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Molecular Diversity of Chrysoviruses in Korean Isolates of A New Fungal Species, *Cryphonectria nitschkei*

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Genetic diversity of the chrysovirus within the four fungal strains was analyzed by comparing the full-length sequences of cloned chrysoviral genes encoding the RNA-dependent RNA polymerase (RdRp) and capsid protein (CP). Because the morphological characteristics of four chrysovirus-infected *Cryphonectria* spp. strains were different, strain identification was conducted via sequence comparison of the internal transcribed spacers (ITSs) of the fungal rRNA gene. Phylogenic analysis of the ITS regions revealed that the four strains were closely clustered with the reference strain of *Cryphonectria nitschkei*, while they were more distantly related to other common *Cryphonectria* species, indicating that they were likely *C. nitschkei.* Sequence comparison among chrysoviruses from Korean *C. nitschkei* strains revealed that similarities of the RdRp and CP genes ranged from 98% to 100% and from 95% to 100%, respectively, at the protein level. Their corresponding nucleotide sequences showed 97% to 100% and 84% to 100% identities, respectively. Compared to RdRp, the CP gene had more divergence, suggesting the presence of genes possessing different evolutionary rates within the chrysovirus genome. Sequence comparisons with other known chrysoviruses showed that the four Korean chrysoviruses were clustered together at the next lineage level. Discovering why two strains (bs131 and bs132) containing identical ITS sequences and chrysoviruses display different phenotypes should prove interesting.

***Keywords*:** *Cryphonectria nitschkei*, ITS, Chrysovirus, dsRNA

Double-stranded RNA (dsRNA) viruses have been described in a wide variety of filamentous fungi and yeasts (Hollings, 1982; Ghabrial, 1998; Dawe and Nuss, 2001; Herrero *et al*., 2009; Pearson *et al*., 2009). Although many other dsRNAs remain unassigned, five families have been formally named and recognized as dsRNA mycoviruses: *Chrysoviridae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae*, and *Totiviridae* (Fauquet *et al*., 2005). In general, mycovirus infections do not cause measurable phenotypic changes in the fungal hosts, and if anything, remain cryptic or latent. In some cases, however, a virus infection can result in considerable morphological and physiological changes, including cytological alterations and changes in colony morphology, spore production, pigmentation, growth rate, and virulence-related phenotypes (Boland, 1992; Lakshman *et al*., 1998; Preisig *et al*., 2000; Dawe and Nuss, 2001; Castro *et al*., 2003; Kwon *et al*., 2007). Among these, the *Cryphonectria parasitica*-hypovirus interaction is one of the best characterized fungal-viral interactions, resulting in reduced fungal pathogenicity, hypovirulence (Van Alfen *et al*., 1975; Anagnostakis, 1982; Nuss, 2005). In addition, the genus *Cryphonectria* comprises a high diversity of mycoviruses (Hillman and Suzuki, 2004; Hillman *et al*., 2004) and has recently been suggested to harbor another viral family, the *Chrysoviridae* (Liu *et al*., 2007), which may confer a range of phenotypes on their fungal hosts. Even though major species of *Cryphonectria*, such as *C. parasitica*, *C. nitschkei*, *C. macrospora*, and *C. radicalis*, are known to occur on woody hosts in East Asia (Myburg *et al*., 2004b), morphological taxonomic identification is still extremely challenging. Therefore, molecular taxonomic studies based on DNA sequence comparisons have been used to resolve many questions (Myburg *et al*., 2004a).

