypovirulent. Category 4 was virulent. Data from Elliston (8, and *personal communication*).

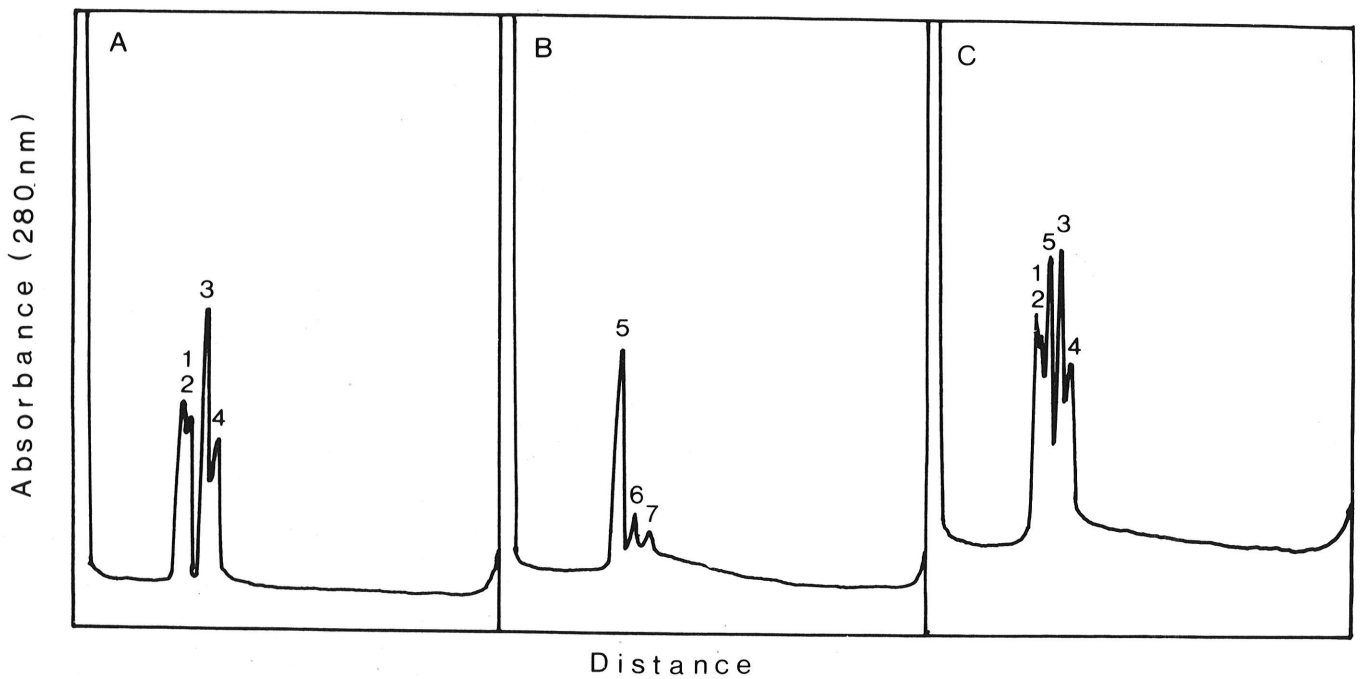


**Fig. 2.** Absorbance profiles of dsRNA from *Endothia parasitica*. **A,** Type 1 dsRNA (EP 113). **B,** Type 3 dsRNA (EP 60). **C,** A mixture of both types of dsRNA. Molecular weights of the dsRNA segments are 1 = $6.2 \times 10^6$, 2 = $5.9 \times 10^6$, 3 = $5.0 \times 10^6$, 4 = $4.6 \times 10^6$, 5 = $5.5 \times 10^6$, 6 = $4.8 \times 10^6$, 7 = $4.3 \times 10^6$. Samples were electrophoresed through 5% polyacrylamide gels at 6mA/gel for 10 hr in 0.04 M Tris, 0.02 M sodium acetate, 0.001 EDTA, pH 7.8.

and type 3 dsRNA. The patterns of dsRNA from the European and North American strains are distinct.

Linear molecules 1.8–3.4 $\mu$m long (MW = 3.9–7.3 $\times$ $10^6$), corresponding in size to the major dsRNA segments detected by gel electrophoresis, made up 49 and 61% of the number (65 and 75% of the total length) of 213 and 112 molecules of type 1 and type 2 dsRNA, respectively (Fig. 3). As expected, a higher relative amount of 3.9–5.5 $\times$ $10^6$ molecules to 5.5–7.3 $\times$ $10^6$ molecules was observed in type 1 dsRNA than in type 2 dsRNA. Electron microscopy did not resolve size clusters of dsRNA as effectively as did gel electrophoresis.

Recoveries of dsRNA from the 12 strains are compared in Table 1 with their relative growth rates and pathogenicities (8). Correlations between either type or amount of dsRNA and either growth rate or pathogenicity are difficult to establish. The type of dsRNA present certainly does not alone determine the biology of a strain; strains which contained a single type of dsRNA, either type 2 or 3, had a variety of growth rates and pathogenicities. The amount of dsRNA in American strains was low compared to most European strains.

## DISCUSSION

Hypovirulence in *E. parasitica* is associated with the presence of dsRNA which is presumed to be of viral origin (5–7), and the potential of hypovirulence for the control of chestnut blight is established (9,10). This study pointed out that there are two basic types of dsRNA associated with hypovirulence; one typical of European hypovirulent strains (type 2) and the other typical of North American hypovirulent strains (type 3). European strains also contained more dsRNA than North American strains. These observations suggest that at least two different viruslike agents may be associated with hypovirulence.

