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SSCP Analysis of Variations in Haplotypes of Citrus Tristeza Virus Isolated from Yuzu (*Citrus junos*) in Geographically Separate Regions of Korea

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Yuzu (*Citrus junos*) trees were examined from six geographically separate provinces in the Republic of Korea, including four islands (Geoje, Namhae, Wan, and Jeju), 1 peninsula (Goheung), and 1 inland area (Boseong). The population of sequence variants of citrus tristeza virus (CTV) was isolated and analyzed by single-strand conformation polymorphism (SSCP) analysis of cDNA from the *p20* gene. SSCP profiles of 65 PCR products showed different band patterns but with similar intensities. Sixteen haplotypes were subgrouped according to their SSCP profiles and severity of symptoms. Their genomes were sequenced and compared. DNA analysis of the *p20* genes revealed nucleotide identities ranging from 88-99.8%. Based on SSCP analysis, the pathologically mild isolates of CTV yielded two to three DNA bands, whereas the most virulent isolates contained more than two bands. Comparisons of these physically separate haplotypes suggest that CTV isolates with multiple SSCP profiles could have arisen as a result of a mixed infection and genetic recombination of two divergent isolates. Plants with severe disease symptoms, such as stem pitting, closely corresponded to a CTV strain showing typical SSCP profiles in Florida (USA) and Japan.

Keywords: citrus tristeza virus, *p20* gene, single-strand conformation polymorphism analysis, viral RNA population

Citrus tristeza virus (CTV) virions are flexuous filaments, ~2000 × 11 nm, with a genomic RNA (gRNA) molecule and two capsid proteins of 25 and 27 kDa, which coat 95 and 5% of the particle length, respectively (Bar-Joseph and Lee, 1989; Pappu et al., 1994; Febres et al., 1996). The CTV gRNA, single-stranded with positive polarity, is 19226 to 19296 nucleotides in size, and is organized into 12 open reading frames. It potentially encodes at least 19 protein products, with untranslated regions (UTRs) of ~108 and 290 nucleotides at the 5′- and 3′-termini, respectively (Karasev et al., 1995; Mawassi et al., 1996; Karasev and Hilf, 1997; Vives et al., 1999; Yang et al., 1999).

CTV is readily propagated by infected buds and is spread locally by several aphid species, mainly *Toxoptera citricida* (Kirkaldy), *Aphis gossypii* (Glover), Aphis *spiraecola* (Patch), and *T*oxoptera *aurantii* (Boyer de Fonscolombe). The most efficient vector is *T. citricida*, followed by *A. gossypii* (Hermoso de Mendoza et al., 1988; Yokomi et al., 1994). A 1995 to 1997 survey of 35 citrus orchards in Jeju Island, Korea, showed that CTV infection rate was 70%, which is higher than for all other citrus viruses, i.e., citrus tatter leaf virus (CTLV) and satsuma dwarf virus (SDV) (Kim et al., 1999). The symptoms of infection include small, abnormally shaped fruits, stem-pitting on the twigs, and reduced plant vigor and yield.

CTV isolates often vary in their biological characteristics, such as the intensity of symptoms manifested by a particular citrus species (Roistacher and Moreno, 1991; Ballester-Olmos et al., 1993) or because of aphid transmissibility (Bar-Joseph and Loebenstein, 1973; Hermoso de Mendoza et al., 1988), suggesting wide genetic variation in CTV populations. This variation might be enhanced because citrus trees often remain in the field for 30 or more years, thereby providing many opportunities for exchanges in the viral population between individual trees through repeated aphid inoculations or as a result of cultural practices, such as top-working to new varieties. However, data on their genetic diversity are still limited and variation in these populations under field conditions is largely unknown.

Because CTV isolates induce such different disease

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phenotypes and severities, efforts have been made to develop molecular techniques that rapidly identify CTV isolates as well as molecular markers related to CTV-induced symptoms. Variations in serological reactivity, peptide maps of the coat protein, double-stranded RNA patterns, restriction fragment length polymorphisms, and single-strand conformation polymorphisms (SSCPs) have been utilized in an attempt to differentiate CTV isolates (Orita et al., 1989; Rubio et al., 1996, 2000; Sambade et al., 2002). Among these methods, nucleotide sequence analysis is the most accurate procedure for identifying and analyzing genetic variants in geographically restricted regions, as well as among countries (Rubio et al., 2001; D'urso et al., 2003).

