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Mitochondrial DNA of *Cryphonectria parasitica*: Lack of Migration Between Vegetatively Compatible Strains

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The mitochondrial (mt)DNA of *Cryphonectria parasitica* is approximately 144 kb, but the size varies among strains because of length polymorphisms. One of the restriction fragment length polymorphisms, a 2.4-kb *Sac*I fragment of the mtDNA of strain EP2001, hybridizes to only a 0.7-kb *Sac*I fragment in strain EP113. Migration of mtDNA between vegetatively compatible strains was examined by hybridization using as a probe a plasmid containing a clone of the 2.4-kb fragment of EP2001. RFLP analysis of isolates from various parts of the colonies and *in situ* colony hybridization procedures showed that the mtDNA of EP2001 does not migrate into strain EP113 when hyphal anastomosis occurs. Migration of dsRNA between the two strains showed that hyphal anastomosis had occurred. A genetic marker indicated that heterokaryons did not form and that hyphae of EP2001 did not grow beyond the zone of colony interaction.

Additional keywords: *Endothia parasitica*, hypovirulence.

Genetic elements can be transferred by anastomosis between vegetatively compatible filamentous fungal strains. This is the basis of the formation of heterokaryons and the movement of viruses within populations of fungi. Although heterokaryons are known to form as the result of the somatic fusion of different fungal strains, evidence suggests that heterokaryon formation is infrequent except when "forced" by complementation of auxotrophs (Cooke and Rayner 1984). Fungal viruses, on the other hand, are known to move readily and rapidly between vegetatively compatible strains (Anagnostakis and Waggoner 1981). Fungal viruses also move between strains of different vegetative compatibility (vc) groups, although with much lower frequency than between strains of the same vc group (Brasier 1983; Anagnostakis 1982).

Little information is available concerning the movement of mitochondrial (mt)DNA between fungal strains. Caten (1972) reported that vegetative death of *Aspergillus amstelodami* (Mangin) Thom & Church, a trait associated with mitochondria, is readily transmitted by hyphal anastomosis. However, mitochondria were reported not to move between strains of *Coprinus cinereus* (Schaeff. *ex* Fr.) S. F. Gray (Casselton and Economou 1985) or *Ophiostoma ulmi* (Buisman) Nannf. (Brasier 1986).

Somatic fusion between strains of the same vc type apparently is common, although the strains may be from different geographic regions or of different sexual mating types. The ease with which double-stranded (ds)RNA viruses move between such vegetatively compatible strains suggests that other cytoplasmic genetic elements such as mtDNA, or plasmids associated with mitochondria, may also move by somatic fusion within populations of filamentous fungi. It is important to determine the extent of such asexual cytoplasmic gene migration, particularly if filamentous fungi containing recombinant DNA are to be released into the environment.

Transmissible hypovirulence of the fungal pathogen *Cryphonectria* (*Endothia*) *parasitica* (Murr.) Barr is a cytoplasmically transmitted trait (Van Alfen *et al.* 1975). Hypovirulent strains of *C. parasitica* are less virulent on susceptible species of chestnut than are the virulent forms of the fungus. Transmissible hypovirulence has been correlated with the presence of dsRNA (Day *et al.* 1977). However, other cytoplasmic elements, including mtDNA, may also be involved in the expression of hypovirulence. The dsRNA has been transmitted successfully only by somatic fusion, so the possibility of cotransfer of other genetic elements along with the dsRNA cannot be ignored.

To examine the extent of mtDNA migration between compatible strains of *C. parasitica*, we have isolated mtDNA from different strains of the fungus, partially characterized the mtDNA, and cloned a restriction fragment for use as a probe to facilitate detection of mtDNA movement. About 70% of the sequences of this fragment are strain-specific, so it was possible to use the cloned fragment to detect movement of mtDNA between strains of the fungus *in situ*. We concluded from these studies that the mtDNA does not move readily between compatible strains of the fungus, although dsRNA moves rapidly between the strains.

