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Research Papers

Characterization of viruses associated with garlic plants propagated from different reproductive tissues from Italy and other geographic regions

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Summary. Garlic is an important crop cultivated worldwide and several different viruses have been associated with propagative material. Garlic is propagated from bulbs and/or from vegetative topsets of the inflorescences known as bulbils. The effects of the geographic origin and the type of the propagative material on the phylogenetic relationships and genetic variability of the coat protein genes of four allium viruses are presented here. *Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), *Garlic virus X* (GVX), and *Garlic common latent virus* (GCLV) were detected in single and mixed infections in plants grown either from bulbils and/or bulbs originating from Italy, China, Argentina, and the U.S.A. OYDV and LYSV fell into five and three well supported clades respectively whereas isolates of GVX and GCLV all clustered into one well-supported clade each. Some of the OYDV and LYSV clades presented evidence of host tissue selection while some phylogenetic structuring based on the geographic origin or host was also observed for some virus clades. Unique haplotypes and novel coat protein amino acid sequence patterns were identified for all viruses. An OYDV coat protein amino acid signature unique to *Chenopodium quinoa*, an uncommon host of the virus, was of particular interest. The type of propagative material affected the population dynamics of all of the viruses. The virus populations in plants propagated from bulbs were more diverse than in plants propagated from bulbils.

Key words: bulbils, bulbs, coat protein, phylogenetic analysis, population diversity.

Introduction

Garlic (*Allium sativum* L.) is an ancient crop that was originally domesticated in Central Asia. Garlic is one of the most important allium crops cultivated throughout the world and global production has been consistently increasing in recent decades. For example, 2.98 million tons were produced in 1972 which increased to 15.8 million tons by 2007 (Etoh and Simon, 2002; FAO, 2009). However, significant reductions in quality and yield have been documented for garlic production due to the many viral diseases associated with this crop (Walkey, 1989; Conci *et al.*, 2003; Cafrune *et al.*, 2006).

Commercial garlic is vegetatively propagated from cloves of bulbs and/or from vegetative topsets of the inflorescences (bulbils). In many cases the plant material used for garlic propagation is not tested for viruses, which results in garlic plants in crops that are often infected with two or more viruses (Pooler and Simon, 1994; Verbeek *et al.*,1995; Takaichi *et al.*,1998; Shiboleth *et al.*,2001). The most common viruses that have been associated with garlic worldwide are members of the *Potyviridae* and *Flexiviridae* families. The potyviruses *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) have been the most common and important

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allium viruses around the world (Takaichi *et al.*,1998; Dovas *et al.*, 2001). Within the *Flexiviridae* family, the allexivirus *Garlic virus X* (GVX) and the carlavirus *Garlic common latent virus* (GCLV) have been commonly detected infecting garlic plants (Takaichi *et al.*, 1998; Tsuneyoshi *et al.*, 1998a; Dovas *et al.*, 2001; Pappu *et al.*, 2008).

Reports of effects of geographic origin and host type on the phylogenetic and/or genetic relationships of the allium viruses have been variable. Reports for OYDV and LYSV have suggested both no and partial correlation between geographic localization and host type of germplasm (Tsuneyoshi *et al.*, 1998b; Chen *et al.*, 2002; Takaki *et al.*, 2005). In a study focused on the classification of allexiviruses in China, Chen *et al.* (2004) concluded that isolates from one region occasionally had similar sequences but there was a weak relationship between sequence type and geographical origin. In the case of carlaviruses, two studies suggested that the probability of geographic variants was greater than for host- or plant-induced sequence variations (Tsuneyoshi *et al.*, 1998a; Chen *et al.*, 2002).