Our study covered four geographically separate islands, 1 peninsula, and 1 inland area of Southern Korea, all important yuzu-producing provinces. We analyzed the variation among CTV isolates by comparing the SSCP profiles of *p20* genes isolated from infected, field-grown trees that differed in their disease durations, symptoms, and environmental conditions. Our objective was to use these profiles as molecular markers for monitoring a cross-protection experiment.

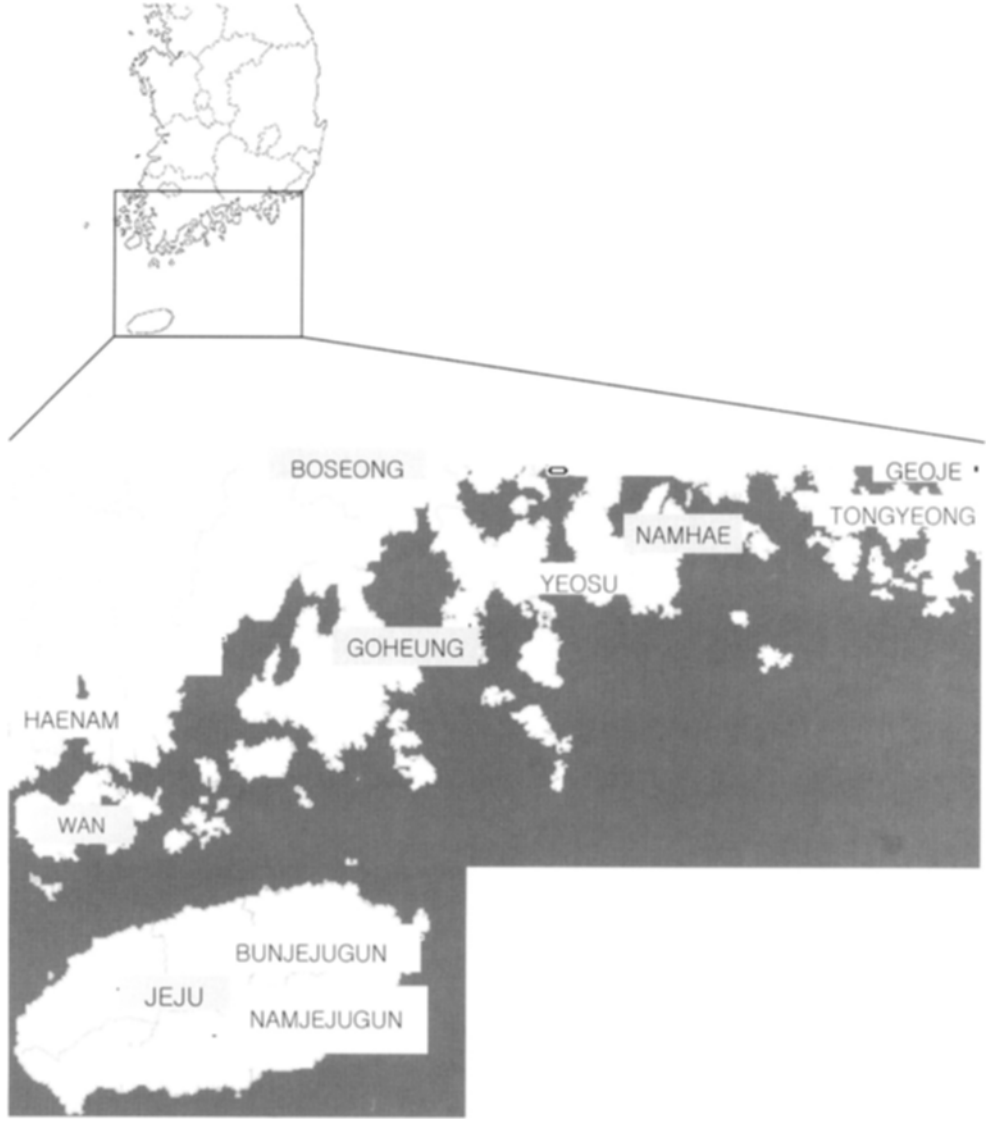


Figure 1. Site map and sample sizes (in parentheses) for SSCP analysis. Jeju Island (39); Geoje island (22); Namhae island (20); Goheung peninsula (21); inland Boseong (20); Wan island (20).

