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# Occurrence of diverse dsRNA in a Korean population of the chestnut blight fungus, *Cryphonectria parasitica*

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ABSTRACT

We analysed 676 isolates from 33 Korean *Cryphonectria parasitica* subpopulations in Korea for dsRNA incidence and diversity. dsRNA was detected in 84 isolates. Although the dsRNA banding patterns varied in several minor bands, infected isolates could be categorized into two groups. The most common banding pattern occurred in 77 isolates and contained a 12.7-kb band indicative of *Cryphonectria hypovirus* 1 (CHV1), and several accompanying minor bands with sizes ranging from 0.9–5 kb. Northern blot analysis revealed that all 12.7-kb fragments in the dsRNA-containing isolates hybridized to probes corresponding to open reading frames (ORFs) A and B from the reference CHV1 strain (GenBank accession no. M57938). In addition, the sequence of a 1.4-kb cDNA fragment from a representative isolate of the most common group showed 99 % sequence similarity to ORF A of CHV1. However, the other group of seven isolates had distinctive bands of 3.5 and 3.3 kb, but not the 12.7-kb band. Sequence comparison showed that cloned fragments of these dsRNAs were similar to those of the coat protein and RNA-dependent RNA polymerase genes of chrysovirus, which indicates the occurrence of chrysovirus in the Korean population. Fungal strain identity was assessed *via* RFLP analysis of the ITS regions. Among the 84 tested isolates, six had different ITS-RFLP patterns (RFLP-II) from that (RFLP-I) of *C. parasitica*, and are believed to be *C. nitschkei*, a sympatric species reported on chestnut trees in Japan. The chrysovirus and CHV1 were detected in strains showing both RFLP patterns. However, the chrysovirus was more frequent in the RFLP-II group.



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## Introduction

The chestnut blight fungus, *Cryphonectria parasitica*, has devastated chestnut forests in North America since the early 20th century. However, strains harboring *Cryphonectria hypovirus* (CHV) show hypovirulence, a phenomenon in which fungal viruses significantly reduce the virulence of *C. parasitica* to chestnut trees (van Alfen *et al.* 1975; Anagnostakis 1982; Nuss 1992), and exhibit other characteristic symptoms, including reduced sporulation and altered pigmentation (Havir & Anagnostakis 1983; Elliston 1985; Rigling *et al.* 1989). *C. parasitica* and its hypovirus constitute one of the best-characterized model systems for the study of host (fungus)–parasite (virus) interactions.

Among fungal viruses, dsRNA genomes are the rule rather than the exception (Hillman *et al.* 2004), and mycoviruses with dsRNA genomes have been detected in a wide variety of filamentous fungi and yeasts (van Alfen 1986; Nuss & Koltin 1990; Wickner 1992). The majority of characterized mycoviruses harbour the dsRNA genome, either encapsulated in isometric particles (Buck 1986), occurring as unencapsidated elements associated with fungal membranous vesicles in the cytoplasm (Hansen *et al.* 1985), or within mitochondrial fractions (Lakshman *et al.* 1998). Typically, mycoviruses cause no visible phenotypic changes in their host fungi (Ghabrial 1998). However, in a few known cases viral infection can result in viral-specific morphological and physiological changes in the host, and hypoviral infection is a well-known example of this phenomenon. CHV is a member of the genus *Hypovirus*, with one to several dsRNA fragments encapsulated in pleomorphic vesicles originating in the host, which persistently attenuate virulence and alter a number of specific biological processes, including the pigment production, sporulation, and mating of its fungal host, *C. parasitica*.