In addition to physical or geographical barriers that separate virus and host populations, several factors can affect the genetic composition of a virus population at the intrahost level. Susceptibility of different tissues, systemic *vs.* localized infections, and invasion of young organs can all affect the genetic composition of a virus population (Magome *et al.*, 1999; French and Stenger, 2003; Sacristan *et al.*, 2003; Li and Roossinck, 2004; Jridi *et al.*, 2006). Previous reports on the distribution of allium viruses within a individual garlic plants have indicated uneven dispersal in different organs, suggesting that bulbils may be the best organ for the propagation of virus-free garlic plants (Ebi *et al.*, 2000; Ramírez-Malagón *et al.*, 2006). However, the effects of these factors on the phylogenetic and/or genetic variability of specific garlic viruses has not been examined.

In the present study, garlic plants grown from different types of propagative material (i.e. bulbils or bulbs) collected from different garlic production regions were tested for the presence of OYDV, LYSV, GCLV, and GVX using different diagnostic protocols. The coat protein gene of the identified viruses was characterized to determine genetic variability and phylogenetic relationships among the detected virus species as affected by geographic origin and/or type of propagative material.

Materials and methods

Garlic material

Bulbils from inflorescences of two Italian garlic varieties, 'Bianco Piacentino' and 'Rosso di Sulmona' (100 bulbils each) were imported under a USDA-APHIS-PPQ permit from the germplasm collection of the Department of Plant Production at the University of Tuscia, Viterbo. Additionally, commercially available bulbs from China, Argentina, and three California (CA) cultivars (CA-organic, CA-non-organic, and 'Elephant') were also used in this study with a total of 12 bulbs from each variety tested for viruses.

The commercially available garlic bulbs were germinated during the spring of 2008 in 16.5×16.5×17.5 cm plastic pots in a potting soil of 1:1 mixture of commercial peat-and soil and a sand based UCR mix. The potted plants were maintained in a temperature-controlled greenhouse at 25–28°C with no supplemental light, and were fertilized once per week with a nutrient solution made with Peters Excel 21-5-20 water soluble fertilizer.

Since the disease status of the imported garlic bulbils was unknown, as a precaution they were not grown in the greenhouse. The bulbils were surface sterilized in a sodium hypochlorite solution (10%, v/v) for 5 min, washed with ethanol (95%), and rinsed with sterile distilled water. They were then germinated *in vitro* on water agar substrate (10%) in a temperature-controlled incubator at 25°C using a 16/8 h (day/night) photoperiod of 1000 lux. After 3–4 d, seedlings were transplanted in 11×11×12 cm plastic pots in the potting mix described above, and grown in the laboratory under natural light at 25°C.

Plant indicators

Biological activity and host range of the viruses was assessed via mechanical inoculation of 2–3 plant indicators each of tobacco (*Nicotiana tabacum* 'Xanthi' and 'Turkish'), tomato (*Lycopersicon esculentum*), and chenopodium (*Chenopodium quinoa*). Indicator plants were grown in a temperature-controlled greenhouse in 11×11×12 cm plastic pots using the potting mix as described above. For mechanical inoculation of the indicator plants, a total of 1.0–1.5 g of fresh garlic leaf tissue was collected from individual plants of each variety and were combined together and macerated in a mortar in 500 μL of sodium phosphate buffer (0.1

M) at pH 8.0. The plants were inoculated by gently rubbing the macerated solution onto carborundum-dusted leaves. Indicator plants were monitored daily for symptom development for a period of 16 weeks.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Enzyme Linked Immunosorbent Assay (ELISA)

Approximately 500 mg of combined leaf samples from individual plants of the different garlic varieties were homogenized in a mortar and pestle using liquid nitrogen in 1 mL Buffard buffer (0.1 M NaCl, 2% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 9.0). The supernatant was collected after 5 min centrifugation at 16,000 ×*g* (Zimmermann *et al.,* 1998). Total RNA was extracted from the supernatant using a phenol-chloroform protocol (Sambrook and Russell, 2001). The nucleic acids were resuspended in diethylpyrocarbonate (DEPC)-treated sterile distilled H_2O (dH_2O), and RNA yield was estimated by spectrophotometry at A_{260} nm.