MATERIALS AND METHODS

CTV Isolates

We sampled 142 yuzu (*Citrus junos*) trees from commercial orchards at six sites within the following regions in Korea: four islands (Geoje, Namhae, Wan, and Jeju), one peninsula (Goheung), and one inland area (Boseong) (Fig. 1). Among them, 65 were infected with CTV based on RT-PCR analysis. At the end of May 2003, a young, fully expanded leaf was collected from each of these infected trees for SSCP and phylogenetic analyses (Table 1). For SSCP characterization, the tissues were frozen at -70°C.

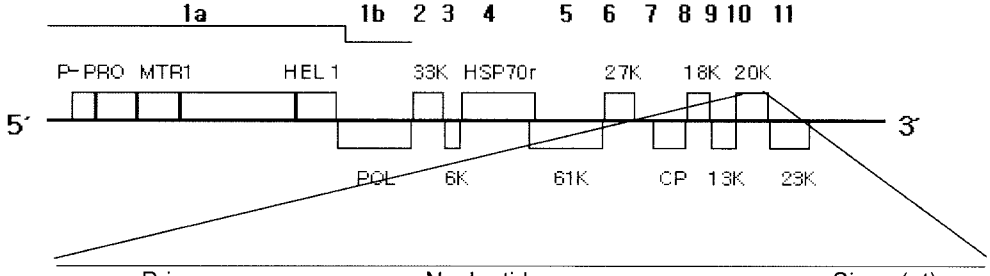
cDNA Synthesis and SSCP Analysis

cDNA was synthesized by reverse transcription and polymerase chain reaction amplification (RT-PCR), using total RNA as template and two sets of primers selected to amplify the *p20* genes (Fig. 2). The PCR product was approximately 520 bp. For cDNA synthesis, 8 μL of the RNA extract was heat-denatured at 93°C for 2 min, then chilled on ice. Single-step RT-PCR was performed in a 20 μL reaction volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, 4 units of RNaseOut®, 25 units of MMLV reverse transcriptase (Promega, USA), and 2 units of *Taq* DNA polymerase (Life Technologies, USA). RT-PCR products were analyzed in a 1% agarose gel stained with ethidium bromide (Lee et al., 2005). For SSCP analysis, 1 μL of the RT-PCR product was mixed with 9 μL of denaturing solution [95% formamide, 20 mM EDTA (pH 8.0), and 0.5 mg mL^{-1} bromophenol blue], heated at 99°C for 10 min, and immediately chilled on ice. DNA strands were separated by electrophoresis in a non-denaturing 7.5% polyacrylamide gel, using 0.5X TBE (89 mM Tris-borate and 2 mM EDTA; pH 8.0) as the electrophoresis buffer and running at 200 V for 18 h at 4°C (Rubio et al., 1996). Gels were

Table 1. Severity of CTV symptoms in yuzu trees, compared among sampling sites[a].

Site	No. of CTV-infected yuzu	Disease parameter		
		Age of trees (year)	Vigor[b]	Stem-pitting[c]
Namhae A	4	10 ~35	W ~S	– ~+++
Namhae B	4	20 ~50	W ~S	– ~+++
Boseong A	5	15 ~20	M	+/-
Boseong B	1	20	W	+++
Geoje A	1	15	S	–
Geoje B	2	30	S	–
Geoje C	1	5	M	+/–
Geoje D	5	15	S	–
Geoje E	1	15	S	+/–
Geoje F	3	15	M	–
Wan A	4	15	M	+/–
Wan B	9	15	M	+/–
Goheung A	2	15	M	+/–
Goheung B	3	15	M	+/–
Goheung C	3	15	M	+/–
Goheung D	2	15	W	++++
Goheung E	1	15	S	+/–
Jeju A	9	20	W ~S	– ~+++
Jeju B	5	30	M	+/–
Total	65	–	–	–

[a]Samples were collected from geographically separate island, peninsular, and inland areas. [b]Vigor: s, Strong; m, moderate; W, weak. [c]Stem-pitting: Symptom intensity was scored from - to ++++.