MATERIALS AND METHODS

Strains. The strains obtained from the American Type Culture Collection (ATCC, Rockville, MD) were EP113

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(ATCC 38771) and EP155 (ATCC 38755). EP113 is a subculture of a French hypovirulent strain (EP3) originally isolated by J. Grente. It is in vc group 10 and contains dsRNA. EP155 is a virulent strain originally isolated in Bethany, Connecticut. It contains no dsRNA and is in vc group 40. EP2001 was obtained from S. Anagnostakis (Connecticut Agricultural Experiment Station, New Haven). It is a single ascospore progeny from a cross of EP67, originally isolated in Italy, and EP294, a laboratory strain developed from a sexual cross of two Connecticut isolates. It contains no dsRNA, is virulent, and is in vc group 10.

Isolation of mtDNA and dsRNA. The fungus was grown as described by Hansen *et al.* (1985). After 5 to 7 days of growth at 25° C, the mycelium was harvested by filtration through Miracloth (Calbiochem, La Jolla, CA). The mycelial pads were cut into small pieces and stored at −70° C. Cellular DNA was isolated following the method of Garber and Yoder (1983). Generally, three cycles of isopycnic centrifugation in cesium chloride-bisbenzimide were necessary to obtain mtDNA free of nuclear DNA. Twenty randomly selected clones of approximately 5 kilobases (kb) each from a library prepared from mtDNA purified in this manner hybridized only to mtDNA fragments when used to probe Southern blots of total DNA (Y. Wang and N. K. Van Alfen, unpublished). This suggests that there is little nuclear DNA present in these mtDNA preparations. The dsRNA was isolated and separated by gel electrophoresis as described by Hansen *et al.* (1985).

Size estimates of mtDNA. The size of the mtDNA was estimated by restriction endonuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN) digestion. Restriction enzymes were used according to the instructions of the manufacturer. The electrophoresis procedures used for size estimation were repeated at least 10 times and standard deviations were determined. The largest fragment from *Kpn*I digestion was isolated from agarose gels and its size estimated. This segment was cut from low melting point agarose gels (International Biotechnologies, New Haven, CT) after electrophoresis. The portion of the agarose gel containing the segment was crushed in the bottom of a microfuge tube in 300 μl of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (TE), and then heated to 65° C for 30 min. If it was not dissolved, the sample was suspended using a vortex mixer and then heated to 37° C. It was extracted twice with TE-equilibrated phenol, once with phenol:chloroform (1:1), and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase using ethanol and resuspended in TE. The *Kpn*I fragment was labeled by nick translation (Maniatis *et al.* 1982) and used to probe a Southern blot of mtDNA that had been double digested with *Kpn*I and *Sac*I. Hybridization was done using Gene Screen Plus (New England Nuclear, Boston, MA) according to the manufacturer's instructions.

Cloning of a specific mtDNA fragment. The 2.4-kb *Sac*I fragment of strain EP155 was isolated from gels using Gene Clean (Bio-101, La Jolla, CA). It was cloned into the polylinker site of pBluescript (Stratagene, La Jolla, CA). The recombinant plasmid, pUV10, was isolated by the boiling lysis miniprep method described by Maniatis *et al.* (1982).

Membrane preparation. When fungal colonies had reached the desired size, the membranes were peeled from the agar plates with tweezers, and the excess agar was removed by scraping with a razor blade. The filters were then washed with 50 mM EDTA (pH 8.0), 2.5% (w/v) β-mercaptoethanol for 30 min followed by a 3-hr incubation at 45° C in 50 mM sodium citrate buffer (pH 5.8), 1.0 M D-sorbitol, 2% (w/v) novozyme 234 (NOVO Biolabs, Wilton, CT). The nucleic acid on the membranes was then denatured by washing in 1.5 M NaCl, 0.5 M NaOH for 5 min and rinsed in 0.5 M Tris-Cl (pH 7.5), 1.5 M NaCl for 5 min. The membranes were blotted between paper towels between each wash. After the final wash, the membranes were air dried, baked in an oven for 2 hr at 80° C, and stored in a desiccator.