Primers for detection and subsequent characterization of the coat protein genes of OYDV, LYSV, GCLV, and GVX were designed with the program Gene Runner v3.05 using conserved regions of sequences available in GenBank (Table 1). Representative full-length coat protein gene sequences of the viruses detected in the present study were deposited in the GenBank (OYDV, GQ475358-60; LYSV, GQ475411-18; GCLV, GQ475419-23; and GVX, GQ475424-26).

Reverse transcription (RT) was carried out using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) in 20 µL reaction volumes in two stages as directed by the manufacturer. The initial RT reaction consisted of 3 µL of RNA extract (300–500 ng per reaction) or water for control, 1 µL of 10 mM dNTPs (Invitrogen), 2 µL of gene specific reverse complementary primer (5 nmole), and 7.8 µL of DEPC dH_2O heated to 65°C for 5 min and then incubated on ice for at 1–2 min. In the second stage a master mix consisting of 4 µL of 5× First-Strand Buffer, 1 µL of (40 U μL^{-1}) RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 1 µL (0.1 M) DTT, and 0.2 µL of SuperScript III RT (200 U μL^{-1}) (Invitrogen), was added to the initial mixture and incubated at 45°C for 60 min.

The RT produced cDNA was amplified by PCR using the primers listed in Table 1 in a 20 µL volume containing 2 µL cDNA template, 10 µL Go Taq Hot Start Master Mix (Promega, Madison, WI), 2 × 0.8 µL of each primer (5 nmole), and 6.4 µL of DEPC dH_2O. PCR cycling parameters included one cycle of 95°C for 2 min, followed by 34 cycles of 95°C for 10 s, 58°C for OYDV, 63°C for LYSV, and 57°C for GVX

Table 1. Newly designed primers based on unique sequences of the coat protein gene of different allium viruses used in the reverse transcription and polymerase chain reactions.

Virus[a]	GenBank[b]	Nucleotide	Designation	Orientation[c]	Sequence (5′ to 3′)	Product Size (bp)[d]
OYDV	NC_005029	9552-9573	1-OYDV	F	GCG GGG GAA GGA GAA GAT GCA G	730
		10281-10261	2-OYDV	R	CCG CAG CTG TGT GTC TTT CCG	
LYSV	AF071525	31-54	1-LYSV	F	ACA AGT AAG AAA CAG AAG GAC AGC	409
		440-417	2-LYSV	R	GAG GTT CCA TTT TCA ATG CAC CAC	
GVX	NC_001800	6875-6894	1-GVX	F	ATG GGC GAT CGG AAC CAA GG	732
		7606-7586	2-GVX	R	TCA GAA TGT GAG CAT AAG GGG	
GCLV	AF228416	1-22	1-GCLV	F	ATG TCA GTG AGT GAA ACA GAG G	960
		960-940	2-GCLV	R	CTA GTC TGC ATT GTT GGA TCC	

[a]OYDV, *Onion yellow dwarf virus*; LYSV, *Leek yellow stripe virus*; GVX, *Garlic virus* X, and GCLV, *Garlic common latent virus*.
[b]Sequence accession numbers.
[c]F, Forward; R, Reverse.

and GCLV for 10 s, and 72°C for 1 min, and a final extension cycle at 72°C for 5 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gels stained with ethidium bromide and visualized under UV light.

PCR products were excised from 0.5% TBE agarose gel and purified by the PureLink™ Quick gel extraction kit (Invitrogen). Purified amplicons were cloned into pGEM-T Easy vector (Promega). Nucleotide sequences of the cloned PCR products were determined using forward and reverse primers by the Core Instrumentation Facility of the Genomic Institute at the University of California, Riverside.

Commercially available DAS-ELISA diagnostic kits for OYDV, LYSV, and GCLV produced by BIOREBA AG (Reinach, Switzerland) were used following the manufacturer's instructions. ELISA plates were read using a Spectra Max 340 PC spectrophotometer. Positive reactions were accepted at three times the OD value of the negative controls.