Primer	Nucleotide sequence	Sizes (nt)
20A (forward)	5'- CGAGCTTACTTTAGTGTTA-3'	520
20B (reverse)	5'- TAATGTCAAACTGACCGC-3'	

Figure 2. Organization of CTV genome and design of specific primers for RT-PCR amplification of CTV in *p20* gene. CP, capsid protein. See Karasev et al. (1995) for details.

Table 2. Severity of CTV symptoms in yuzu trees, compared among haplotypes.

Haplotype	Disease parameter			Sites[c]
	Age of trees (year)	Vigor[a]	Stem-pitting[b]	
a	10~15	W ~M	++	Namhae
b	15~20	M~S	+/–	Namhae, Boseong
c	15~35	W ~M	++	Namhae, Boseong
d	20~40	W ~M	+++	Namhae
e	15~30	M ~S	–	Geoje
f	15~30	M ~S	–	Geoje, Goheung
g	5~15	M	+/–	Geoje, Goheung
h	15~20	S	–	Geoje, Jeju
i	15~20	M	+/–	Wan, Jeju
j	15~20	M	+/–	Wan
k	15~20	M	+/–	Wan
l	15~20	M	+/–	Wan
m	15~20	W ~M	+	Wan, Goheung, Jeju
n	15~20	M	+/–	Goheung, Jeju
o	15~20	M	+/–	Goheung
p	15~20	W	+++	Goheung, Jeju

[a]Vigor: S, strong; M, moderate; W, weak. [b]Stem-pitting: Symptom intensity was scored from - to ++++. [c]Samples were collected from geographically separate island, peninsula, and inland areas.

stained with silver nitrate (Beidler et al., 1982).

Phylogenetic Analysis

The sequence data were analyzed by the neighbor-joining method (Saito and Nei, 1987). PCR products from our 65 samples were cloned and 3 to 5 colonies per sample were chosen for sequence analysis. A comparative analysis, including multiple alignments of the *p20* gene sequences for CTV, was done according to the Clustal V method (Higgins and Sharp, 1988). Databases were searched using the BLAST program. Numbers at the nodes indicated the levels of bootstrap support based on a neighbor-joining analysis of 1000 re-sampled data sets.

RESULTS AND DISCUSSION

SSCP Analysis

RT-PCR and SSCP analysis were performed for the *p20* gene of the CTV isolates (Fig. 3). Variation analysis of the CTV population was preliminarily assessed for 65 infected yuzu trees in six regions of Korea (Table 1). By comparing their manifested symptoms and the viral RNA diversities revealed by SSCP analysis, these 65 trees could be subclassified into 16 different haplotypes for *p20*, designated as "a" through "p" (Table 2). Profiles for "a", "c", "d", "m" and "p" demonstrated severe symptoms, such as weak vigor and stem-pitting. Many of their SSCP patterns showed more than two bands (Fig. 3), indicating that various predominant haplotypes were present in the geographically separate areas. This SSCP analysis was originally developed to analyze viral genome diversity because the procedure combines simplicity, low cost, and the potential for use with many samples (Sambade et al., 2002). Additionally, it has enough sensitivity to detect a single nucleotide difference in relatively large DNA fragments (up to 700 nucleotides) (Rubio et al., 1996); these differences among profiles are consistent if the experimental conditions are carefully maintained.

Guerri et al. (1991) have compared the dsRNA patterns from 125 randomly selected CTV-infected citrus trees, and have found up to 16 different profiles, which is indirect evidence of genetic variation based on the population of D-RNAs. SSCP analysis by Orita et al. (1989) of complementary DNA from two CTV genes has also been used to characterize the population of sequence variants for several CTV isolates (Ayllón et al., 1999). This method can also detect

