Incidence and Diversity of Double-Stranded RNAs Occurring in the Chestnut Blight Fungus, *Cryphonectria parasitica*, in China and Japan

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ABSTRACT

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Isolates of the chestnut blight fungus, *Cryphonectria parasitica*, were randomly sampled from 10 subpopulations in China and 8 subpopulations in Japan and screened for the presence of double-stranded (ds) RNA using an immunoblot procedure with a monoclonal antibody specific for dsRNA. The overall incidence of dsRNA in *C. parasitica* was 2 and 6% in China and Japan, respectively, much lower than the 28% found previously in North American populations. Genetic relatedness of dsRNAs within and among populations in China and Japan was examined using RNA-RNA hybridizations with labeled-dsRNA probes. The majority of Chinese and Japanese dsRNAs were members of a single hybridization group, related to *Cryphonectria hypovirus* 1 (CHV1) from Europe, and are referred to as CHV1-type dsRNAs. No evidence was obtained for genetic differentiation between CHV1-type dsRNAs sampled in China and Japan. Five Japanese isolates contained two genetically distinct dsRNAs. The larger segments (approximately 12 kilobases [kb]) were

members of the CHV1 hybridization group, while the smaller segments (approximately 3 kb) did not hybridize with any known dsRNA from *C. parasitica* including the 2.7-kb dsRNA from isolate NB631 from New Jersey or dsRNA from isolate RC1 from Michigan. Two small dsRNA segments (approximately 1.8 and 2 kb) from one isolate sampled from Liaoning Province in northeastern China did not hybridize with any of the dsRNA probes tested including several described dsRNAs of similar size from *C. parasitica* in North America. Three dsRNAs from Anhui Province, China, hybridized to *Cryphonectria hypovirus* 2 (CHV2)-specific probes and are thus referred to as CHV2-type dsRNAs. Sequence analysis of 1,627 base pairs of these three CHV2-type dsRNAs from Anhui revealed that they were identical to each other in the region sequenced and very closely related to CHV2-NB58, isolated from New Jersey. We speculate that CHV2-NB58 may have been introduced into North America from this part of China. This is the first record of a North American *C. parasitica* dsRNA that is genetically related to a dsRNA from Asia.

Additional keywords: biological control, *Castanea* spp., *Endothia parasitica*, hypovirulence, population structure.

Recent research on chestnut blight has been largely motivated by the potential for biological control of this disease with transmissible hypovirulence (1,12,21,28,39,40). Hypovirulence refers to the reduced virulence of isolates of the chestnut blight fungus, *Cryphonectria parasitica*, that are infected with double-stranded (ds) RNA viruses. These viruses, and the corresponding hypovirulent phenotype, can be transmitted from infected to uninfected isolates by hyphal anastomosis and mixing of cytoplasm (2,16,20) and, therefore, have the potential to spread in fungal populations as biological control agents (1,12).

Two dsRNA viruses in *C. parasitica*, *Cryphonectria hypovirus* 1 (CHV1)-EP713 and CHV2-NB58, have been recently classified in the family *Hypoviridae*. CHV3-GH2 has also been tentatively included as a member of this family (13). In addition to these recognized hypoviruses, various other dsRNAs and dsRNA viruses have been found in *C. parasitica* including dsRNAs from isolate SR2 (9), reovirus-like dsRNAs from isolate C18 from West Virginia (8), dsRNA in isolate RC1 from Michigan (30), and dsRNA viruses in the mitochondria of isolate NB631 from New Jersey (33). Peever et al. (32) recently discovered additional dsRNAs in

C. parasitica isolates from New Jersey that did not cross-hybridize with any other known dsRNAs in this fungus.

Some dsRNAs in *C. parasitica* are widespread geographically, while others have more limited distributions. CHV1 (CHV1-type dsRNA) has been found only in Europe (12,17,30) and China (19, 34). In North America, the geographic distribution of hypoviruses and dsRNAs was recently studied by Peever et al. (32). They found only three dsRNA hybridization groups in a sample of dsRNAs from 525 isolates collected in nine different locations throughout eastern North America. One hybridization group, termed SR2-type, was widespread in six different states in the United States. The next most common group was CHV3, found only in Michigan isolates and in one site outside of Michigan where CHV3 had been released 10 years previously for biological control. In another study, CHV3 was also recently found in a population in Ontario, Canada (22). A third hybridization group was identified by Peever et al. (32) exclusively in isolates from New Jersey. Other dsRNAs that were previously identified in the United States (8,9,30,33) were not found in this study, presumably because of their rarity, limited geographic distributions, or both.