Phylogenetic and population genetics analyses

The sequences were edited using BioEdit (version 5.0.9) and aligned using Clustal X (version 1.81) (Thompson *et al.*, 1997; Hall, 1999). BLAST searches were performed for all viruses identified and representative sequences were downloaded from the NCBI website so that comparisons could be made between the viruses found in this study with similar viruses found in garlic from other geographic regions. The alignments were manually edited in MacClade 4.03 (Maddison and Maddison, 2001) with ambiguous sites removed, and were subjected to neighbour joining (NJ) analyses and maximum parsimony (MP) analysis using the heuristic search procedure with 100 random-addition-sequence replicates and tree-bisection-reconnection branch swapping using PAUP* (version 4.0 beta 10) (Swofford, 2002). Gaps were treated as missing data. Confidence in tree topology was examined using bootstrap with 1,000 replicates for NJ and 1,000 replicates under the fast step-wise addition option. Protein amino acid sequence similarities were analyzed using the program GeneDoc v2.6.002 (Nicholas and Nicholas, 1997).

The population genetics parameters, number of polymorphic sites (S), nucleotide diversity per site (π), number of unique haplotypes, and haplotype diversity (H) were used to describe the genetic variation of virus populations from bulbils and bulbs. All parameters were calculated with equations from Nei (1987) using the DNA Sequence Polymorphism (DnaSP) software version 3.50 (Rozas and Rozas, 1999). DnaSP was also used to determine haplotypes in which gaps were treated as missing data. The categorical data for polymorphic sites and unique haplotypes were tested for significant differences using χ^2 and Fisher's Exact Test when counts were fewer than five. Nucleotide and haplotype diversity were analyzed using t-tests. All statistical tests were applied at 0.05 probability level using the SigmaStat 3.00 software (Copyright 1992-2003 SPSS Inc).

Results

Symptom observations and laboratory testing of garlic and plant indicators

The bulbil germination rate of the Italian varieties 'Bianco Piacentino' and 'Rosso di Sulmona' was 9 and 13%, respectively. All garlic plants grown from bulbils developed general yellowing leaf symptoms with deformed growth (Figure 1, a and b). The bulb germination rate ranged from 50% (China) and 66% (Argentina) to 91% (CA-organic) and 100% ('Elephant' and CA-non-organic). All garlic plants grown form bulbs developed leaf symptoms with yellow to light green stripes with streaks and yellow mottling (Figure 1, c–e).

RT-PCR detected different viruses in mixtures and single infections in garlic plants growing from the two types of propagative tissues tested. The viruses OYDV, LYSV, and GVX were detected in the Italian varieties growing from bulbils as well as from the CA-non-organic bulbs. The viruses OYDV, GVX, and GCLV, were detected from plants growing from bulbs of the Chinese and CA-organic varieties. OYDV and LYSV were detected as single infections of Argentinian and 'Elephant' varieties respectively (Table 2). The ELISA tests for LYSV gave erratic results even for the positive controls. Therefore, no reliable serological data could be presented. On the other hand, ELISA results for OYDV and GCLV were reliable and consistent with RT-PCR (Table 2).

The tobacco and tomato indicators inoculated mechanically with crude garlic plant extracts did not express any symptoms and tested negative with RT-PCR and ELISA for all the targeted allium viruses. On the contrary, *C. quinoa* developed local lesions and/or tested positive for at least one allium virus