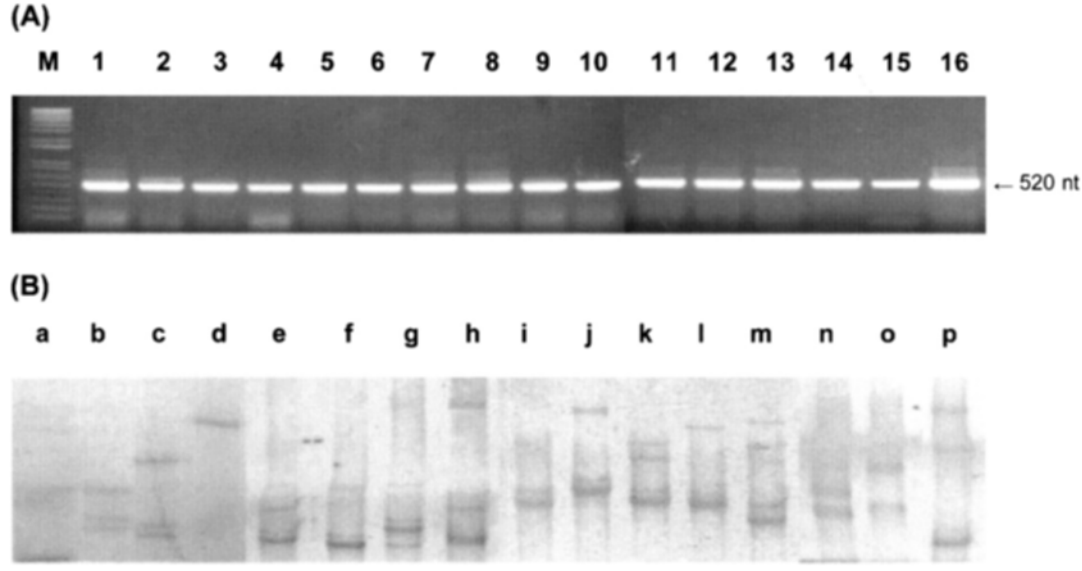


Figure 3. Typical SSCP pattern for *p20* gene from citrus tristeza virus. **(A)** PCR-amplified product from yuzu at different sites, expressing 16 haplotypes by SSCP analysis. **(B)** SSCP analysis of cDNA obtained by RT-PCR using specific primers: M, 1-kb marker; 1, haplotype a; 2, haplotype b; 3, haplotype c; 4, haplotype d; 5, haplotype e; 6, haplotype f; 7, haplotype g; 8, haplotype h; 9, haplotype i; 10, haplotype j; 11, haplotype k; 12, haplotype l; 13, haplotype m; 14, haplotype n; 15, haplotype o; 16, haplotype p. SSCP analysis was performed by electrophoresis under non-denaturing conditions in 7.5% acrylamide gels, at 4°C and 200 V for 18 h.

variations within these populations after aphid transmission (D'urso et al., 2000) or host passage (Rubio et al., 2000). Kong et al. (2000) have characterized the population structure and genetic diversity of five California CTV isolates. Finally, Rubio et al. (2001) have reported no correlation between geographical origin and nucleotide distance in their comparison of two groups of CTV isolates from Spain and California (USA).

Sequences and Comparisons among 16 Haplotypes

The nucleotide sequences for our 16 CTV haplotypes ("a" through "p") were aligned with CTV strains that were previously reported and analyzed: T309P1 (AF356319), T398P2 (AF356327), T340P1 (356322), T311P1 (356320), T385 (Y18420), T300P1 (AF-356317), 190 (AF203081), T315P1 (AF356321), T362P2 (AF356324), T346P1 (AF356322), T373P1 (AF356325), T405P2 (AF356329), T398P1 (AF-356326), 405P1 (AF356328), DS2-CT (AY263361), Seedling Yellows (SY, AB046398) T36 (strain from Florida), EGYPT (AY340974), T308P1 (AF356318), 386P1 (AG356314), 65P1 (AF203073) and 519P1 (AF-356316). Sequence identities ranged from 87.6 to 99.6% (Table 3), being the greatest (97.1-99.6%) for haplotypes "a", "c", and "m", which matched severe CTV strains DS2-CT, SY, and T36, respectively. Among our 16 haplotypes, identities ranged from 88.6 to 99% (Table 4).

The number of different *p20* haplotypes at each sampling site varied from two to six (Table 5), with the highest diversity found on the Goheung peninsula and the lowest in the Boseong inland area. In Korea, yuzu was first cultivated on the Goheung peninsula in 1962. Currently, yuzu production on the Goheung peninsula and Wan Island accounts for over 35% of the Korean total (MAF, 2002). The main reason for such a high diversity of haplotypes at Goheung could be the many opportunities for changes in the viral population within individual trees, due to repeated aphid inoculation or because of cultural practices, such as top-working to new varieties (D'urso et al., 2003).