The origin of dsRNAs in *C. parasitica* in North America and Europe is not known. The simplest hypothesis is that they were introduced from Asia with their fungal host. *C. parasitica* is native to east Asia (38) and was probably introduced into North America from Japan on imported *Castanea* seeds or seedlings (3). There is also a documented introduction of *C. parasitica* from China

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to the Washington, DC, area in 1912 (37), after the epidemic in the eastern United States was well underway. This latter introduction appears to have had a negligible effect on the genetic structure of *C. parasitica* populations in the United States, which are similar to those found in Japan but unlike populations in China (25). Two European populations were found to be genetically indistinguishable from populations in North America (25), and therefore, the possibility exists that *C. parasitica* was introduced into Europe from either Japan or North America.

A test of the hypothesis that hypoviruses and other dsRNAs in *C. parasitica* were introduced from Asia requires knowledge of the diversity of dsRNAs in Asia. However, there is currently little information available about dsRNAs present in *C. parasitica* in Asia. In a sample of 160 isolates from China, Liang et al. (19) found only two with dsRNA. These two dsRNAs and two more from eastern China were found to hybridize with CHV1, but not with dsRNAs from isolates EP868 and EP915 from the United States (18,34). dsRNAs from isolates EP868 and EP915, derived from isolates EP60 and EP700, respectively, cross-hybridize with each other (17); dsRNA from EP60 cross-hybridizes with CHV3-GH2, another Michigan hypovirus (Y.-C. Liu and M. G. Milgroom,

unpublished data). Although it seems plausible that CHV1-type dsRNAs may have originated from Asia, only a small sample of dsRNAs from China have been investigated. Further, there is currently no information available on dsRNAs in *C. parasitica* in Japan, which is the more likely source population of *C. parasitica* in North America and Europe (3,25).

Given the paucity of information on hypoviruses or dsRNAs in *C. parasitica* in Asia, we had three objectives in this study: (i) to estimate the incidence of hypoviruses in China and Japan; (ii) to determine how much diversity exists for dsRNA hybridization groups within and between populations of *C. parasitica* in China and Japan; and (iii) to determine whether hybridization groups found in Asia are also found in Europe and North America.

MATERIALS AND METHODS

Samples of *C. parasitica* dsRNAs. *C. parasitica* isolates (172 from 10 subpopulations in the People's Republic of China and 95 isolates from 8 subpopulations in Japan) were randomly selected from a culture collection and screened for dsRNAs (Table 1). Most of these isolates were from subpopulations sampled previously for

TABLE 1. Incidence of double-stranded (ds) RNA in isolates of *Cryphonectria parasitica* in China and Japan and identification of dsRNAs used in Northern hybridizations

