Quasispecies of *Chrysanthemum stunt viroid* in different chrysanthemum cultivars

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Viroids are the smallest infectious agents and consist of a short and single strand of RNA, which does not encode any protein. Chrysanthemum stunt viroid (CSVd) is a member of the family Pospiviroidae and causes chrysanthemum stunt disease. Here, we report the genomic variations of CSVd to understand the quasispecies of CSVd in different chrysanthemum cultivars. We randomly sampled 36 different chrysanthemum cultivars and examined the infection of CSVd in each cultivar by RT-PCR. Eleven cultivars were infected by CSVd. Cloning followed by Sanger sequencing successfully identified a total of 271 CSVd genomes derived from 12 plants composed of 11 cultivars. They were further classified into 105 CSVd variants. The individual single chrysanthemum plant has a different set of CSVd variants. Moreover, different single plants from the same cultivar have a different set of CSVd variants; however, the consensus genome sequences were identical between them. A phylogenetic tree using 12 consensus genome sequences revealed three groups of CSVd genomes, while six different groups were defined by the phylogenetic analysis using 105 variants. Based on the consensus CSVd genome, by combining all variant sequences, we identified 99 single-nucleotide variations (SNVs) as well as three nucleotide positions showing high mutation rates. Although 99 SNVs were identified, most CSVd genomes in this study were derived from variant 1, which is identical to known CSVd SK1 showing pathogenicity. Taken together, this study is the first report providing evidence of the quasispecies of CSVd in a single chrysanthemum plant as well as different cultivars.

Quasispecies of *Chrysanthemum stunt viroid* in different chrysanthemum cultivars

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Abstract

Viroids are the smallest infectious agents and consist of a short and single strand of RNA, which does not encode any protein. *Chrysanthemum stunt viroid* (CSVd) is a member of the family *Pospiviroidae* and causes chrysanthemum stunt disease. Here, we report the genomic variations of CSVd to understand the quasispecies of CSVd in different chrysanthemum cultivars. We randomly sampled 36 different chrysanthemum cultivars and examined the infection of CSVd in each cultivar by RT-PCR. Eleven cultivars were infected by CSVd. Cloning followed by Sanger sequencing successfully identified a total of 271 CSVd genomes derived from 12 plants composed of 11 cultivars. They were further classified into 105 CSVd variants. The individual single chrysanthemum plant has a different set of CSVd variants. Moreover, different single plants from the same cultivar have a different set of CSVd variants; however, the consensus genome sequences were identical between them. A phylogenetic tree using 12 consensus genome sequences revealed three groups of CSVd genomes, while six different groups were defined by the phylogenetic analysis using 105 variants. Based on the consensus CSVd genome, by combining all variant sequences, we identified 99 single-nucleotide variations (SNVs) as well as three nucleotide positions showing high mutation rates. Although 99 SNVs were identified, most CSVd genomes in this study were derived from variant 1, which is identical to known CSVd SK1 showing pathogenicity. Taken together, this study is the first report providing evidence of the quasispecies of CSVd in a single chrysanthemum plant as well as different cultivars.

38 **Introduction**

39

40 Viroids are the smallest pathogen that infect plant species (Diener, 1974). The genomes of the
41 viroid are composed of a circular single-stranded RNA, which does not encode a protein (Tabler
42 & Tsagris, 2004). The size of viroid RNAs ranges from 246 to 401 nucleotides (nt) (Ding, 2010).
43 So far, more than 30 viroid species have been identified in a wide range of plants, and they are
44 further divided into two families: *Pospiviroidae* and the *Avsunviroidae* (Di Serio *et al.*, 2014). Five
45 genera, *Apscaviroid*, *Cocadviroid*, *Coleviroid*, *Hostuviroid* and *Pospiviroid*, are members of the
46 family *Pospiviroidae*, while three genera, *Avsunviroid*, *Elaviroid* and *Pelamoviroid*, are members
47 of the family *Avsunviroidae*. The viroids in the family *Pospiviroidae* with a rod-like conformation
48 replicate in the nucleus, whereas the viroids in the family displaying highly branched structures
49 with self-cleaving ribozymes replicate in the chloroplast (Ding, 2009).