In previous studies on the diversity of dsRNA in Korean *Cryphonectria* spp., the presence of dsRNA representing chrysoviruses within Korean populations has been confirmed (Park *et al*., 2008). In addition, based on the morphological characteristics and restriction fragment length polymorphism of the internal transcribed spacer of the nuclear ribosomal RNA subunit (ITS-RFLP), the presence of *Cryphonectria nitschkei*, a sympatric species reported in Japan on chestnut trees, was suggested as well (Park *et al*., 2008). Among 676 previously screened isolates, four were tentatively identified as *C. nitschkei* that were infected by chrysoviruses. Moreover, these four strains showed different phenotypes on culture plates. Therefore, in this study, four atypical *Cryphonectria* strains showing different cultural characteristics and containing chrysoviruses were taxonomically identified using DNA sequence comparisons of their ITS regions. Then, the di-

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Table 1. Specimens of *Cryphonectria* species and GenBank accession numbers used in this comparison

Species identification	Strain designation	Origin/Sources	ITS-GenBank no.	CP-GenBank no.	RdRp-GenBank no.
C. parasitica	EP155/2	ATCC 38755	AY309482		
C. macrospora	CRY1543	Venter *et al*. (2002)	AF368331		
C. nitschkei	OB5-11	ATCC:MYA-4105	EU331074	DQ865187	DQ865185
C. nitschkei	BS122	KACC44426[a]	GQ290653[b]	GQ290645	GQ290649
C. nitschkei	bs131	KACC44425	GQ290654	GQ290646	GQ290650
C. nitschkei	bs132	KACC44424	GQ290655	GQ290647	GQ290651
C. nitschkei	BS321	KACC44423	GQ290656	GQ290648	GQ290652
Diaporthe ambigua	CMW5587	Myburg *et al*. (1999)	AF543818		
Fusarium solani	SUF704	Nogawa *et al*. (1996)		D55669	

[a] Accession number of the Korean Agricultural Culture Collection (KACC)
[b] GenBank accession number of the corresponding gene from the mycovirus infected in the designated fungal strain

versity of the chrysoviruses was examined among the four strains to analyze the correlation of fungal phenotype with a particular mycovirus. We confirmed strain identities using the ITS sequences and examined the diversity of chrysoviruses in *C. nitschkei* by comparing two representative viral genes encoding RNA-dependent RNA polymerase (RdRp) and capsid protein (CP).

Materials and Methods

Fungal strains and growth conditions

The CHV1-713-containing hypovirulent *C. parasitica* strain UEP1, its isogenic virus-free strain EP155/2 (ATCC 38755), and isolates from a previous study (Park *et al*., 2008) were maintained on potato dextrose agar supplemented with methionine (100 mg/L) and biotin (1 mg/L) (PDAmb), then incubated under constant low light at 25°C until the culture phenotypes were distinctive (Kim *et al*., 1995). Liquid mycelial cultures were grown in EP complete medium (Day *et al*., 1971). The methods of preparing the primary inoculum for liquid cultures and culture conditions have been described previously (Kim *et al*., 1995; Park *et al*., 2008). Detailed information on the strains is provided in Table 1.

dsRNA isolation and Northern blot analysis

A miniprep method for purifying dsRNA was applied as described previously (Park *et al*., 2008). To verify 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 individual dsRNA fragments were resolved by electrophoresis on 1.0% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized by ethidium bromide staining.

For Northern blot analysis of dsRNA, denaturation and renaturation of resolved dsRNA followed by transfer to nylon membranes (GE Healthcare, USA) via capillary blotting were conducted as described previously (Park *et al*., 2008). Labeling, hybridization, and autoradiography were carried out as described previously (Park *et al*., 2004). A representative hypovirus, CHV1-EP713, was incorporated as a control.

Cloning and sequencing of the ITS regions

Sequence comparisons based on the ITS of the nuclear ribosomal RNA subunit were conducted to confirm that the dsRNA-containing strains were *C. parasitica*, as previously described (Myburg *et al*., 1999, 2004a; Liu *et al*., 2003). The ITS-1, 5.8S, and ITS-2 regions were amplified via the polymerase chain reaction (PCR) using the ITS1 and ITS4 primers (White *et al*., 1990). The amplified gene was then cloned into a pGEM-T Easy vector (Promega, USA), analyzed with the restriction enzymes *Alu*I and *Cfo*I, and then sequenced via chain termination methods (Sanger *et al*., 1977). For each reaction, at least three different bacterial colonies were selected for plasmid isolation, and both strands of the entire region were bidirectionally sequenced.