Population	Collection date	dsRNAs detected by immunoblot ^a			Abnormal colony morphology ^b		Total dsRNA-positive
		<i>n</i>	dsRNA (isolate no.)	Proportion + ^c	<i>n</i>	Isolate no. ^d	
China							
Shucheng, Anhui	11/88	5	0	0.00	...	...	0
Xiuning, Anhui	11/92	31	2 (09503, 09505)	0.06	1	09544	3
Wuxian, Jiangsu	11/87	...	...	...	2	09027, 09102	2
Nanjing, Jiangsu	12/85	20	0	0.00	...	...	0
Yixing, Jiangsu	11/87	...	...	...	2	09059, 09077	2
Huairou, Beijing	06/91	...	...	...	5	09225, 09228, 09235, 09238, 09248	5
Donggou, Liaoning	06/91	...	...	...	3	09271, 09276, 09280	3
Kuandian, Liaoning	06/91	...	...	...	1	09289	1
Fengcheng, Liaoning	06/91	9	1 (09269)	0.11	1	09250	2
Heyuan, Guangdong	02/92	12	0	0.00	...	...	0
Guixi, Jiangxi	02/92	10	1 (09367)	0.10	3	09372, 09374, 09379	4
Jinhua, Zhejiang	03/92	...	...	...	3	09410, 09413, 09419	3
Ninghua, Fujian	02/91	...	...	...	1	09207	1
Jianyang, Fujian	03/93	14	0	0.00	...	...	0
Shanghang, Fujian	03/93	3	0	0.00	...	...	0
Liyang, Jiangsu	10/92	38	0	0.00	...	...	0
Taian, Shandong	11/92	30	0	0.00	1	09316	1
Total		172	4	0.02	23		27
Japan							
Chiyoda, Ibaraki	10/92	17	2 (JA7, JA16)	0.12	...	...	2
Yasato, Ibaraki	10/92	19	2(JA67, JA80)	0.11	...	...	2
Iwama, Ibaraki	10/92	12	1 (JA28)	0.08	...	...	1
Yasui, Ibaraki	10/92	12	0	0.00	...	...	0
Yachiyo, Ibaraki	10/92	11	1 (JA91)	0.09	...	...	1
Sowa, Ibaraki	10/92	3	0	0.00	...	...	0
Sanwa, Ibaraki	10/92	8	0	0.00	...	...	0
Morioka, Iwate	10/92	13	0	0.00	...	...	0
Total		95	6	0.06		0.00	6

^a dsRNAs sampled from isolates of *C. parasitica* in China and Japan. Isolates were screened for the presence of dsRNA using the immunoblot procedure as described in Materials and Methods and Peever et al. (32). Isolates identified as dsRNA-positive by immunoblot are in parentheses.
^b Isolates were selected for dsRNA analysis because of abnormal colony morphology
^c Proportions of isolates screened by immunoblotting that were scored as dsRNA-positive.
^d Isolate numbers assigned to Chinese isolates and stored at Nanjing Agricultural University, Nanjing, China.

TABLE 2. Oligonucleotide primers used for reverse transcriptase polymerase chain reaction and sequencing of double-stranded RNAs from Xiuning, Anhui, China

Oligonucleotide number	Sequence ^a	Position ^b
NB58-50	AGAATCGTGGTGGCGG	8,477–8,492, upper
NB58-45	ATCAAGTTTAGCTAAG	9,327–9,312, lower
NB58-44	AAGGAAGATTCTGAAG	8,779–8,794, upper
NB58-38	ACAACCTACCCAATG	9,721–9,736, lower
NB58-51	TTCTCTGTACTTCTCG	9,702–9,717, upper
NB58-52	AAGAAAGTGGATTTCG	10,205–10,190, lower

^a Sequences are provided 5' to 3'.
^b Oligonucleotide positions are relative to the upper or lower strand of the *Cryphonectria hypovirus* 2-NB58 double-stranded RNA sequence (14).

a study of the worldwide population genetic structure of *C. parasitica* (25). These isolates were sampled from cankers on *Castanea* spp. Most isolates from China were sampled from *Castanea mollissima*, while those from Japan and from Liaoning Province in north-eastern China were sampled from *Castanea crenata*. All isolates from Japan that were available in our collection were screened. In China, we sampled 30 or more isolates from three populations to make within-population estimates of incidence; samples from other populations were smaller, but covered a wider geographical distribution.

dsRNA screening. All 267 isolates were screened for the presence of dsRNA with an immunoblot procedure that used a monoclonal antibody specific for dsRNA (35) as described by Peever et al. (32). In addition to immunoblot screening of randomly selected isolates, we analyzed 24 additional dsRNA-containing isolates that were identified by abnormal cultural morphology and maintained in the culture collection at Nanjing Agricultural University, Nanjing, China (Table 1). dsRNA was purified from isolates that were dsRNA-positive by immunoblot screening using a modification (32) of the procedure of Morris and Dodds (26). We also attempted to isolate dsRNA from six isolates from Japan that had slightly abnormal colony morphology. The reliability of the immunoblot screening method was evaluated by testing 42 randomly selected *C. parasitica* isolates from Japan that were dsRNA-negative by immunoblot. These isolates were screened for dsRNA using the rapid extraction of multiple samples miniprep method described by Morris et al. (27) with the following modifications: 75 mg of lyophilized, ground mycelium was used; no bentonite was used in lysis; and 0.1 g of CF11 cellulose was added to each sample. Control isolates for this method were EP102, which contains SR2-type dsRNA (32), and two Italian isolates with CHV1 (Y.-C. Liu and M. G. Milgroom, unpublished data).