Previous estimates of the molecular weights of the dsRNAs in *E. parasitica* (6) must now be considered to be low because the standards used had molecular weights lower than those of *E. parasitica* dsRNA, and the relationship between dsRNA mobility in gel electrophoresis and molecular weight has been shown to be nonlinear (3). The values have now been more accurately estimated by gel electrophoresis to be between 4.3 and 6.2 $\times$ $10^6$ and linear molecules of similar size were detected by electron microscopy. The conclusion in a previous study (6) that a common dsRNA segment (MW = 3.3 $\times$ $10^6$) was present in all hypovirulent strains must now be challenged. In the present study, it was demonstrated that the major dsRNA segments in North American strains and European strains are not the same. The new interpretation points out the importance of analyzing mixtures of components with similar mobilities to confidently detect differences between them. The 3.3 $\times$ $10^6$ segment previously reported in EP 3 (now called EP 113) has been resolved as two segments (MW = 6.2 $\times$ $10^6$ and 5.9 $\times$ $10^6$). The 3.2 $\times$ $10^6$ segment previously reported in EP 4 has now been estimated to have a molecular weight of 5.5 $\times$ $10^6$ and it has been detected in strains EP 40, 50, and 51 in which it was previously overlooked. The sensitive staining obtained with ethidium bromide has revealed additional previously undetected minor segments.

The three types of segmented dsRNAs detected, though qualitatively different, have exceptionally high molecular weights. This property sets these viruslike agents apart from segmented dsRNA fungal viruses (2). The dsRNA of the Hm9 VLP (2,3) with a similar molecular weight is the exception, but it is not segmented. The dsRNA associated with hypovirulent strains of *E. parasitica* therefore represents a much larger viruslike genome than those typically found in virus-infected fungi.

There is almost a continuous range of pathogenicities from avirulent to virulent among strains which contain dsRNA segments with molecular weights of 4.0–7.0 $\times$ $10^6$ (8). The knowledge that more than one type of dsRNA is associated with hypovirulence is not in itself sufficient to explain the variation. Variation among strains with the same type of dsRNA suggests that: the viruslike genomes can exist as numerous strains, different fungal genetic backgrounds can influence the effects of the dsRNA, or both operate to determine the biology of a hypovirulent strain. The usual result of the interaction appears to be hypovirulence, but occasionally (EP 103) there is no measurable effect on virulence.

The existence of strains of viruslike genomes, or the effects of the host on viral genome expression, could be determined by transmitting a single set of dsRNA segments into different fungal genetic backgrounds or by transmitting different dsRNA genomes into a single fungal genetic background. Studies of this kind have been attempted with EP 113, which has the most complex pattern of major dsRNA components, type 1 (1). It was found that some converted strains contained type 1 dsRNA but a variety of types of dsRNA were detected in others, including some which were similar to type 2 dsRNA. Therefore, the two apparently distinct types of dsRNA in European strains may be related. This indicates the need for further studies on identity and relatedness of dsRNAs from hypovirulent strains of *E. parasitica*. A full understanding of the nature and effects of these dsRNAs should assist in the selection of hypovirulent strains for the control of chestnut blight in the field.

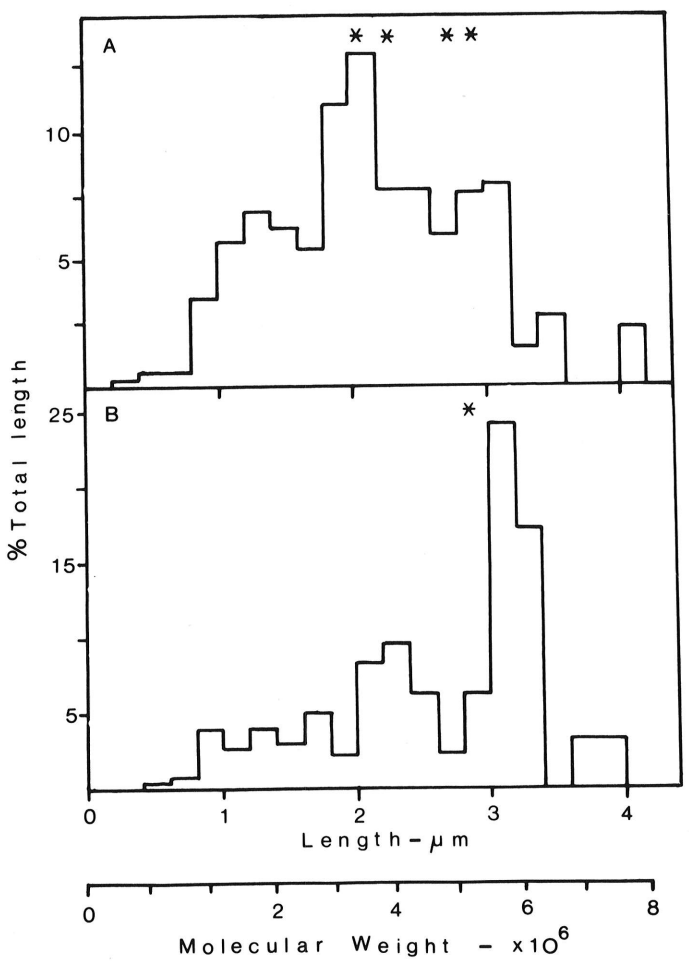


**Fig. 3.** Lengths and molecular weights of dsRNA molecules purified from hypovirulent strains of *E. parasitica*. **A,** Type 1 dsRNA (EP 113) and **B,** Type 2 dsRNA (EP 51). The total length of dsRNA molecules of each size class is expressed as a percentage of the cumulative length of all molecules. The molecular weights estimated by polyacrylamide gel electrophoresis for the major dsRNA segments are indicated by stars.

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