Cloning and sequencing of genes encoding the capsid protein (CP) and the RNA-dependent RNA polymerase (RdRp) of chrysoviruses

A library of cDNA clones for the dsRNA detected in the BS122 strain was constructed (Park *et al*., 2008). To acquire cDNA clones that corresponded to the full-length CP and RdRp of the chrysoviruses, gaps between non-overlapping cDNA clones were connected using RT-PCR and sequence-specific primers. In addition, for clones containing the terminal sequences of the dsRNA segments, dsRNAs were polyadenylated with yeast poly(A) polymerase (USB Co., USA), and RT-PCR was conducted using primers with a sequence-specific primer and a primer containing a 3'-dT_{25} tail with the last base degenerated (Covelli *et al*., 2004).

Based on the dsRNA sequence information from the reference BS122 strain, RT-PCR of dsRNAs from other isolates was carried out using sequence-specific primers, and then the PCR products were cloned and sequenced as described above using universal primers or sequence-specific primers.

Sequence analysis

The sequences of the ITS regions and the dsRNA were aligned using the program CLUSTAL W (Thompson *et al*., 2002) and sequence similarity searches were conducted using the BLAST program (Altschul *et al*., 1997). Phylogenetic analysis based on the neighbor-joining method was performed using the program PAUP 4.0 (Swofford, 2002).

Results

Strain confirmation of chrysovirus-containing *Cryphonectria* spp.

Previous studies on the occurrence of dsRNA in Korean strains of *Cryphonectria* spp. suggested the presence *C. nits-*

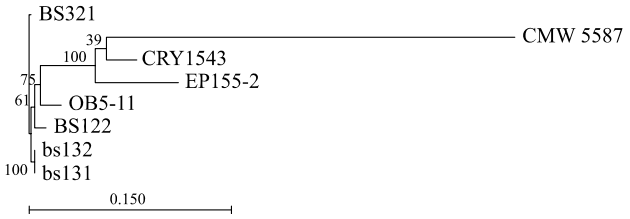

Fig. 1. Phylogenetic tree indicating the relationships among Korean strains and other commonly known species of *Cryphonectria* based on sequences of the ITS regions. An evolutionary matrix was generated by Henikoff and Henikoff (1992), and the branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate a bootstrap value of 1,000 resampled data sets. Sequence information and strain designation are provided in Table 1. *Diaporthe ambigua* (CMW 5587) was used as the outgroup (Myburg *et al*., 1999).

chkei based on ITS-RFLP (Park *et al*., 2008). Among six dsRNA-containing strains that showed the ITS-RFLP patterns of *C. nitschkei*, four proved to contain chrysoviruses and displayed different colonial morphology. Therefore, these four strains were selected to examine the diversity of the chrysoviruses. Strain identification was further confirmed using sequence comparisons of the cloned ITS regions of all four strains. Although the sizes of the ITS sequences differed in that they were 669 bp, 667 bp, 667 bp, and 670 bp for strains BS122, bs131, bs132, and BS321, respectively, the ITS sequences showed high similarity with each other (>99%). Among those four, the two ITS sequences from strains bs131 and bs132 were identical and the sequence identities of bs131 to the two other strains, BS122 and BS321, were 665/669 and 667/670, respectively. In addition, the identity of the ITS sequence between BS122 and BS321 was 663/670. All four ITS sequences showed high similarity to that of *C. nitschkei* in that they were all more than 97.7% (595/606) identical to the known sequence of *C. nitschkei*. When using the available ITS sequence data, phylogenetic analysis of the four strains relative to the reference strains of other commonly known species of *Cryphonectria*, including *C. nitschkei*, *C. macrospora*, and *C. parasitica*, revealed that the four strains grouped more closely with the reference strain of *C. nitschkei*, indicating that they were likely *C. nitschkei* (Fig. 1).