Northern hybridizations. To determine the diversity of dsRNAs and their similarities to known hybridization groups, dsRNAs were purified and Northern blots were probed with a variety of labeled dsRNAs. Northern blots of column-purified dsRNA were probed with ³²P end-labeled dsRNA as described previously (32). Our strategy was first to test all unknown dsRNAs with probes of known hybridization groups, and then to follow up by probing with selected dsRNAs from different *C. parasitica* populations in Asia. dsRNA probes from known hybridization groups were purified from CHV1-EP113 or CHV1-EP43 (France), CHV2-NB58 (New Jersey), CHV3-GH2 (Michigan), SR2 (West Virginia), NB631 (New Jersey), and RC1 (Michigan). Purified dsRNA controls from known hybridization groups were included on most Northern blots. These included CHV1-EP113 and CHV1-EP43, which are identical to CHV1-EP713 (13); CHV2-NB58; CHV3-GH2; and isolate SR2 as described by Peever et al. (32). Additional dsRNAs used as controls on some gels were purified from isolates NB631 (33) and RC1 (30).

dsRNAs that did not hybridize with any known dsRNA probe from *C. parasitica* were tested to confirm that they were dsRNA. dsRNA is degraded by RNase in low salt but, unlike single-stranded RNA, is resistant to RNase in high salt (26). We treated all dsRNAs with RNase-free DNase (Gibco BRL, Gaithersburg, MD) prior to treatment with RNase A (Amersham Corp., Arlington Heights, IL) in low (0.125 M NaCl) or high (0.375 M NaCl) salt.

Nucleotide sequencing of selected dsRNAs. For a more detailed comparison of Chinese dsRNAs that hybridized to CHV2, complementary DNA to column-purified dsRNA was synthesized using random hexamer primers and Moloney murine leukemia virus reverse transcriptase as described by Chung et al. (5). Using the methods and conditions described in Chung et al. (5), we amplified three fragments representing a total of 1,729 contiguous nucleotide residues corresponding to positions 8,477 to 10,205 of CHV2-NB58 dsRNA (literature citation 66 of Hillman et al. [14], figure 2 of Chung et al. [5]). Individual polymerase chain reaction (PCR) fragments were amplified with primers NB58-50 and -45, -44 and -38, or -51 and -52 (Table 2), ligated into pGEM-T (Promega Corp.,

Madison, WI), and cloned into DH5α cells. From each transformation, three clones were chosen for sequencing. Plasmids were recovered and sequenced using either the Fidelity DNA sequencing system (Oncor, Inc., Gaithersburg, MD) or Sequenase Ver 2.0 sequencing kit (United States Biochemical Corp., Cleveland). CHV2-NB58-specific primers listed in Table 2, or the T7 and SP6 promoter primers (United States Biochemical Corp.), were used to prime sequencing reactions. The consensus sequence among clones for each dsRNA was used for comparisons with CHV2-NB58 and among different isolates.

Fig. 1. Hybridization analysis of double-stranded (ds) RNAs sampled from *Cryphonectria parasitica* isolates from China and Japan probed with *Cryphonectria hypovirus* 1 (CHV1)-specific dsRNA probe. Ethidium bromide-stained agarose gel with dsRNAs sampled from **A**, Beijing, Liaoning, Jiangxi, and Xiuning provinces in China and **C**, Jiangsu, Fujian, Shandong, and Zhejiang Provinces in China and in Ibaraki Prefecture in Japan. **B and D**, Autoradiographs of Northern blots of dsRNAs shown in **A and C**, respectively, probed with ³²P-labeled CHV1 dsRNA from isolate EP113 from France. Size markers at left indicate positions of dsRNA segments of known size from CHV1-EP713 (12.7 kilobases [kb]) (36) and NB631 dsRNA (2.7 kb) (33) or estimated size from CHV3-GH2 (3.5 kb) (30). **A and B**, Isolates in lanes 1 to 4 (from left to right): from known hybridization groups; and in lanes 5 to 22: 09225, 09228, 09235, 09238, 09248, 09250, 09269, 09271, 09276, 09280, 09289, 09367, 09372, 09374, 09379, 09503, 09505, and 09544. **C and D**, Isolates in lanes 1 to 6: 09027, 09059, 09077, 09102, 09207, and 09316; and in lanes 8 to 16: 09410, 09413, 09419, JA7, JA16, JA28, JA67, JA80, and JA91 (lane 7 is blank).