50 The chrysanthemum (*Dendranthema X grandiflorum*) is famous for its flowers, and
51 numerous cultivars have been developed and cultivated worldwide. Due to the clonal propagation
52 of chrysanthemum cultivars, such as through cutting, the rates of viruses and viroids in cultivated
53 chrysanthemums are very high, resulting in a severe reduction of the quality and quantity in
54 chrysanthemum flower production (Chung *et al.*, 2005). So far, nine viruses and two viroids are
55 known to infect chrysanthemum species. Interestingly, the chrysanthemum is susceptible to two
56 different viroids: *Chrysanthemum stunt viroid* (CSVd) and *Chrysanthemum chlorotic mottle viroid*
57 (CChMVd) (Cho *et al.*, 2013).

58 Viruses and viroids show a high level of genetic diversity in the infected host by
59 replicating with strong mutation rates (Sanjuan *et al.*, 2010). Therefore, viral populations in the
60 host are composed of diverse variants of viruses and viroids, which are called viral quasispecies,

rather than a single unique viral genome (Domingo *et al.*, 2012). Viral quasispecies affect the genetic diversity and pathogenicity of viruses and viroids. The nature of viral quasispecies has been previously characterised in RNA and DNA viruses infecting plants (Duffy & Holmes, 2008; Schneider & Roossinck, 2001). In addition, viroids exhibit quasispecies. For example, the quasispecies of CSVd and CChMVd in the chrysanthemum host have been characterised (Codoñer *et al.*, 2006). However, the quasispecies of CSVd in different chrysanthemum cultivars, their genetic variations, and the association of genetic variants with the host have not been reported.

In this study, we analysed the genetic variations of CSVd genomes in different chrysanthemum cultivars in order to elucidate the genetic diversity and quasispecies of CSVd by cloning-based Sanger sequencing.

Materials and methods

Plant samples

All chrysanthemum plants in this study were purchased from the Gangnam flower market, Seoul, on January 22, 2015. Leaf samples were harvested from a single plant for each cultivar and frozen immediately using liquid nitrogen. All frozen leaf samples were kept at −80°C for further experimentation.

RNA isolation and RT-PCR

The frozen leaf samples were ground in liquid nitrogen with a mortar and pestle. The total RNAs were extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total RNAs were subjected to RT-PCR using CSVd-specific primers (5′-AAAGAAATGAGGCGAAGAAGTC-3′ and 5′-TTCTTTCAAAGCAGCAGGGT-3′) (Choi *et al.*, 2015).

Cloning and sequencing

The amplified PCR products from CSVd-infected plant samples were cloned using pGEM-T-Easy Vector (Promega, WI, USA). For each plant sample, at least 20 clones were subjected to Sanger sequencing. The obtained CSVd genome sequences were deposited in GenBank with their respective accession number. The accession numbers of the CSVd genome sequences for the individual chrysanthemum plant are listed in Table 1.

Sequencing analysis and phylogenetic analysis

A total of 271 clones were sequenced. We analysed all 271 CSVd genome sequences and identified 105 variants. From each single plant, we generated a consensus CSVd genome sequence. The twelve CSVd consensus genome sequences were subjected to the construction of a phylogenetic tree using the MEGA6 programme (Tamura *et al.*, 2013). For the construction of the phylogenetic tree, the genome sequences were aligned by ClustalW implemented in the MEGA6 programme with default parameters (Thompson *et al.*, 2002). Using the neighbour-joining method, a phylogenetic tree was constructed. In the construction of the phylogenetic tree for 105 CSVd variants, the aligned sequences were converted into the NEXUS file format using MEGA6, and then the nexus file was imported into the SplitsTree4 programme (Huson & Bryant, 2006). Finally, the unrooted phylogenetic tree was constructed by SplitsTree4 using the neighbour-joining method.

Analysis of single-nucleotide variations and recombination analysis

In order to examine the SNVs of the CSVd genome in a single chrysanthemum plant, we generated a consensus CSVd genome sequence from each plant. We aligned each genome sequence against the generated consensus CSVd genome sequence derived from each chrysanthemum plant. To identify the SNVs within the 105 identified variants, the sequences for the 105 variants were aligned against a consensus CSVd genome sequence that was obtained by combining all 105 variants. The aligned sequences for the 105 variants were subjected to recombination analysis using the RDP programme with default parameters (Martin *et al.*, 2015).