Hybridization. Desiccated membranes were rewetted in 1% Triton X-100, blotted between Whatman 3MM paper (Whatman 3030917, Maidstone, England), and placed in hybridization bags (BRL-8278BA, Bethesda Research Laboratories, Gaithersburg, MD). The membranes were incubated overnight at 42° C in a prehybridization solution consisting of deionized formamide (45% v/v), 20× SSC (25% v/v [3.0 M NaCl, 3.0 M sodium citrate pH 7.0]), 50× Denhardt's solution (10% v/v [0.05% Ficoll, 0.05% polyvinylpyrrolidone, 0.05% BSA pentax fraction V]), sodium phosphate buffer (5% v/v [1.0 M, pH 6.8]), 20% sodium dodecyl sulfate (SDS) (2.5% v/v), dextran sulfate (10% w/v), sheared salmon sperm DNA (0.1 mg/ml) (Maniatis *et al.* 1982). The plasmid pUV10 was labeled with ^{32}P dCTP by nick translation (Maniatis *et al.* 1982). Following prehybridization, the labeled probe was added to the hybridization bags and the membranes incubated overnight at 42° C. The membranes were washed following standard procedures with two final washes for 20 min each in 0.05× SSC plus 0.1% SDS at 65° C.

RESULTS

Isolation of mtDNA. To obtain mtDNA free of nuclear DNA, it was necessary to run two or three successive isopycnic gradients in the presence of the intercalating dye bisbenzimide (20 hr, 55K rpm at 20° C in a Beckman 80 Ti rotor). For optimum separation, the initial concentration of the cesium chloride was 1.3 g/ml.

Size of mtDNA and restriction fragment length polymorphisms. The mtDNA of *C. parasitica* is large. Most restriction endonucleases used (*Bam*HI, *Eco*RI, *Pst*I, *Xba*I, and *Hpa*I) cut the mtDNA into too many fragments to use for size estimates. *Kpn*I and *Sac*I proved to be the most useful enzymes for obtaining a reasonable number and size of fragments. Figure 1 shows the gel pattern and fragment sizes of *Kpn*I-digested mtDNA of strain EP155. The largest fragment was extracted from this gel and labeled by nick translation for use as a probe. The mtDNA was double digested with *Kpn*I and *Sac*I, the fragments separated by electrophoresis, and then Southern blotted and probed with the single-cut *Kpn*I fragment. The size of the largest fragment is estimated at 78.7 kb. The size

of the mtDNA of strain EP155 is thus estimated to be 145.6 ± 4.4 kb.

Figure 2 shows the gel pattern and fragment sizes of mtDNA of EP155 digested with *Sac*I. The size estimate of the mtDNA using this enzyme is 143.2 ± 4.3 kb. This size is within the range of the size estimated by digestion with *Kpn*I. A 0.7-kb fragment discovered when mtDNA of EP113 was probed with pUV10 is not shown on these gels. This fragment is present in strain EP113 but not in strains EP155 and EP2001. The fragment was not detected in the gels shown in Figure 2 because of its small size.

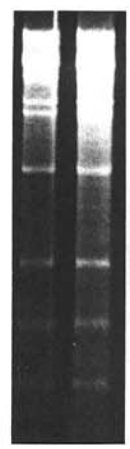
Fragment Size, Mean kb	± Std dev kb
78.7*	
17.5	1.0
14.2	1.0
11.3	0.7
11.3	0.7
4.8	0.3
4.3	0.6
3.5	0.1
145.6	4.4

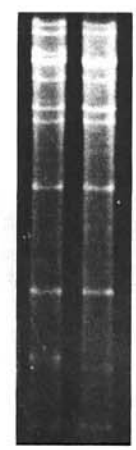
Fig. 1. Gel pattern and fragment sizes of mtDNA of strain EP155 digested with *Kpn*I. The standard deviations are based upon at least 10 replications of size estimates from 1% agarose gels run with molecular mass markers. The gel shown was stained with ethidium bromide. The size of this fragment was assessed by double digestion after it was isolated from a gel.

Restriction fragment length polymorphisms (RFLPs) are common in the fungus mtDNA. Figure 2 shows RFLPs that distinguish strains EP155, EP113, and EP2001 from each other. RFLPs observed in this fungus are length polymorphisms. EP155 shows a 16.8-kb fragment, but strains EP2001 and EP113 do not show one of corresponding size. These strains have three fragments that are about 14.7 kb, whereas EP155 has only two of this size. Both EP155 and EP2001 have a 2.4-kb fragment, while EP113 has a fragment that appears to correspond to this one but is slightly smaller (2.3 kb).