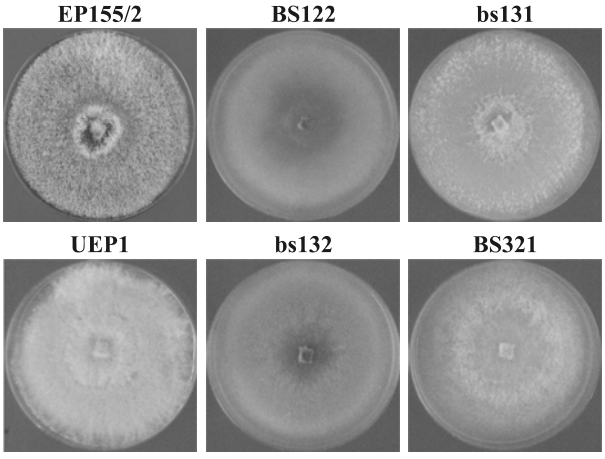

Fig. 2. Colony morphology of cultures on PDAmb plates. Strains are indicated at the top of panels and photographed 10 days after inoculation.

Characteristics of the colonial morphology of chrysovirus-containing *C. nitschkei*

To compare cultural characteristics of the four chrysovirus-containing *C. nitschkei* strains, at least ten different single-spored isolates from each strain were cultured to observe the morphological colony characteristics. Morphological characteristics of each strain were maintained among all single-spored isolates (Fig. 2). Compared to that of *C. parasitica*, the four chrysovirus-containing strains demonstrated thinner growth and their aerial mycelia were not as distinctive as those of *C. parasitica*. In addition, the four strains produced less pigment than *C. parasitica*, which is generally more orange in color and larger in area (Fig. 2). However, differences existed in the colonial morphology among the four strains. As shown in Fig. 2, strain bs131 displayed almost no pigmentation even after prolonged incubation, resulting in peripheral hyphal aggregates as the culture matured. Strain BS122 showed very little, if any, pigmentation, but exhibited vigorous aerial mycelial growth compared to the other three strains. Among the other two strains, the production of pigment was greatest in BS122, followed by the

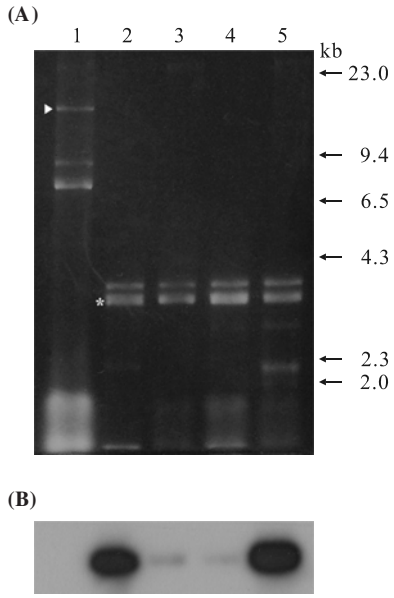

Fig. 3. (A) Ethidium bromide-stained gel showing representative dsRNA patterns. Lanes: 1, contains a dsRNA preparation from the representative isolate UEP1 (CHV1-EP713), which contains the characteristic 12.7 kb viral genome of CHV1; 2~5, contain dsRNAs from the four Korean *C. nitschkei* strains BS122, bs131, bs132, and BS321. Arrowhead and asterisk indicate the characteristic 12.7 kb CHV1 genome and multiple bands with similar sizes of chrysoviral genome segments, respectively. Numbers on the right are band sizes in kb. (B) Northern blot analyses of the corresponding gel using a full-length cDNA clone of CP from strain BS122 as a probe.

bs132 strain, and the pigmentation was less orange in color and mostly restricted to the center of the colony. Note that the two strains, bs131 and bs132, had identical ITS sequences but showed different colonial phenotypes. Compared to bs132, strain bs131 was less-pigmented with peripheral hyphal aggregates containing pycnidia.