Results

Identification of CSVd-infected chrysanthemum plants from commercial chrysanthemum cultivars

We sampled 36 different chrysanthemum cultivars from the market and examined the infection of CSVd in obtained chrysanthemum cultivars by reverse transcription polymerase chain reaction (RT-PCR) using CSVd-specific primers. In this study, we did not consider the disease symptoms caused by CSVd. Out of 36 cultivars, at least 11 cultivars were infected by CSVd (data not shown). Among the CSVd-infected chrysanthemum cultivars, the Shinma cultivars were derived from two different regions: Jeju, Korea, and an unknown city in China. The 12 plant samples were named based on their cultivar name and cultivation region (Table 1 and Fig. 1A). Five cultivars were derived from Jeju and four cultivars were grown in Icheon. Except the Isokuk belonging to *Dendranthema pacificum*, all other cultivars are members of *Dendranthema x grandiflora*.

Amplification of CSVd genomes from 12 different chrysanthemum plants

In order to examine the genetic variations of CSVd genome sequences in a single chrysanthemum plant, we conducted RT-PCR again using CSVd-specific primers and sequenced the amplified CSVd genomes by cloning-based Sanger sequencing. We only used the total RNAs extracted from a single plant representing an individual chrysanthemum cultivar except the Shinma cultivar, which was further divided into SMJ and SMC based on origin (Table 1). From each cultivar, at least 20 clones were sequenced, resulting in a total of 271 genome sequences (Table S1). Interestingly, the sizes of all sequenced CSVd genomes were identical at 354 (nt) in length. The identified number of variants in each cultivar ranged from six to 20 (Table 1). The Froggy cultivar

possessed only six variants, while the Disk Club cultivar displayed at least 20 variants.

Comparison of identified CSVd variants in 12 chrysanthemum plants

We analysed all obtained 271 CSVd genome sequences and identified 105 CSVd variants (Table S2). The dominant CSVd variant in each cultivar was variable (Fig. 1B). For example, CSVd variant 1 (v1) was dominant in the Ford, Yes Song, Shinma from China, Disk Club, and Froggy. In Borami, Vatican and Shinma from the Jeju cultivars, v2 was dominant, whereas v3 was dominant in the Fire Pink and Pink This Plus cultivars.

We examined the number of sequenced CSVd genomes for each variant (Table S3 and Fig. 1C). The v1 (66 genomes) was dominant, followed by v2 (34 genomes), v3 (25 genomes), and v4 (13 genomes). Eleven variants contained more than two genomes. Based on the number of each variant, v1 represents 24%, followed by v2 (13%), v3 (9%) and v4 (5%). The four major variants represent 51% of all CSVd variants.

Phylogenetic relationships of identified CSVd genomes

Whether the cultivation region is an important factor for CSVd genome variation might be of interest. For this, we generated a phylogenetic tree using CSVd consensus genome sequences from 12 plants (Fig. 1D). The phylogenetic tree displayed three groups of CSVd genomes. Group I contains seven cultivars, including the Shinma cultivar from China and Jeju, Korea. Group II contains the Yellow Cap, Borami and Vatican cultivars. Group III has only two cultivars: Fire Pink and Pink This Plus.

We generated an unrooted phylogenetic tree for 105 CSVd variants using SplitsTree to reveal the phylogenetic relationships among 105 CSVd variants (Fig. 2A). The phylogenetic tree

revealed at least six groups of CSVd variants. Group A was the largest, containing 24 variants, including v1 and v11. Group C was the second largest group with 21 variants, including v3 and v8. Group B was the third largest group, possessing 18 variants, including v2. Group D, group E and group F consist of 13, 6 and 8 variants, respectively. Some variants, such as v68 and v96, which belong to group A and group C, respectively, were distinct from other variants in the same group. Based on the phylogenetic tree, it seems that v1 to v6 were the main CSVd genomes in each group and several other CSVd variants might be derived from the genomes of v1 to v6.