We cloned the 2.4-kb fragment from EP155 into pBluescript. The resultant recombinant plasmid (pUV10) was used to probe Southern blots of *Sac*I-digested mtDNA of EP155, EP2001, and EP113. The 2.4-kb fragment of EP155 does not hybridize to the 2.3-kb fragment of EP113, but it does hybridize to the corresponding fragment from EP2001 (see Fig. 3). The only hybridization of pUV10 to *Sac*I restriction fragments of EP113 was to a 0.7-kb fragment that had not been detected previously because it routinely ran off gels used to separate the larger fragments. The 2.4-kb fragment of EP155 and EP2001 thus contains sequences not present in EP113.

Lack of movement of mtDNA between vegetatively compatible strains. The mtDNA of EP2001 can be distinguished from EP113 by the presence of the 2.4-kb *Sac*I fragment in EP2001 and the absence of most of this





EP155		EP2001		EP113	
Mean kb	± Std dev kb	Mean kb	± Std dev kb	Mean kb	± Std dev kb
30.5	1.2	30.4	1.4	30.5	1.2
24.3	1.5	24.9	1.6	24.3	1.5
16.8*	0.4	14.7	0.2	14.7	0.1
14.7	0.1	14.7	0.2	14.7	0.1
14.7	0.1	14.2	0.2	14.1	0.3
12.0	0.3	12.0	0.4	12.0	0.3
9.1	0.2	9.1	0.2	9.1	0.2
8.2	0.1	8.2	0.2	8.2	0.1
5.4	0.1	5.4	0.1	5.5	0.1
3.3	0.1	3.3	0.1	3.3	0.1
2.4	0.1	2.4	0.1	2.3*	0.1
1.8	0.1	1.8	0.1	1.8	0.1
143.2	4.3	141.1	4.8	140.5	4.4

Fig. 2. Gel pattern and fragment sizes of mtDNA isolated from strains EP155, EP2001, and EP113 and digested with *Sac*I. The standard deviations are based upon at least 10 replications of size estimates from 1% agarose gels run with molecular mass markers. The gels were stained with ethidium bromide. These fragments are polymorphic with respect to the other strains.

fragment from EP113 (Fig. 3). Strains EP2001 and EP113 are of the same vc group (vc10). Our experience has shown that dsRNA can readily pass between these strains when the colonies make contact on agar plates. The colony pairing depicted in Figure 3 did not result in a formation of heterokaryons, and the hyphae of the two strains did not intermingle beyond the immediate contact zone. These results were shown using the *met* marker of EP2001. Isolations were made from the locations shown in Figure 3A, and the cultures grown from these isolates were checked for *met*, presence of dsRNA, and mtDNA type. The dsRNA was found to migrate between the colonies. Using pUV10 as a probe of Southern blots of mtDNA prepared from the cultures showed that the mtDNAs of EP2001 and EP113 did not migrate beyond the zone of interaction of the two colonies (Fig. 3).

The possibility exists that the mtDNA of one strain moves into some parts of the adjacent colony when somatic fusion occurs, but is not uniform in its distribution. This would not have been detected easily in the above experiment because tests for mtDNA migration were made only in a few discrete locations within the colonies. To test the possibility that nonuniform mtDNA migration may have occurred, colonies of EP2001 and EP113 were paired on agar plates and hybridization membranes were placed over the colonies. The hyphae that penetrated the membrane were lysed, and the *in situ* location of mtDNA of the EP2001 type was determined by probing with pUV10 (Fig. 4). The probe containing the 2.4-kb fragment present in EP2001 and absent from EP113 showed that the mtDNA of EP2001 did not move into the EP113 colony. The border between the two colonies is easily distinguished because of color differences in the colonies. The dsRNA, however, readily moved from EP113 into EP2001 as was detected by colony blot hybridizations (R. M. Martin and N. K. Van Alfen, unpublished).