The genetic diversity observed in our study suggests that CTV isolates among geographically separate populations drift due to the intense traffic of CTV-infected propagative citrus materials between distant regions (Roistacher and Moreno, 1991). Mixed infections should be expected in woody plants that may live in fields for more than 15 years and be exposed to multiple aphid inoculations (Rubio et al., 2001). The duration of the infection, successive inoculations by

Table 3. Sequence comparisons for 16 haplotypes among different strains of citrus tristeza virus.

	o	n	e	i	b	h	P	g	d	A	m	c	j	l	k	f
T309	98.2	98.2	98.0	92.9	92.2	92.0	91.8	92.4	92.4	91.2	92.4	92.2	89.8	88.4	88.8	88.8
T398P2	98.0	98.0	97.8	92.7	92.0	91.8	91.6	92.2	92.2	91.0	92.2	92.0	89.6	88.2	88.6	88.6
T340	98.2	98.2	98.0	92.9	92.2	92.0	91.8	92.4	92.4	91.2	92.4	92.2	89.8	88.4	88.8	88.8
T311	98.2	98.2	98.2	92.9	92.2	92.0	91.8	92.4	92.4	91.2	92.4	92.2	89.8	88.4	88.8	88.8
T385	98.2	98.2	98.0	92.9	92.2	92.0	91.8	92.4	92.4	91.2	92.4	92.2	89.8	88.4	88.8	88.8
T300	98.2	98.2	98.0	92.9	92.2	92.0	91.8	92.4	92.4	91.2	92.4	92.2	89.8	88.4	88.8	88.8
190	98.0	98.0	97.8	92.9	92.2	92.0	91.8	92.4	92.4	91.0	92.2	92.0	89.4	88.0	88.6	88.4
T315	98.4	98.4	98.4	93.1	92.4	92.2	92.0	92.7	92.7	91.4	92.7	92.4	90.0	88.6	89.0	89.0
T362P1	98.4	98.4	98.4	93.1	92.4	92.2	92.0	92.7	92.7	91.4	92.7	92.4	90.0	88.6	89.0	89.0
T346	92.4	93.3	93.3	98.8	98.2	98.4	97.8	99.0	98.8	92.2	92.0	92.2	91.8	91.0	91.0	91.8
T373	92.2	93.1	93.1	98.6	98.0	98.2	97.6	98.8	98.8	92.0	91.8	92.0	91.6	90.8	90.8	91.6
T405P2	92.7	93.3	93.3	98.8	98.2	98.4	97.8	99.0	98.8	92.2	92.0	92.2	91.8	91.0	91.0	91.8
T398P1	92.7	93.3	93.3	98.8	98.2	98.4	97.8	99.0	98.8	92.2	92.0	92.2	91.8	91.0	91.0	91.8
T405P1	92.2	92.9	92.9	98.2	98.8	98.0	97.3	98.6	98.4	91.8	91.6	91.8	91.2	90.4	90.4	91.2
DS2-CT	93.1	93.1	93.1	93.1	92.4	92.4	93.3	92.4	92.2	97.6	99.6	99.0	93.1	91.0	91.2	92.7
SY	92.9	93.7	93.3	92.9	92.8	92.0	92.7	92.7	92.4	97.1	99.0	99.2	92.7	90.6	90.8	92.2
T36	92.7	93.5	93.1	93.1	92.0	92.2	92.9	92.9	92.7	97.3	99.2	99.4	93.1	91.0	91.2	92.7
EGYPT	88.2	88.6	88.2	90.8	89.4	90.2	90.4	90.2	98.8	90.2	91.4	91.2	94.3	93.1	92.9	94.1
T308	87.6	88.2	87.8	90.6	98.2	90.0	90.2	90.0	98.6	90.0	91.0	90.8	93.9	92.7	92.4	93.7
386P1	90.0	89.4	89.8	92.4	91.0	91.8	92.4	92.4	92.0	90.4	91.4	91.2	98.0	97.8	97.6	98.4
65P1	89.2	88.6	89.0	92.2	90.8	91.6	92.2	92.2	91.8	90.4	91.4	91.2	97.8	97.1	96.9	98.2
519P1	89.6	89.0	89.4	92.7	91.2	92.0	92.0	92.7	92.2	90.6	91.6	91.4	98.0	97.3	97.1	98.4