Generation of a consensus CSVd genome sequence and analysis of single-nucleotide variations

In order to examine SNVs for identified CSVd genomes, we generated a consensus CSVd genome combining all CSVd genome sequences. The consensus CSVd genome was perfectly identical to the previous known CSVd strain: SK1 (accession number: AB679193.1). After the sequence alignment of all variants against the consensus CSVd genome sequence revealed 99 SNVs the CSVd genome in 354 nt in length (Table S4). In general, most nucleotides in the CSVd genome were highly conserved among the 105 variants in this study. However, three nucleotide positions showed a high level of sequence variations (Fig. 2B). The three SNVs were localised at 298 (A to U with 50.47%), 256 (C to U with 77.14%) and 156 (A to G with 80%). We further analysed the recombinant events among the 105 CSVd variants using the RDP4 programme, and no recombinants were detected.

Discussion

In this study, we examined genetic variations of the CSVd genome in a single chrysanthemum plant. For this, the infectivity of CSVd in different chrysanthemum cultivars, which were randomly selected, was investigated. Finally, CSVd genomes amplified from twelve plants composed of eleven cultivars were sequenced. As we expected, each chrysanthemum plant had a different set of CSVd variants. Although a large number of chrysanthemum cultivars have not been investigated, our results suggest that genetic variations of the CSVd genome in a single plant might be influenced by the genetic differences of the chrysanthemum plant. Furthermore, we found that different environmental conditions could be a factor that determines the genetic variations of the CSVd genome in a single plant. For instance, the compositions of the CSVd variants were different in the Shinma from Jeju, Korea, and the Shinma from China, although the host was identical. Interestingly, the consensus CSVd genome sequences in the two Shinma plants were identical. This result showed that some nucleotides in the CSVd genome could be changed; however, the dominant CSVd genome sequence in a certain chrysanthemum cultivar might be highly conserved.

The phylogenetic tree using 12 consensus CSVd genomes revealed three groups of CSVd genomes. This result demonstrated that at least three dominant CSVd variants are present in chrysanthemum plants grown in Korea. In fact, the result of the phylogenetic relationship is highly correlated with the results for the composition of CSVd variants in the individual plant. For instance, the dominant variant in group I was v1, while v2 and v3 were dominant in groups II and III, respectively. We theorise that the chrysanthemum cultivars in each group might have a common genetic background that can control the genetic variations of the CSVd genome. To verify this, a large number of plants from different regions should be examined.

206 The unrooted phylogenetic tree using 105 CSVd variants revealed at least six groups of
207 CSVd variants. The advantage of this phylogenetic tree is that it can show the dominant CSVd
208 variant as well as its sister variants, which were derived from the dominant CSVd variant. For
209 example, among the 24 CSVd variants in group A, v1 is the dominant CSVd genome, while the
210 other 23 variants were derived from v1.

211 Interestingly, the consensus CSVd genome sequences as well as v1 were identical to the
212 previously identified CSVd SK1 genome (Yoon *et al.*, 2014). In addition, v2 and v3 display only
213 one nucleotide and three nucleotide substitutions compared with CSVd SK1. SK1 was previously
214 identified in the chrysanthemum showing stunt disease symptoms and was also used for the
215 construction of an infectious clone for CSVd (Yoon *et al.*, 2014). Therefore, the majority of CSVd-
216 infected chrysanthemum plants in this study might be pathogenic, leading to CSVd disease
217 symptoms. Therefore, the potential risk caused by CSVd in the chrysanthemum production in
218 Korea might be very high.

219 According to a previous study, there are genome sequences for at least 117 CSVd isolates
220 worldwide, and only 24 isolates divided into six variants have been sequenced in Korea (Yoon &
221 Palukaitis, 2013). In this study, we sequenced 271 CSVd genomes; this number is high enough to
222 obtain the genetic variations of CSVd genomes in Korea. Based on our results, it seems that the
223 number of identified CSVd variants could be increased as the number of sequenced genomes is
224 increased. However, the consensus genome sequences for CSVd in Korea could be identical,
225 suggesting the conservancy of the CSVd genome.