RESULTS

Based on immunoblot screening of randomly sampled isolates, the overall incidence of dsRNA (across all subpopulations) was 2% (4/172) in China and 6% (6/95) in Japan (Table 1). dsRNAs were found in only three of the ten subpopulations in China and in four of the eight subpopulations in Japan. Incidence within populations in China, estimated from sample sizes of 30 or more, was 6% (2/31) in Xiuning, Anhui, but no dsRNAs were detected in Liyang, Jiangsu ($N = 38$), or Taian, Shandong ($N = 30$). Overall incidence of dsRNA was lower in Asian *C. parasitica* populations compared with North America (28%) (32). None of the six Japanese isolates that were selected for abnormal colony morphology contained dsRNA (data not shown). Similarly, none of the 43 Japanese isolates that were dsRNA-negative by immunoblot screening contained dsRNA in the miniprep assay (data not shown).

Fig. 2. Hybridization analysis of double-stranded (ds) RNAs sampled from *Cryphonectria parasitica* isolates from China and Japan probed with *Cryphonectria hypovirus* (CHV)2-specific dsRNA probe from Chinese isolate 09503. Ethidium bromide-stained agarose gel with dsRNAs sampled from **A**, Beijing, Liaoning, and Jiangxi provinces in China and **C**, Xiuning, Jiangsu, Fujian, Shandong, and Zhejiang Provinces in China and from Ibaraki Prefecture in Japan. **B and D**, Autoradiographs of Northern blots of dsRNAs shown in **A** and **C**, respectively, probed with ^{32}P -labeled dsRNA from isolate 09503 from Xiuning, China. Weak hybridization signal to CHV2-NB58 is because very little dsRNA was loaded on the gel. Size markers at left indicate positions of dsRNA segments of known size from CHV2-NB58 (12.5 kilobases [kb]) (15). **A and B**, Isolates in lanes 8 to 12 (from left to right): 09228, 09235, 09238, 09248, and 09250; in lanes 14 to 16: 09271, 09276, and 09280; and in lanes 19 to 21: 09372, 09374, and 09379. **C and D**, Isolates in lanes 1 to 6: 09503, 09505, 09544, 09027, 09059, and 09077; in lanes 8 to 12: 09207, 09316, 09410, 09413, and 09419; and in lanes 14 to 18: JA16, JA28, JA67, JA80, and JA91.

Fig. 3. Nuclease analyses of double-stranded (ds) RNAs from Chinese isolate 09269 (Fengcheng, Liaoning) and Japanese isolates JA7, JA16, JA28, JA67, JA80, and JA91. dsRNAs were treated with RNase A in high salt (0.375 M NaCl, labeled RNase HS) or RNase A in low salt (0.125 M NaCl, labeled RNase LS), electrophoresed in an agarose gel, and stained with ethidium bromide. All dsRNAs were treated with DNase prior to RNase treatment. dsRNA from isolate EP43 was used as a control.

Fig. 4. DNA fingerprints of isolates containing *Cryphonectria hypovirus* 2 from Xiuning, China, and isolate NB58 from New Jersey. DNA was digested with *Pst*I and probed with pMS5.1 as described in Milgroom et al. (24). Isolates in lanes 1 to 4 are NB58, 09503, 09505, and 09544, respectively.