Table 4. Nucleotide diversity of SSCP haplotypes within a citrus tristeza virus population.

	o	n	e	i	b	h	p	g	d	A	m	c	j	l	k	f
o		98.0	98.0	93.5	92.9	92.7	92.9	92.7	92.7	91.4	92.7	92.4	90.2	89.2	89.6	89.6
n			98.8	93.7	93.1	92.9	92.7	93.3	93.3	92.4	93.1	93.3	90.0	88.6	89.0	98.0
e				93.7	93.1	92.9	92.7	93.3	93.3	92.4	93.1	92.9	90.4	89.4	89.8	89.4
i					98.6	98.8	98.2	99.0	98.8	92.9	92.7	92.9	92.7	91.8	91.8	92.7
b						99.0	98.4	98.4	98.2	92.2	92.0	91.8	91.2	90.4	90.4	91.2
h							98.6	98.6	98.4	92.4	92.2	92.0	92.0	91.2	91.2	92.0
p								98.0	97.8	92.7	92.9	92.7	92.7	91.8	91.8	92.7
g									99.0	92.7	92.4	92.7	92.7	91.8	91.8	92.7
d										92.4	92.2	92.4	92.2	91.0	91.0	92.2
a											97.6	97.1	92.0	90.0	90.2	91.4
m												99.0	93.1	91.0	91.2	92.7
c													92.9	90.8	91.0	92.4
j														98.0	97.8	98.4
l															99.0	98.0
k																97.8
f																

aphids carrying different CTV variants, and, perhaps, time of year (D'urso et al., 2003) also might affect their distribution within infected populations and, therefore, the haplotypes observed.

Table 5. Distribution of SSCP haplotypes *p20* gene among and within sites. Yuzu trees were sampled from geographically separate island, peninsula and inland areas.

Site	Haplotype (*p20* gene)																
	Total	a	b	c	d	e	f	g	h	I	j	k	l	m	n	o	p
Namhae	8	2	1	2	3	-	-	-	-	-	-	-	-	-	-	-	-
Boseong	6	-	4	2	-	-	-	-	-	-	-	-	-	-	-	-	-
Geoje	13	-	-	-	-	3	2	3	5	-	-	-	-	-	-	-	-
Wan	13	-	-	-	-	-	-	-	-	3	1	5	2	2	-	-	-
Goheung	11	-	-	-	-	-	1	2	-	-	-	-	-	1	1	5	1
Jeju	14	-	-	-	-	-	-	-	4	3	-	-	-	3	1	-	3
Total	65	2	5	4	3	3	3	5	9	6	1	5	2	6	2	5	4