226 Based on a consensus CSVd genome sequence, 99 SNVs were identified from 105
227 variants; however, no indel (insertion and deletion) was identified. The 99 SNVs were scattered
228 through the CSVd genome. This result is consistent with a previous study that analysed all

available CSVd genome sequences (Yoon & Palukaitis, 2013). However, the mutation rates for most SNVs were very low, suggesting the high conservancy level of the CSVd genome, which is different from CChMVd, which displayed high mutation rates (Gago *et al.*, 2009). We found three nucleotide positions that displayed strong mutation rates among 105 variants. A previous study identified seven positions (47, 49, 50, 64, 65, 254 and 298) that showed strong sequence variations among examined 80 variants of CSVd (Yoon & Palukaitis, 2013). Interestingly, position 298 was identified by our study. At position 294, v1, v4, v5 and v6 had A, while v2, v3, v7, v8, v9 and v10 had U. Although specific sequences in the tetraloop of CChMVd associated with pathogenicity have been revealed (De la Peña *et al.*, 1999), the specific nucleotides or regions for the pathogenicity of CSVd have not been determined. Thus, it might interesting to examine the functional roles of the 294 nucleotide associated with the CSVd life cycle, such as replication, pathogenicity and movement, in the near future.

Taken together, we revealed the quasispecies of CSVd in a single chrysanthemum showing different genetic variations of the CSVd sequence in diverse chrysanthemum cultivars. Although 105 variants and 99 SNVs were identified from 271 CSVd genomes, the CSVd genomes in chrysanthemum plants grown in Korea were highly conserved.

Acknowledgments

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296 **Table 1.** Detailed information on chrysanthemum plants used in this study

297 Cultivar name, scientific name and cultivated geographical location are provided. In the case of the Shinma cultivar, the plants were
298 obtained from two different locations: China (SMC) and Jeju, Korea (SMJ). From each plant, at least 20 clones were sequenced, and
299 their sequences were deposited in GenBank with their respective accession number. The number of variants in each plant is also
300 indicated.

Index	Abbreviation	Cultivar Name	Scientific Name	Location	No. of Clones	No. of Variants	Length	Accession No.
1	BRJ	Borami	*Dendranthema x grandiflora*	Jeju	20	12	354	KX096347 - KX096366
2	FDJ	Ford	*Dendranthema x grandiflora*	Jeju	22	12	354	KX096408 - KX096428
3	YSI	Yes Song	*Dendranthema x grandiflora*	Icheon	22	8	354	KX096555 - KX096576
4	YCI	Yellow Cap	*Dendranthema x grandiflora*	Icheon	23	14	354	KX096533 - KX096554
5	SMC	Shinma	*Dendranthema x grandiflora*	China	24	8	354	KX096472 - KX096491
6	DCI	Disk Club	*Dendranthema x grandiflora*	Icheon	25	20	354	KT005803 - KT005827
7	VCS	Vatican	*Dendranthema x grandiflora*	Siheung	20	10	354	KX096512 - KX096532
8	FGI	Froggy	*Dendranthema x grandiflora*	Icheon	22	6	354	KX096429 - KX096449
9	FPJ	Fire Pink	*Dendranthema x grandiflora*	Jeju	24	14	354	KX096388 - KX096407
10	PTG	Pink This Plus	*Dendranthema x grandiflora*	Gangneung	23	15	354	KX096450 - KX096471
11	ISJ	Isokuk	*Dendranthema pacificum*	Jeju	23	14	354	KX096367 -

12	SMJ	Shinma	*Dendranthema x grandiflora*	Jeju	23	15	354	KX096387 KX096492 - KX096511

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302

303 **Figure legends**

304

305 **Fig. 1.** Analysis of genetic variations for CSVd genomes derived from 12 chrysanthemum plants
306 composed of 11 different cultivars.
307 (A) Images of 12 chrysanthemum plants infected by CSVd used for amplification of CSVd genome
308 sequences. (B) Number of identified variants in individual chrysanthemum plant. (C) Distribution
309 of identified CSVd variants based on number of clones in individual chrysanthemum plant. (D)
310 The phylogenetic tree for 12 consensus CSVd genomes constructed using the neighbour-joining
311 method with 1000 bootstrap replicates.