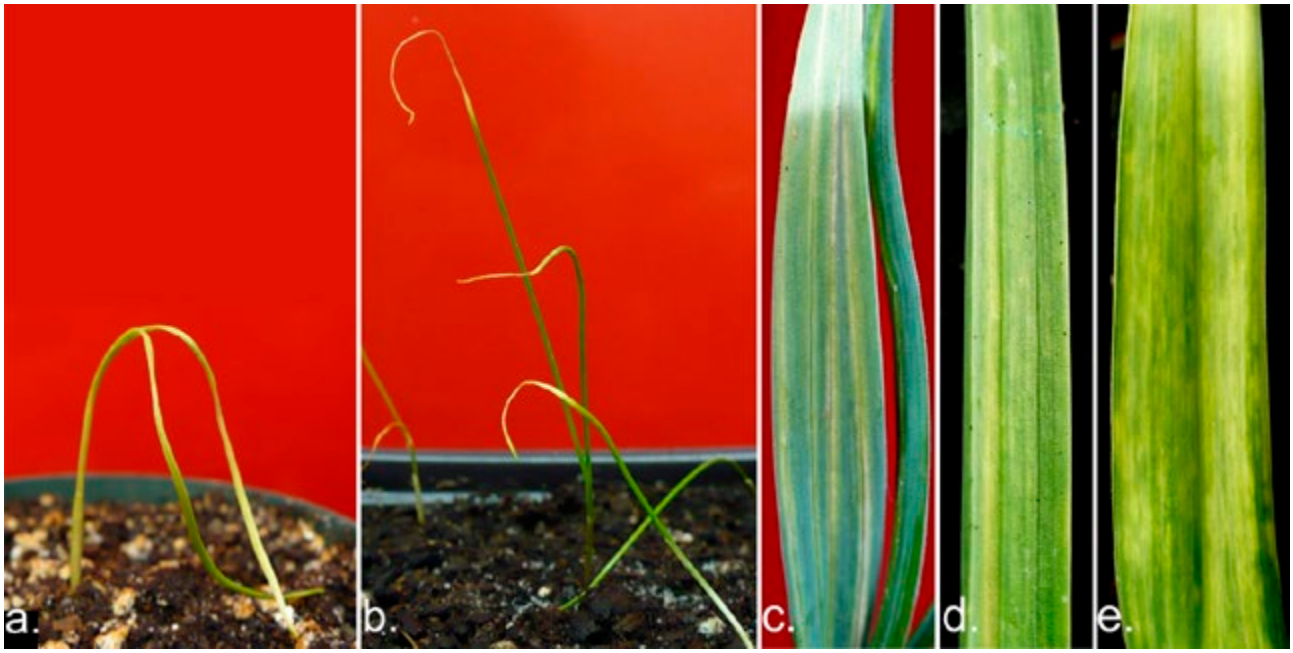

Figure 1. a, 'Bianco Piacentino', and b, 'Rosso di Sulmona' Italian varieties grown from bulbils, expressing yellowing and inhibited growth. c, yellow stripes observed in plants growing from bulbs originating from California (non-organic). d, yellow streaks in plants growing from bulbs originating from China, Argentina, and California (organic). e, yellow mottling observed in plants growing from bulbs originating from California ('Elephant').

for all garlic samples (Table 2). Of particular interest was the detection of OYDV from symptomatic *C. quinoa* inoculated with extracts of the Argentinian garlic variety, since *C. quinoa* has been classified as non-susceptible to OYDV (Table 2) (Büchen-Osmond, 2006).

Molecular characterization of the coat protein gene of the detected viruses

Onion yellow dwarf virus (OYDV)

NJ and MP analyses produced similar trees for all viruses. Therefore, only the MP analysis is presented. OYDV phylogenetic analysis was based on 74 coat protein gene sequences. In total, 730 characters were analyzed which had 48 parsimony-uninformative and 244 parsimony-informative characters. Figure 2 shows one of the 192 parsimony trees found with a length of 904, Consistency Index (CI) = 0.462 and a Retention Index (RI) = 0.873.

Overall, five terminal clades were found that consisted of OYDV viruses that were detected from garlic in this study. Clade A was well supported (97%) and consisted of bulbil isolates from the Italian 'Bianco Piacentino' variety. A total of seven unique haplotypes were found with five consisting of singletons and two that were recovered four times each (total, n = 13). Clade B did not have any bootstrap support but consisted of isolates from California non-organic garlic, and all were found to be unique haplotypes (n = 6). Clade C was well supported (89%) and all viruses were from the Italian 'Rosso di Sulmona' variety. Three unique haplotypes were found; two were singletons and the other was recovered four times from different samples (total, n = 6).