Genetic diversity of chrysoviruses using a comparison of the sequences encoding RdRp and CP

Even though a total of four strains may not comprise a large sample size, differences in the colonial morphology among the four chrysovirus-infected *C. nitschkei*, especially the two having identical ITS sequences, were of sufficient interest to warrant examining the diversity of the chrysoviruses. In addition, Northern blot analysis using a full-length cDNA clone of CP from strain BS122 as a probe suggested sequence divergence among the chrysoviruses in the four *C. nitschkei* strains because the intensity of the hybridizing bands was not correlated with the RNA sample loading; i.e., the intensity of the hybridzing band of BS321 was stronger than that of bs132 even though more bs132 was loaded compared to BS321 according to visual inspection of the ethidium bromide-stained agarose gel (Fig. 3). We then examined the sequence divergence of two genes encoding the CP and RdRp of the chrysoviruses.

The assembly of 60 random clones from the dsRNAs of the representative strain BS122, and PCR using an anchored primer and internal gene-specific primers for RdRp and CP based on previous studies (Park *et al*., 2008) resulted in two contig sequences of 2,922 bp and 3,306 bp, respectively. Analysis of the 3,306 bp contig sequence revealed that it comprised one large open reading frame (ORF) of 906 amino acid residues and hybridized to the lower 3.3 kb band. Sequence comparison of this contig using a BLASTX search revealed a high level of homology with the CP of several chrysoviruses, including *C. nitschkei* chrysovirus 1, *Penicillium chrysogenum* virus, *Helminthosporium victoriae* 145S virus, Amasya cherry disease associated chrysovirus, and Cherry chlorotic rusty spot associated chrysovirus. Among these, the contig was most similar to the available sequence

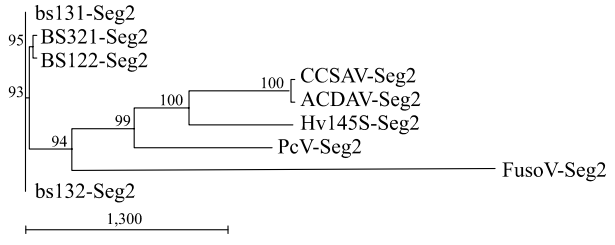

Fig. 4. Phylogenetic tree indicating the relationships among chrysoviruses from Korean *C. nitschkei* strains and other full-length sequenced representative chrysoviruses based on sequences of capsid protein. An evolutionary matrix was generated by Henikoff and Henikoff (1992), and the branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate a bootstrap value of 1000 resampled data sets. Sequence information and strain designation are provided in Table 1. *Fusarium solani* mycovirus 1 (FusoV-seg2) was used as the outgroup (Coutts *et al*., 2004).

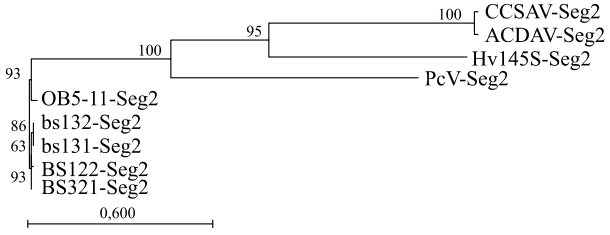

Fig. 5. Phylogenetic tree indicating the relationships among chrysoviruses from Korean and Japanese *C. nitschkei* strains and other representative chrysoviruses based on the available sequences of capsid protein. An evolutionary matrix was generated by Henikoff and Henikoff (1992), and the branching pattern was generated by the neighbor-joining method. The numbers at the nodes indicate a bootstrap value of 1,000 resampled data sets. Sequence information and strain designation are provided in Table 1.

data for *C. nitschkei* chrysovirus 1, showing 97.5% (153/157) identity with the sequenced N-terminus of the CP of *C. nitschkei* chrysovirus 1. The 2,922 bp contig comprised one ORF of 962 amino acid residues and hybridized to the higher 3.5 kb band. Sequence comparison of this contig revealed an even higher homology with the RdRp of other chrysoviruses. Among these, it was most similar to *C. nitschkei* chrysovirus 1, showing amino acid identities of 93.6% (88/94) and 96.9% (124/128), respectively, with the sequenced N-terminus and C-terminal proximal region of the RdRp.