312

313 **Fig. 2.** Phylogenetic relationship and identification of single-nucleotide variations for 105 CSVd
314 variants.
315 (A) The unrooted phylogenetic tree for 105 CSVd variants was constructed using the SplitsTree4
316 programme with the neighbour-joining method. Each number indicates an individual variant. The
317 top ten variants are indicated by yellow and red with bold characters. (B) Identification of single-
318 nucleotide variations in the consensus CSVd genome. The secondary structure of the CSVd
319 genome was adapted from a previous study (Yoon & Palukaitis, 2013). Number indicates the
320 position of the nucleotide. The three nucleotide positions showing high mutation rates are indicated
321 by red lines.

322

323 **Supplementary Materials**

324 **Table S1.** Name and detailed genome sequence of each clone derived from 12 different
325 chrysanthemum plants.

326 **Table S2.** Classification of sequenced clones based on 105 CSVd variants.

327 Number of clones, corresponding genome sequence and names of clones for each variant are

328 described.

329 **Table S3.** Distribution of identified CSVd variants in each single plant.

330 **Table S4.** Identification of single-nucleotide variation in each variant.

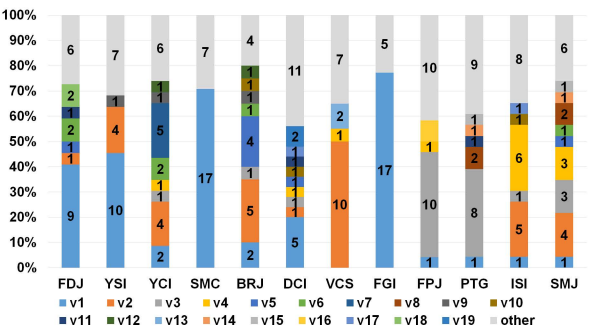
331

Figure 1(on next page)

Analysis of genetic variations for CSVd genomesderived from 12 chrysanthemum plants composed of 11 different cultivars.

(A) Images of 12 chrysanthemum plants infected by CSVd used for amplification of CSVd genome sequences. (B) Number of identified variants in individual chrysanthemum plant. (C) Distribution of identified CSVd variants based on number of clones in individual chrysanthemum plant. (D) The phylogenetic tree for 12 consensus CSVd genomes constructed using the neighbour-joining method with 1000 bootstrap replicates.


A
Ford
Yes Song
Yellow Cap
Shinma_China
Borami
Disk Club
Vatican
Froggy
Fire Pink
Pink This Plus
Isokuk
Shinma_Jeju


B
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
FDJ
YSI
YCI
SMC
BRJ
DCI
VCS
FGI
FPJ
PTG
ISI
SMJ
v1
v2
v3
v4
v5
v6
v7
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v9
v10
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v12
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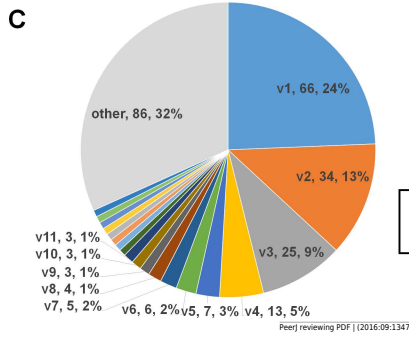
v14
v15
v16
v17
v18
v19
other


C
v1, 66, 24%
v2, 34, 13%
v3, 25, 9%
v4, 13, 5%
v5, 7, 3%
v6, 6, 2%
v7, 5, 2%
v8, 4, 1%
v9, 3, 1%
v10, 3, 1%
v11, 3, 1%
other, 86, 32%