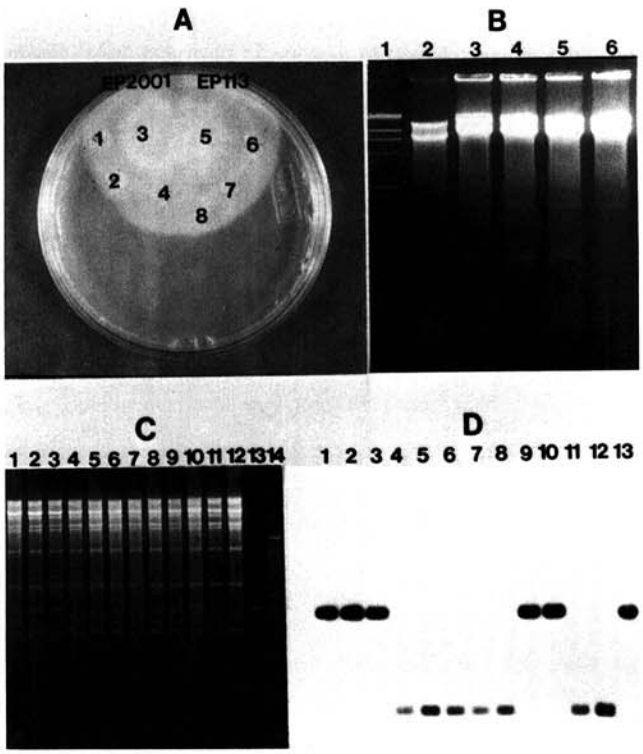


Fig. 3. A, Interacting colonies of strains EP2001 and EP113. The numbers indicate where isolations were made. **B**, Agarose gel showing double-stranded (ds)RNA isolated from cultures grown from different parts of the colonies. The dsRNA was stained with ethidium bromide. Lane 1, lambda DNA cut with *Hin*dIII; lane 2, dsRNA standard from EP113; lane 3, dsRNA, position 1; lane 4, dsRNA, position 2; lane 5, dsRNA, position 6; and lane 6, dsRNA, position 7. **C**, Agarose gel stained with ethidium bromide showing mtDNA cut with *Sac*I. Lanes 1–8, mtDNA isolated from cultures grown from positions 1–8, respectively; lane 9, mtDNA standard from EP2001; lane 10, mtDNA standard from EP155; lanes 11 and 12, mtDNA standard from EP113; lane 13, 2.4-kilobase (kb) mtDNA insert cut from pUV10; and lane 14, uncut pUV10. **D**, Southern blots of the gel shown in panel **C** probed with pUV10. The larger fragment is 2.4 kb and the smaller fragment is 0.7 kb.

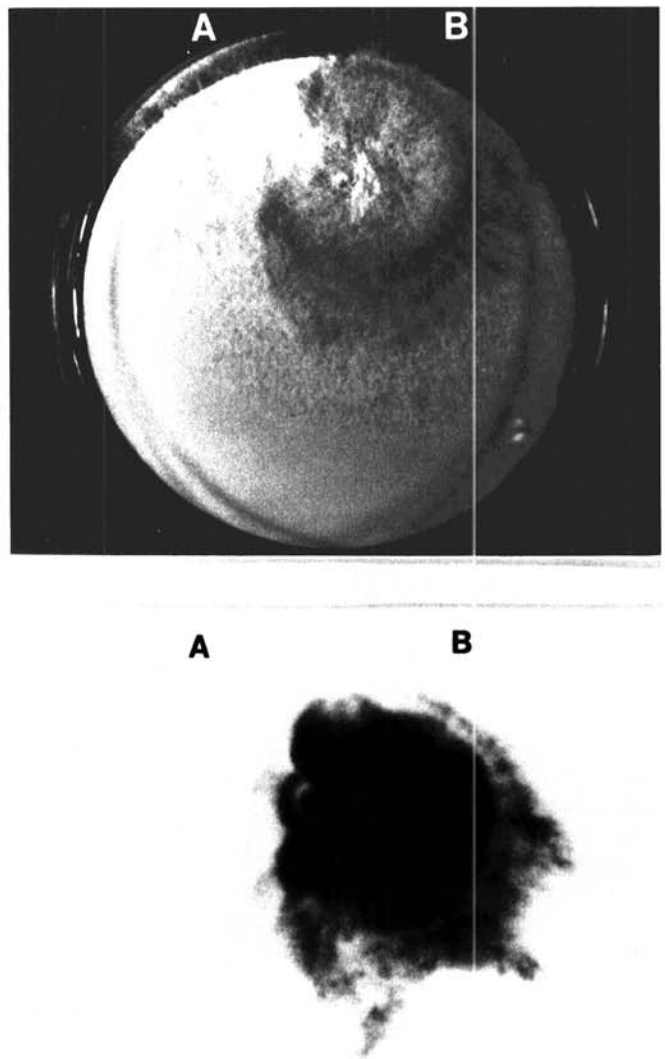


Fig. 4. Colony blot showing mtDNA of EP2001 hybridizing to pUV10. The upper photograph shows EP113 (**A**) and EP2001 (**B**) growing adjacent to each other on a hybridization filter placed on a potato-dextrose agar plate. The lower photograph shows the filter after it was processed and probed with pUV10. Hybridization and film exposure conditions were optimized to reduce visualization of the hybridization of pUV10 to the 0.7-kilobase portion of mtDNA of EP113.