Based on the sequence information of the chrysovirus from the BS122 strain, cloning of full-length CP and RdRp genes from the other three chrysoviruses was conducted using RT-PCR with the primer pairs corresponding to the N-terminal and C-terminal regions of the full-length ORF sequences. Multiple alignments of the CP genes of the chrysoviruses from the four *C. nitschkei* strains revealed that although they were very similar among each other, showing more than 95% and 84% identities at the amino acid and nucleotide levels, respectively, sequence divergence had occurred (Fig. 4). Note, however, that no difference between the chrysoviruses from strains bs131 and bs132 were observed. Multiple alignments of the RdRp genes among those four strains revealed that they were even more similar among each other, showing more than 98% and 97% identities at the amino acid and nucleotide levels, respectively. Again, the sequences of the chrysoviruses from the bs131 and bs132 strains were identical.

Phylogenic analysis of CP with other chrysoviruses having full-length sequence data demonstrated that chrysoviruses from Korean *C. nitschkei* strains clustered roughly with *P. chrysogenum* virus 1, whereas Amasya cherry disease associated chrysovirus and Cherry chlorotic rusty spot associated chrysovirus clustered roughly with *H. victoriae* 145S virus (Fig. 4). Phylogenetic analysis using a partial sequence of a chrysovirus from *C. nitschkei* strain OB5-11 and its corresponding regions from other chrysoviruses revealed that the chrysovirus from *C. nitschkei* strain OB5-11 grouped closely with those from the Korean *C. nitschkei* strains (Fig. 5). Phylogenetic analysis of RdRp using full-length as well as partial sequence data revealed similar patterns of clustering (data not shown).

Discussion

Our previous studies on the isolation of a fungus with orange stromata typical of *Cryphonectria* species revealed the high incidence of *C. parasitica* on necrotic areas of chestnut trees and the presence of a few abnormal colonies with less-pigmented and less-aerial mycelia (Ju *et al*., 2002). In a recent study on the occurrence of dsRNA in our culture collection (Park *et al*., 2008), we suggested that abnormal colonies containing a newly described chrysovirus represent another species of *Cryphonectria*, *C. nitschkei*, based on the RFLP data of the ribosomal DNA region. In East Asia, including China and Japan, the majority of *Cryphonectria* species known to occur on woody hosts appear to be *C. parasitica*, *C. radicalis*, *C. nitschkei*, and *C. macrospora*. In Korea, the presence of *C. nitschkei* has been recorded on *Quercus* spp. (Lee *et al*., 1992), although no further studies have been conducted. Firm identification of these *Cryphonectria* species based on morphology is challenging because the range of spore sizes commonly overlaps and the ascospores are not always fully developed, resulting in variable measurements (Myburg *et al*., 2004a). In addition, without complete information on the effects of chrysovirus infection on fungal morphology, conclusively identifying abnormal strains of *Cryphonectria* species based on morphology is even harder. However, recent taxonomic studies based on sequence comparisons and RFLP analysis of the ITS region have resolved several questions pertaining to the identification of *Cryphonectria* species (Myburg *et al*., 1999, 2004b; Liu *et al*., 2003). Although , the present study based on DNA sequence comparisons showing that four chrysovirus-containing strains grouped closely to *C. nitschkei* but separately from other commonly known species of *Cryphonectria* indicates that these strains represent *C. nitschkei*.