Most, but not all, dsRNAs found in China and Japan were 12 to 13 kilobases (kb) in size (Figs. 1 and 2), similar in size to those associated with previously described hypoviruses from North America (15,30,32) and Europe (6,11,36). Some isolates had multiple dsRNA segments (Figs. 1 and 2) as found in some strains of CHV1 in Europe (11,36). One isolate from Fengcheng, Liaoning, China (isolate 09269), contained two dsRNA segments of approximately 2 and 1.8 kb (Figs. 1A and 3). Five of six Japanese isolates (all but JA91) had two distinct sizes of dsRNA segments, one that was approximately 12 kb and another that was approximately 3 kb (Fig. 3). The dsRNA nature of the smaller segments from isolate 09269 and five Japanese isolates was verified by demonstrating their resistance to RNase in high salt, but not in low salt (Fig. 3) (26).

All Northern blots were probed with dsRNAs specific to CHV1, CHV2, and CHV3, as well as with dsRNA probes from isolates SR2 and 09269 (Table 3). Most of the Chinese and Japanese dsRNAs hybridized with CHV1 including multiple dsRNA segments in the 12- to 13-kb range similar to those of CHV1-EP43 (Fig. 1). dsRNAs from four Chinese isolates and the 3-kb dsRNA segments in the Japanese isolates did not hybridize with CHV1 (Fig. 1). Of the four Chinese dsRNAs that did not hybridize with CHV1, three from Xiuning, Anhui, hybridized with CHV2 (Fig. 2) and had similar culture morphology to that of isolate NB58, originally isolated in New Jersey (15). The remaining dsRNA, from isolate 09269 from Fengcheng, Liaoning, did not hybridize with any dsRNA probe from *C. parasitica* from China or Japan (Table 3). To determine whether the small dsRNA segments found in Chinese isolate 09269 (Liaoning) or the Japanese isolates would hybridize with other hybridization groups found in North America, they were probed with dsRNA from isolates NB631 (33), SR2 (9), RC1 (30), and NJ128-1 (32). None of these probes hybridized to any Asian dsRNA tested (data not shown).

Sequence analysis was performed on dsRNAs from three isolates from Xiuning that hybridized with CHV2. Consensus sequences of the three dsRNAs were identical to each other in the region sequenced. They differed from CHV2-NB58 (14) at seven nucleotide residues representing five codons. Of the five altered codons, three encoded different amino acid residues in the Xiuning isolates. Comparisons of consensus sequences of the Xiuning isolates with CHV2-NB58 are summarized in Table 4. In addition to the 7 consensus residue differences, a total of 16 differences were observed on one of the three clones for each isolate.

Because of the small number of nucleotide differences observed between Xiuning dsRNAs and CHV2-NB58, DNA fingerprinting was conducted as described by Milgroom et al. (24) to confirm that these isolates of *C. parasitica* were truly Chinese in origin. All three Xiuning isolates containing CHV2 had DNA fingerprints with only two bands (Fig. 4), which is typical of *C. parasitica* isolates from China (24,25). In contrast, isolate NB58 from New Jersey had a fingerprint pattern with multiple bands, typical of North American isolates (Fig. 4).

TABLE 4. Summary of nucleotide substitutions at each position in the 1,644-base pair region sequenced of Cryphonectria hypovirus 2-NB58 double-stranded (ds) RNA and dsRNA from three isolates from Xiuning, China

Nucleotide position ^a	Nucleotides		Position in codon ^b	Amino acids	
	NB58	Xiuning		NB58	Xiuning
8,596, 8,597, 8,598	AAG	GGT	1, 2, 3	Lysine	Glycine
8,985	A	G	3	Threonine	Threonine
9,092	A	G	2	Glutamic acid	Glycine
9,187	T	C	1	Cysteine	Arginine
9,639	A	G	3	Leucine	Leucine

^a Nucleotide numbers from the 5'-end of upper strand (14).

^b Assumes translation of open reading frame B (14).