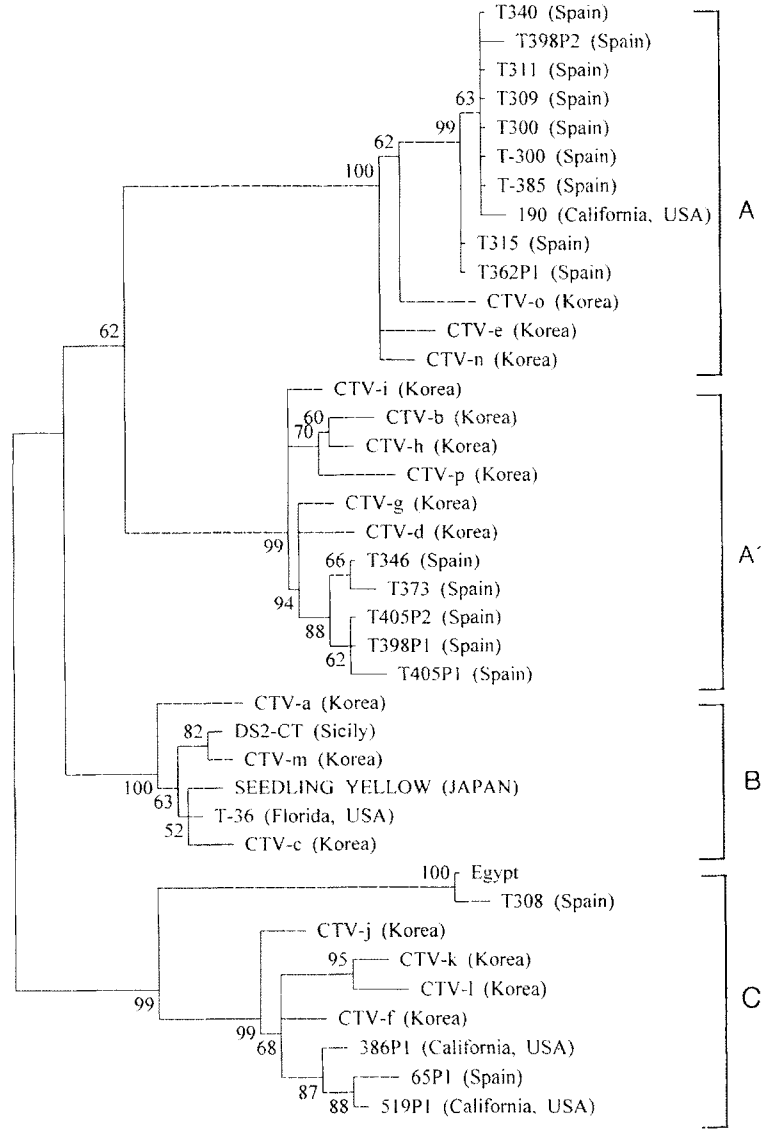


Figure 4. Phylogenetic tree based on comparison of *p20* gene sequences for citrus tristeza virus. Branching pattern was generated by neighbor-joining method (Saito and Nei, 1987). Numbers at nodes indicate levels of bootstrap support based on neighbor-joining analysis of 1000 re-sampled data sets. Numbers on branches are percentages of bootstrap analyses supporting the grouping of each branch.

Phylogenetic Analysis

To determine the relationship between our 16 haplotypes and other known CTV strains, we performed phylogenetic analyses with the PAUP 4.0 program. Phylogenetic trees derived from these *p20* haplotypes were compared with the previously described strains (Fig. 4). They were then divided into three large groups (A, B, and C) and one subgroup (A') in terms of their CTV *p20* gene sequences. Group A comprised the haplotypes "o", "e", and "n"; Group B, "a", "c", and "m"; Group C, "j", "f", "k", and "l"; and Group A', "b", "h", "p", "i", "d", "g" (Fig. 4). Phylogenetic analysis of nucleotides indicated that the Group A isolates were closely related to mild CTV strains T340, T398P2, T311, T309, T300, T385, 190, T315, and T362P1; Group A', to strains T346, T373, T405P2, T398P1, and T405P1; and Group B, to the most virulent strains SY, T-36, and DS2CT. Group C, related to strains 386P1, 65P1, and 519P1, was relatively remote from the A, A', and B groups. Based on this analysis, we determined that our haplotypes in Groups A and A' were genetically similar to those of CTV isolates from Spain, whereas those of Group B were most similar to isolates in Florida (USA) and Japan. Haplotypes from Group C included strains found in Egypt, Spain, and California (USA) (Fig. 4).

Because of their error-prone RNA replications, large populations, and short replication times, RNA viruses have great potential for genetic variation (Ayllón et al., 1999). We studied the variation in a natural CTV population in Korea by comparing the SSCP pattern of 3′ UTRs in randomly selected, geographically separate yuzu trees. Our SSCP profiles showed that the pathologically mild isolates (haplotypes "o", "n", and "e") usually yielded two DNA bands, whereas the most virulent isolates ("a", "c", and "m") had more than two.

Comparison of these haplotypes with the DNA profiles of other isolates indicated that some seem to have arisen as the result of a mixed infection of two divergent isolates (Sambade et al., 2002). Plants with critical stem-pitting corresponded to those isolates with typical SSCP profiles for severe CTV symptoms (Fig. 4). This indicates that the primers designed for SSCP analysis could be used to distinguish mild strains from severe strains (Sambade et al., 2002). Therefore, SSCP analysis may be a very helpful tool for early selection of mild cross-protecting isolates, thereby restricting field trials to only a select group of mild isolates and providing some means for impeding the multiplication of more severe isolates (Gago-Zachert et al., 1999; Sambade et al., 2002).

Tristeza is a complex disease, in part, because of the great potential for CTV isolate-vector-host interactions. Numerous isolates with distinct biological and molecular characteristics have been reported from various regions of the world. Changes in such characteristics can alter host traits, symptom severity, or/and aphid transmissibility.

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