Clade D was moderately supported (64%) and formed a sister group with clade C that was supported by 100% bootstrap support. All viruses in clade D were from the Argentina samples and a total of four unique haplotypes were found. Two were singletons and the other two consisted of two and three recovered isolates (total, n = 7). Clade E was well supported (100%) and consisted of viruses from the Italian 'Rosso di Sulmona' variety (bulbil) and from bulb material obtained from China and California organic samples. A total of nine unique haplotypes were

Table 2. Symptom observations and results of biological and laboratory tests for the detection of allium viruses in garlic plants propagated from different types of reproductive tissues.

Garlic variety	Garlic plants					Indicator plants[a]				
	Symptoms[c]	RT-PCR/ELISA[b]				Symptoms[c]	RT-PCR/ELISA			
		OYDV[d]	LYSV	GVX	GCLV		OYDV	LYSV	GVX	GCLV
Bulbil										
'Bianco Piacentino'	Y & IG	+/+[e]	+/[f]	+/[g]	-/-	-	-/-	+/[e]	+/[f]	-/-
'Rosso di Sulmona'	Y & IG	+/+	+	+	-/-	-	-/-	+	+	-/-
Bulb										
China	YSK	+/+	-	+	+/+	LL	-/-	-	+	+/+
Argentina	YSK	+/+	-	-	-/-	LL	+/+	-	-	-/-
Californi-organic	YSK	+/+	-	+	+/+	-	-/-	-	+	+/-
Californi-non-organic	YSP	+/+	+	+	-/-	-	-/-	+	+	-/-
'Elephant'	YM	-/-	+	-	-/-	LL	-/-	+	-	-/-

[a]Results presented only for *Chenopodium quinoa. Nicotiana tabacum* ("Xanthi" and "Turkish") and *Lycopersicon esculentum* did not express any symptoms and tested negative with RT-PCR and ELISA for all the targeted allium viruses.
[b]RT-PCR, Reverse Transcription Polymerase Chain Reaction/ ELISA: Enzyme Linked Immunosorbent Assay.
[c]Symptoms: Y & IG, yellowing and inhibited growth; YSK, yellow streaks; YSP, yellow stripes; YM, yellow mottling, and LL, local lesions.
[d]OYDV, *Onion yellow dwarf virus*; LYSV, *Leek yellow stripe virus*; GVX, *Garlic virus X*, and GCLV, *Garlic common latent virus*.
[e]Positive (+) or negative (-) virus detection with RT-PCR, on the left, and ELISA, on the right, of the slash, respectively.
[f]LYSV ELISA results were erratic for the positive controls, no valid test.
[g]No ELISA diagnostic kit was commercially available for GVX detection.

found with eight being singletons and the remaining haplotype consisting of 12 recovered viruses found from all thee garlic sources.

The minimum similarity of the coat protein nucleic and amino acid sequences of the detected OYDV in comparison to GenBank deposits was 80 and 86% respectively (OYDV from this study, GenBank: GQ475358-60 and OYDV from The Netherlands, GenBank: AB000836), falling within the demarcation limits for the genus (Fauquet *et al.*, 2005). The amino acid alignment of the coat protein revealed a total of 36 variable sites. The majority (29/36 or 80%) were located in the N-terminal region while the core (around residue 130) and C-terminal regions appeared conserved.

The number of polymorphic sites and the nucleotide diversity of the OYDV bulb isolates were significantly greater ($P \leq 0.001$) than the bulbil isolates. No significant differences were observed for the number of haplotypes or the haplotype diversity of the OYDV bulbil and bulb isolates (Table 3).

OYDV isolates from Chenopodium quinoa

Chenopodium quinoa has been generally reported as a non-susceptible host for OYDV (Bos *et al.*, 1978; Marys *et al.*, 1994; Büchen-Osmond, 2006). However, OYDV was detected from *C. quinoa* inoculated with extracts of the Argentinian variety (Table 2). Full length cDNA clones of the OYDV coat protein gene from the Argentinian garlic (OYDV 5) (GenBank: GQ475372-78 and GQ475386) and the *C. quinoa* (OYDV 12) inoculated with the Argentinian garlic extracts (GenBank: GQ475379-85 and GQ475387-88) were analyzed. The nucleotide sequence homology between the consensus sequence of OYDV recovered from garlic and *C. quinoa* was 81% and a total of 142 mutations occurred throughout the 769–771 nucleotides of the coat protein gene. From the nucleotide mutations monitored, the majority were silent since only 22 major mutated sites were monitored in the amino acid sequence comparison of garlic and *C. quinoa* isolates (Figure 3).