TABLE 3. Hybridization of double-stranded (ds) RNAs from 12 subpopulations of *Cryphonectria parasitica* in China and 4 subpopulations in Japan with ³²P-labeled dsRNA probes

Population	n	Probe dsRNA ^a						
		CHV1	CHV2	CHV3-GH2	09269	SR2	RC1	NJ128-1
China								
Xiuning, Anhui	3	0/3 ^b	3/3	0/3	... ^c	0/1	...	...
Wuxian, Jiangsu	2	2/2	0/2	0/2	...	0/2	...	...
Yixing, Jiangsu	2	2/2	0/2	0/2	0/1	0/2	...	...
Huairou, Beijing	5	5/5	0/5	0/5	0/1	0/5	...	...
Guixi, Jiangxi	4	4/4	0/4	0/4	0/1	0/4	...	...
Taian, Shandong	1	...	0/1	...	...	...	...	...
Jinhua, Zhejiang	3	3/3	0/3	0/2	0/1	0/2	...	...
Donggou, Liaoning	3	3/3	0/3	0/2	...	0/2	...	...
Fengcheng, Liaoning ^d	2	1/2	0/2	0/2	1/2	0/2	0/1	0/1
Kuandian, Liaoning	1	1/1	0/1	0/1	...	0/1	...	...
Ninghua, Fujian	1	1/1	0/1	0/1	...	0/1	...	...
Total	27	22/26	3/27	0/26	1/6	0/26	0/1	0/1
Japan								
Chiyoda, Ibaraki	2	2/2 ^e	0/2	0/2	0/2	0/2	0/2	0/2
Yasato, Ibaraki	2	2/2	0/2	0/2	0/2	0/2	0/2	0/2
Iwama, Ibaraki	1	1/1	0/1	0/1	0/1	0/1	0/1	0/1
Yachiyo, Ibaraki	1	1/1	0/1	0/1	0/1	0/1	0/1	0/1
Total	6	6/6	0/6	0/6	0/6	0/6	0/6	0/6

^a Cryphonectria hypovirus 1 (CHV1)-specific dsRNA probes were prepared from dsRNA purified from three different *C. parasitica* isolates: EP113 and EP43, both of which contain CHV1-EP713 from France, and JA91 from Japan. All three probes gave identical results. CHV2-specific probes were prepared from two *C. parasitica* isolates: NB58 from New Jersey (15) and isolate 09503 from Xiuning, China; both probes gave identical results. CHV3-specific probe was prepared from isolate GH2 from Michigan (30). Additional probes were prepared from dsRNA from isolates 09269 from Liaoning, China; SR2 from West Virginia (9); RC1 from Michigan (30); and NJ128-1 from New Jersey (32).

^b Number of isolates hybridizing/number of dsRNAs probed.

^c Not tested.

^d This population contained two isolates containing dsRNAs; one contained dsRNA approximately 12 kilobase (kb) in size that hybridized with CHV1, while the other contained two dsRNA segments approximately 1.8 and 2 kb in size that did not hybridize with any other probes.

^e Five of the six dsRNA-containing isolates from Japan (all but JA91) also had smaller dsRNA segments (approximately 3 kb) that did not hybridize with any of the other dsRNA probes.

DISCUSSION

The incidence of dsRNA in Asian populations of *C. parasitica* was much lower than in North America. In China, only 2% (4/172) of randomly sampled isolates had dsRNAs detectable by immunoblotting, whereas in Japan, 6% (6/95) contained dsRNA. A comparable study, using the same immunoblot procedure, with almost 600 isolates from eastern North America showed that approximately 28% of the isolates had dsRNAs. Incidence of dsRNAs in Asian populations of *C. parasitica* may be underestimated, because of failure to detect dsRNAs by immunoblot screening (32). However, among 6 isolates analyzed because of abnormal colony morphology and 42 randomly chosen isolates that were dsRNA-negative by immunoblot screening, none contained dsRNA. Our results are also consistent with a previous study in China that reported low dsRNA incidence; among 160 isolates examined, only 2 contained dsRNA (18,19). dsRNAs from culture collection isolates, which were selected because of their abnormal colony morphologies, were not included in our estimate of incidence, because abnormal isolates were preferentially saved in the culture collection and may bias the estimate. Abnormal colony morphology has often been interpreted in *C. parasitica* as a symptom that isolates contain dsRNA (7). However, our results with six Japanese isolates with abnormal colony morphology demonstrate that colony phenotype may not always be a good indicator of the presence of dsRNA. In addition, an estimate of incidence based on screening isolates with abnormal morphology will fail to account for those dsRNAs that have little or no effect on *C. parasitica*'s morphology or virulence (9,41).