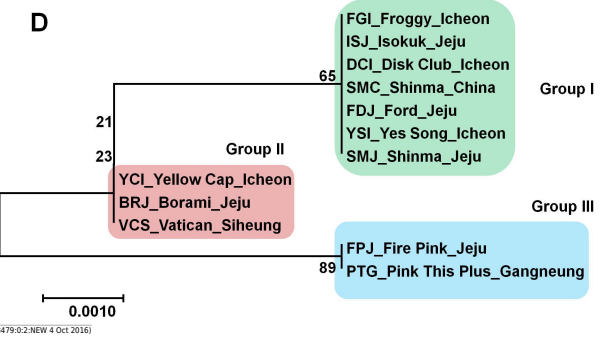
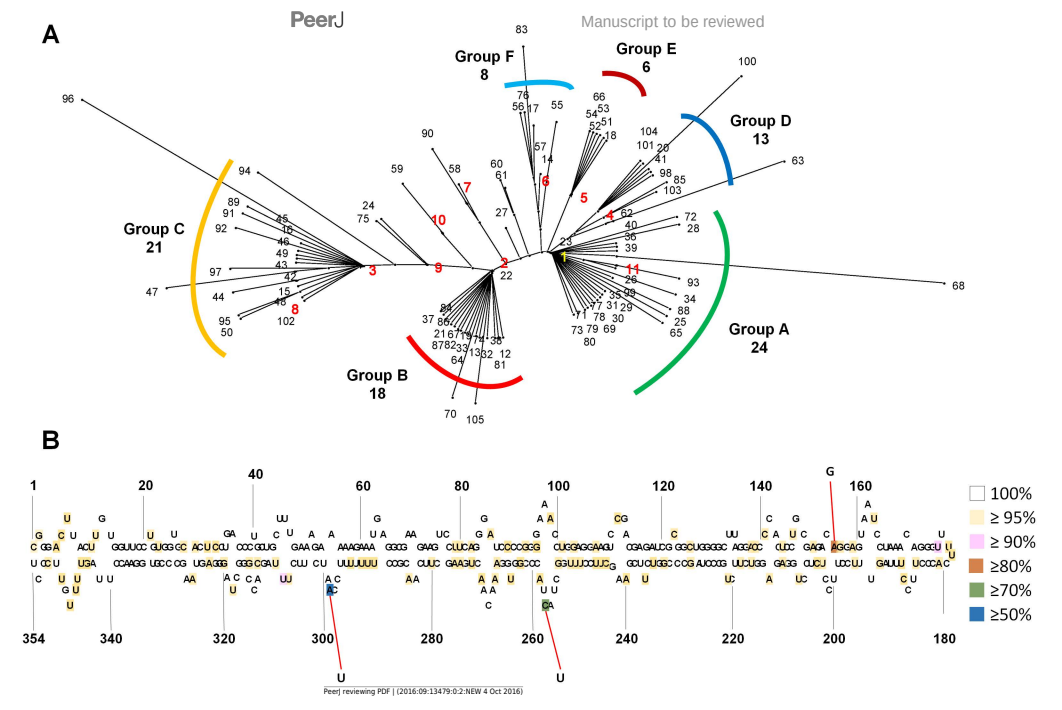

D
FGI_Froggy_Icheon
ISJ_Isokuk_Jeju
DCI_Disk Club_Icheon
SMC_Shinma_China
FDJ_Ford_Jeju
YSI_Yes Song_Icheon
SMJ_Shinma_Jeju
Group I
65
21
23
Group II
YCI_Yellow Cap_Icheon
BRJ_Borami_Jeju
VCS_Vatican_Siheung
Group III
89
FPJ_Fire Pink_Jeju
PTG_Pink This Plus_Gangneung
0.0010

Figure 2(on next page)

Phylogenetic relationship and identification of single-nucleotide variations for 105 CSVd variants.

(A) The unrooted phylogenetic tree for 105 CSVd variants was constructed using the SplitsTree4 programme with the neighbour-joining method. Each number indicates an individual variant. The top ten variants are indicated by yellow and red with bold characters. (B) Identification of single-nucleotide variations in the consensus CSVd genome. The secondary structure of the CSVd genome was adapted from a previous study (Yoon & Palukaitis, 2013). Number indicates the position of the nucleotide. The three nucleotide positions showing high mutation rates are indicated by red lines.


A
Group C
21
Group F
8
Group E
6
Group D
13
Group A
24
Group B
18
B
100%
≥ 95%
≥ 90%
≥80%
≥70%
≥50%