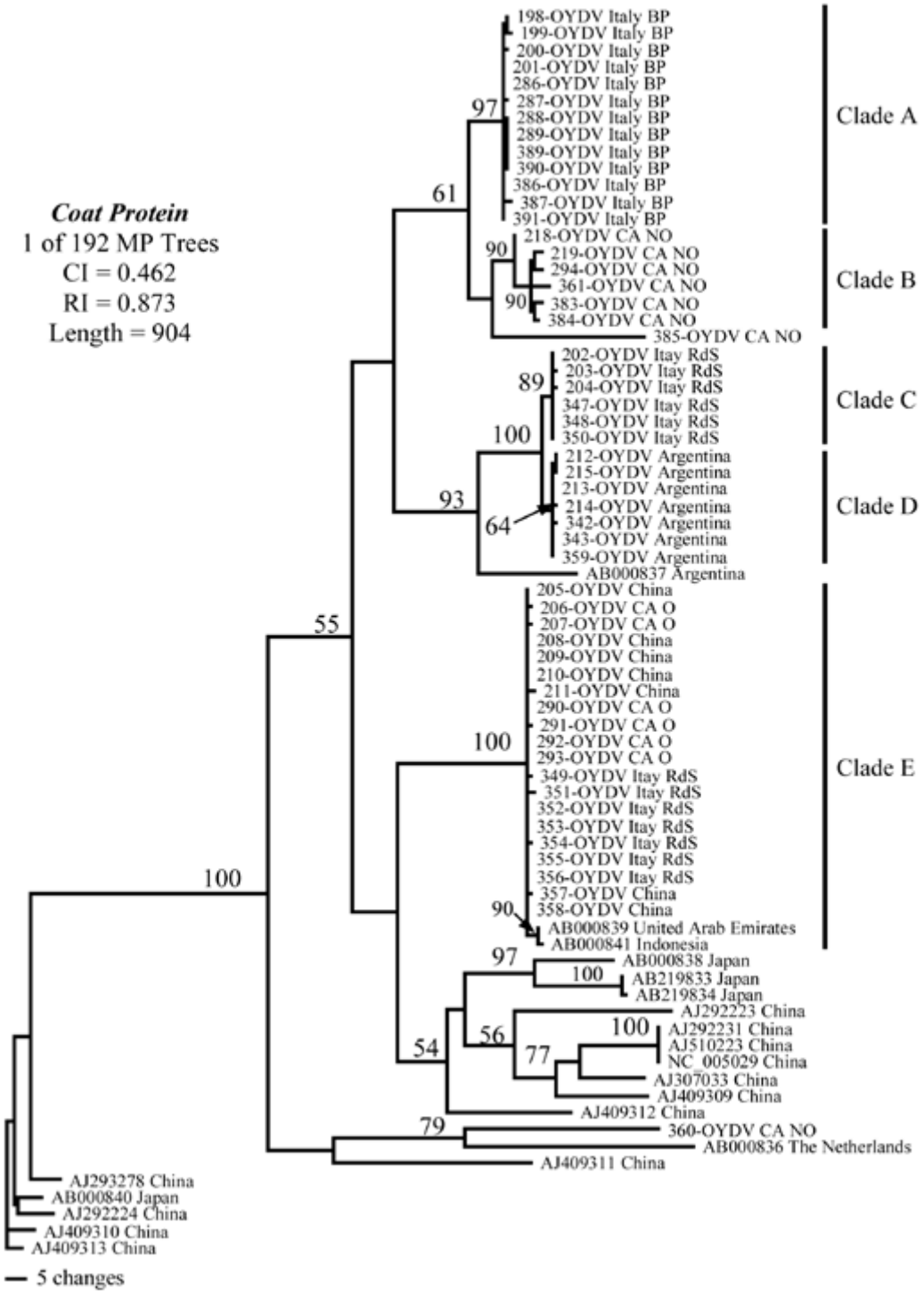

Figure 2. Maximum parsimony analyses of the coat protein gene for *Onion yellow dwarf virus* (OYDV) isolates obtained in this study, as well as sequences from GenBank. Bootstrap support of 50% and above is indicated above nodes based on 1,000 replicates. 198-391: Serial numbering of OYDV cDNA clones, BP: 'Bianco Piacentino', RdS: 'Rosso di Sulmona', CA: California, O: Organic, and NO: Non-organic. Sequences have been deposited in GenBank under accession numbers HQ873761-3806 and GQ475362-69.

Table 3. Estimation of coat protein gene nucleic acid sequence polymorphism and diversity for detected allium viruses in garlic bulbils and bulbs.

Coat protein gene polymorphism and diversity	OYDV[a]		LYSV		GVX	
	Bulbil	Bulbs	Bulbil	Bulbs	Bulbil	Bulbs
Nucleotide sites						
Total	730	776	410	410	732	732
Polymorphic	130	192***	34	47	9	16
Nucleotide diversity	7.5±0.66[b]	9.1±0.88***[c]	4.1±0.54	5.8±0.72***	0.1±0.05	0.2±0.04***
Haplotypes						
Total	26	28	15	11	19	29
Unique	14	18	4	7	7	13
Haplotype diversity	92.6±2.80[b]	91.5±4.40	69.5±8.10	89.1±7.40***	54.4±1.36	70.2±9.60***

[a]OYDV, *Onion yellow dwarf virus*; LYSV, *Leek yellow stripe virus*, and GVX, *Garlic virus X*.
[b]Mean and standard deviation (square root of the variance) of the nucleotide and haplotype diversity multiplied by 100.
[c]***, Significant difference at $P \leq 0.001$. Polymorphic sites and unique haplotypes were tested for significant differences using χ^2 and Fisher's Exact Test when counts were fewer than five. Nucleotide and haplotype diversity were analyzed using t-test.