Almost all dsRNAs sampled from *C. parasitica* subpopulations in China and Japan hybridized with CHV1-specific probes. These results are consistent with previous hybridization studies in which dsRNA from two isolates from China hybridized with CHV1 (18, 34). To date, CHV1 is the only hypovirus identified from European populations of *C. parasitica* (11) and has not been found in North America (32). The widespread occurrence of CHV1 in both China and Japan makes Asia the most logical origin of this virus for European populations, since *C. parasitica* is native to east Asia but was only recently introduced into Europe and North America. We can only speculate on the lack of CHV1 in North America. Despite repeated intentional introductions of CHV1 from Europe (21), it has not become established (32). CHV1 may also have been introduced into North America directly from Asia, but failed to become established.

Based on the genetic differentiation of *C. parasitica* between China and Japan (25), we predicted that viruses infecting the fungus in Asia might also be genetically differentiated, since fungal viruses cannot be dispersed in the absence of their hosts. However, no differentiation of dsRNAs between Japan and China was detected in this study. The hybridization techniques used here provide a useful first look at virus diversity; however, it is impossible to make any further conclusions on genetic differentiation without additional data. As with a similar study in North America (32), identification of hybridization groups is a necessary requirement before finer resolution population genetic analyses, such as those done by Chung et al., (5), can be done with these dsRNAs.

The finding of CHV2-containing isolates in Xiuning, Anhui, China, represents the first identification of viruses of this type outside of the original site of discovery in New Jersey (5,15). Nucleotide sequences were identical among the three Xiuning dsRNAs and differed from CHV2-NB58 by only seven nucleotide residues over the 1,644 base pairs sequenced. More sequence differences in the same region of the CHV2 genome were observed among isolates within a single population in New Jersey (5); however, experimental differences between these two studies preclude direct comparison. The close similarity between Xiuning sequences and CHV2-NB58 was surprising, since numerous nucleotide substitutions would be expected to occur over time. More dsRNAs will

need to be sampled in Xiuning and other Chinese populations to assess the diversity of CHV2 in Asia.

Two dsRNA segments from Fengcheng, Liaoning, China, that were much smaller than other dsRNAs sampled from Asia appear to be different from the dsRNAs in *C. parasitica* characterized to date. These dsRNAs did not hybridize with any of the CHV1 or CHV2 probes. When the Fengcheng dsRNAs were used to probe Northern blots of Chinese and Japanese dsRNAs, they did not hybridize with any other dsRNA from Asia including the 3-kb segments detected in Japanese isolates. They also failed to hybridize with any North American dsRNA in the same size range including the 2.7-kb mitochondrion-associated dsRNA purified from isolate NB631 from New Jersey (33), the smaller dsRNAs of CHV3-GH2 from Michigan (30), or dsRNA from isolate RC1 (30). These two dsRNA segments in 09269 are similar in size to those of partiti-viruses, which have two unrelated dsRNA segments 1.4 to 2.2 kb in size (10,29). Further investigation will be needed to determine if the dsRNAs in 09269 are related to partiti-viruses.

Chestnut blight was first observed in Europe in 1938 (12), 34 years after it was recorded in North America (23). From chestnut introduction records and population genetic data, it seems most likely that *C. parasitica* was introduced into North America from Japan rather than China (3,25). There is one documented case of an introduction of *C. parasitica* from China, but it apparently failed to have an impact on the population genetic structure of *C. parasitica* populations in North America (25). The source of the introduction of *C. parasitica* into Europe is not clear, although it has been speculated that it was introduced into Italy from Japan (4,31). European, North American, and Japanese populations of *C. parasitica* are all genetically similar (25), making it difficult to determine whether the fungus was introduced into Europe from Japan or from North America. The existence of CHV1 in Europe, Japan, and China, but not in North America, suggests that the dsRNAs likely were introduced directly from Asia to Europe. The discoveries of CHV2-type dsRNAs in Xiuning, Anhui, China, and New Jersey suggest that CHV2 may have been introduced into North America from China. This introduction may have occurred after the initial introduction of the fungus from Japan, when Chinese chestnuts were imported for their resistance to chestnut blight (3). However, more extensive sampling of dsRNAs in Asia and North America is necessary to draw this conclusion with confidence.

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