Hypovirulent *C. parasitica* field isolates exhibit a wide range of variability in their virulence levels and in the magnitude and constellation of other hypovirulence-associated traits. Therefore, the diversity of hypoviruses and other *C. parasitica* dsRNAs have been evaluated in North America, China, Japan, and Europe, *via* either Northern blot or RFLP analysis (Peever *et al.* 1997, 1998; Allemann *et al.* 1999). Four different species of CHV (CHV1, CHV2, CHV3, and CHV4) were identified based on differences in genome structure, sequence similarity, and symptoms caused in the fungal host (Smart *et al.* 1999). Although all hypoviruses harbour monopartite genomes, the presence of several dsRNAs representing defective genomic dsRNAs is not uncommon in *C. parasitica*. In addition, recent studies have reported the presence of reoviruses (Hillman *et al.* 2004) and chrysoviruses (Liu & Milgroom 2007) in *C. parasitica*.

Korean chestnut trees (*Castanea crenata*) are native to the Korean peninsula and are intensively cultivated in many orchards due to the economic value of their edible nuts. The cultivation area of chestnut trees in Korea is estimated to be 52 609–59 446 ha, producing approximately 72K tons of chestnuts (Park *et al.* 2007). Although the presence of the chestnut blight fungus *C. parasitica* in the Korean peninsula was first reported in 1927 (Sung & Han 1986), there has been little research on chestnut blight caused by *C. parasitica* in Korea until very recently (<10 years ago). In a previous study regarding the incidence of *C. parasitica* on necrotic regions of Korean chestnut trees, we reported a high frequency (25.6 %) of orange stromata-forming *Cryphonectria* spp. (Ju *et al.* 2002). In addition, variations in colony morphology, virulence, and phenol oxidase activity were observed among Korean *C. parasitica* isolates, which suggests that *C. parasitica* could be fatal to Korean chestnut trees that were thought to be resistant to the pathogen (Ju *et al.* 2002). Although our previous study examined fungal incidence and potential diversity among Korean isolates, no studies on the occurrence of mycovirus in Korean *C. parasitica* isolates have been conducted. As a supplement to these studies, we have begun to assess the presence of dsRNA in fungal hosts and variations, if any, among mycoviruses.

## Materials and methods

### *Fungal strains and growth conditions*

We collected a total of 676 *Cryphonectria parasitica* isolates from 33 Korean subpopulations between 1998 and 2000, following the methods of Bissegger *et al.* (1997). All samples were isolated from the bark of Korean chestnut trees (*Castanea crenata*), showing one or more yellow to orange stromata, a characteristic typical of *Cryphonectria* species. Fungal colonies typical of *Cryphonectria* on 2 % water agar were transferred to potato–dextrose agar (PDA) supplemented with methionine (100 mg $l^{-1}$) and biotin (1 mg $l^{-1}$), and then incubated under constant low light at 25 °C until the culture phenotypes were distinct. The isolates were named according to their sampling locations (Fig 1). Representative strains are held in the Culture Collection of Institute of Molecular Biology and Genetics at Chonbuk National University.

### *dsRNA isolation and northern blot analysis*

A miniprep method for the detection of dsRNA (Morris *et al.* 1983) was applied with slight modifications. In brief, the isolates were grown on top of cellophane overlays, and the mycelia were stripped from the overlays, transferred to 2 ml Eppendorf tubes, and lyophilized for 24 h. The dried mycelium was ground to a fine powder, and the dsRNA was isolated from 400 mg mycelial powder *via* CF-11 cellulose column chromatography (Morris *et al.* 1983). The dsRNA was dissolved in 40 μl RNase-free water. To verify the presence of dsRNA, the purified RNA was digested with either RNase III or RNase A under high (0.375 M NaCl) or low (0.125 M NaCl) salt conditions (Peever *et al.* 1998). The presence of individual dsRNA fragments was analysed by electrophoresis on 0.7 % agarose gels in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8).

For northern blot analysis of dsRNA, dsRNAs resolved on 0.7 % TAE agarose gel were denatured by soaking the gel in TAE buffer containing 40 % formamide and 17 % formaldehyde for 60 min at 65 °C, followed by gentle shaking of the gel in 50 mM NaOH and 100 mM NaCl for 45 min at room temperature. The gels were neutralized twice with 20 × SSC (3 M NaCl, 300 mM sodium citrate, pH 7) for 30 min each. The RNA was transferred to nylon membranes (Hybond,