Detecting differences in cultural phenotypes among Korean chrysovirus-infected strains is notable because no differences among Japanese isolates were observed (Liu *et al*., 2007). The cultural characteristics of each strain appeared to be genetically stable because characteristics of each strain were maintained among all single-spored progenies obtained from different successive culture plates. Among those single-spored progenies, at least ten isolates for each strain were randomly selected for the presence of dsRNA. All isolates showed the presence of dsRNA with the same banding pattern as that of the parental strain, indicating stable inheritance of the dsRNA during asexual conidiation.

Complete sequencing of the four chrysoviruses revealed that the viral genome consists of four segments, each of which contains a single deduced ORF (Covelli *et al*., 2004; Jiang and Ghabrial, 2004; Ghabrial and Caston, 2005). In addition, sequence comparisons indicated the presence of an identifiable homolog to any given dsRNA segment of each of the chrysoviruses suggesting that the chrysovirus genome structure is conserved. In this study, the four dsRNA segments characteristic of chrysoviruses did not resolve electrophoretically on our agarose gel. However, the strong intensity of the lower band on ethidium bromide-stained gels suggested that it represented multiple bands of similar size. In addition, we were able to obtain cDNA clones assigned to other contigs representing two other genome segments (data not shown). These results suggest that the dsRNA from the Korean *C. nitschkei* strains appear to be a four-segmented genome of a newly described chrysovirus of the family *Chrysoviridae* in *Cryphonectria*. The two contig sequences showing a high similarity to the RdRp and CP of chrysoviruses represented two segments of the four-segmented genome.

Sequence comparisons among chrysoviruses from the four Korean *C. nitschkei* strains indicated that although a high level of similarity existed and two of them were identical, sequence divergence had occurred. In addition, different rates of evolution seem to apply to different genes within the same genome based on the differences in the diversity level of the RdRp and CP genes. Compared to RdRp, the identity among CP genes decreased to 95% at the deduced amino acid level, and further decreased to 84% at the nucleotide sequence level.

Since mycoviral transmission is limited to intracellular mechanisms under natural conditions (Buck, 1998), horizontal movement of the mycovirus to other fungal hosts is likely rare. This is in a good agreement with our phylogenic study on chrysoviruses. Phylogenic analysis indicated that chrysoviruses from *C. nitschkei* strains clustered together, suggesting a natural barrier to viral transmission between distantly related fungal species. In addition, geographic isolation tends to exist within the same fungal species because of the closer relationship among chrysoviruses from the Korean *C. nitschkei* strains compared to the chrysovirus from the Japanese *C. nitschkei* strain. Due to the greater sequence divergence inferred from the partial genome sequence data of existing *C. nitschkei* virus 1 (CnV-1) (Liu *et al*., 2007), determining whether those chrysoviruses in the four Korean *C. nitschkei* strains represent another species of chrysovirus will be of great interest.

Considering both the genetic background as evidenced by the identical chromosomal ITS sequences and cytoplasmic constituents as evidenced by the identical RdRP and CP sequences of the chrysovirus, strains bs131 and bs132 appeared to be clonal. In contrast, both strains showed different colonial morphology and those corresponding differences were maintained through successive culturing of single-spored progenies. These discrepancies can be explained by the assumption that differences exist in the fungal genome outside of the ITS region or that dissimilarities are present in the chrysoviral genome other than that observed for the CP or RdRp genes. The presence of chrysovirus confers little, if any, measurable effect on the fungus (Liu *et al*., 2007). However, in this study, differences in cultural characteristics such as pigmentation and mycelial growth were observed among the four Korean *C. nitschkei* strains and the chrysovirus infection did not appear to cause pleiotropic effects on the fungus based on the observation that the single-spored progenies showed no changes from their parental strain as well as among each other. Therefore, obtaining chrysovirus-free and -containing isogenic isolates to examine the effects of the chrysovirus on *C. nitschkei* will be of great interest. In addition, complete genome sequencing of the chrysovirus from *C. nitschkei* should prove to be enlightening.

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