DISCUSSION

Evidence is ample for mtDNA gene flow as a consequence of sexual crosses in filamentous fungi (Rowlands and Turner 1976). There is little evidence, however, to assess the frequency of mtDNA migration as a consequence of somatic fusion of vegetatively compatible hyphae. The genomes of fungal viruses, dsRNAs, are able to migrate rapidly between vegetatively compatible fungal strains (Anagnostakis and Waggoner 1981; Brasier 1986). These dsRNA viruses are not infectious, but depend upon the somatic fusion of hyphae to move between fungal strains (Buck 1986). The cytoplasmic bridges that form as a consequence of somatic fusion may also allow migration of mitochondria and nuclei between compatible strains. Recent evidence does not support the notion that nuclei commonly cross such cytoplasmic bridges with the consequent formation of a heterokaryon (Rayner *et al.* 1983). The ability to force heterokaryons by the complementation of auxotrophs, however, clearly shows that somatic fusions can result in nuclear migration (Van Alfen *et al.* 1975).

The purpose of this study was to determine if mtDNA migration frequently occurs as a consequence of somatic fusion between hyphae of different strains. The role of cytoplasmic gene migration between strains of *C. parasitica*, which results in the biological control of chestnut blight (Van Alfen 1988), makes this an important question. The dsRNAs that are associated with transmissible hypovirulence move readily between vegetatively compatible strains and also between strains which are vegetatively incompatible, although at a lower frequency (Anagnostakis 1982). It is not known, however, if mtDNAs also migrate with the dsRNAs across the cytoplasmic bridges that form between strains during somatic fusion.

To address this question, it was necessary to characterize the mtDNAs and determine if RFLPs were present that could be used to distinguish between strains. The mtDNA of *C. parasitica* is among the largest reported in filamentous fungi. The sizes reported, however, are estimates only until a map of the mtDNA is completed. Although the sizes estimated from fragments cut by two different enzymes were within the standard deviations of fragments cut with each enzyme, there is a possibility of error using this method. It is also clear that the mtDNAs of different strains vary in size. We have detected a length polymorphism between several strains of the fungus that is the consequence of an approximately 1.7-kb piece of DNA present in EP2001 and EP155 and absent from EP113 (Fig. 3). The other RFLPs that have been detected also appear to result from insertions or deletions rather than single base changes in enzyme recognition sites. RFLPs of the mtDNA appear to be common in this fungus. We are currently examining the correlation of RFLPs to the geographic regions in which the fungi are found.

The results of our studies indicate that mtDNA does not readily move between strains of *C. parasitica*, even when the strains are of the same vc group. The dsRNA does move rapidly between these strains, indicating that cytoplasmic bridges exist between the strains. Although we have used hybridization probes to determine if mtDNA migration occurs, small numbers of mitochondria that are below the level of detection by probing may migrate. We assume that something similar occurs during the forcing of heterokaryons. A relatively few nuclei probably migrate and are detected only by selective conditions imposed on the paired colonies (Van Alfen *et al.* 1975).

The lack of frequent mitochondrial movement between strains has implications for those interested in using mitochondrial plasmids as vectors for filamentous fungi. Our studies suggest that such plasmids would rarely move from the strain into which they are introduced.

These studies also suggest that the dsRNAs responsible for hypovirulence are not dependent upon specific mtDNA types for replication or expression of the transmissible hypovirulence phenotype. That the dsRNAs are responsible for hypovirulence is based upon correlative evidence, so it was important to show that specific mtDNA types are not also associated with this transmissible disease of *C. parasitica*. Until a cellfree method is devised for infecting fungal cells with dsRNA, the search for other cytoplasmic elements associated with dsRNA in the induction of the hypovirulent phenotype should continue.

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