The majority of the amino acid mutations (66.7% or 14/21) were located in the first 105 residues of the N-terminal region. A single OYDV clone recovered from *C. quinoa* (573-OYDV12, GenBank: GQ475381) had a distinctively different amino acid sequence between residues 3 and 13 in comparison with the other clones recovered from the same host. The residues 130-180 of the amino acid sequence was conserved among the different clones recovered from both hosts. A single OYDV clone recovered from garlic (565-OYDV5, GenBank: GQ475386) acquired the same mutations on 19 out of the 22 mutated sites of the OYDV *C. quinoa* isolates. Of the three remaining mutated sites only one (K_{65}) was common to the garlic isolates (OYDV5) while two (A_{34} and K_{78}) were unique to the 565-OYDV5 (GenBank: GQ475386) clone (Figure 3).

Leek yellow stripe virus (LYSV)

LYSV phylogenetic analysis was based on 44 coat protein sequences. In total, 410 characters were analyzed which had 64 parsimony-uninformative and 142 parsimony-informative characters. Figure 4 shows one of the 40 parsimony trees found with a length of 351, CI = 0.746, and RI = 0.907. Three well supported (94–100%) clades were found that consisted of LYSV isolates identified in this study. Clade A contained isolates from the Italian cultivars Rosso di Sulmona and Bianco Piacentino, which was most closely related to an Israeli isolate (accession No. AF071525) but significantly different from this virus sequence based on 93% bootstrap support. Two haplotypes were found in this clade with one consisting of a singleton (isolate 224 from Italian 'Bianco Piacentino'). The other five sequences belonged to a single haplotype that contained isolates from both Italian bulbil cultivars. Clade B (97%) consisted of isolates from California non-organic bulb samples as well as LYSV isolates from Japan and New Zealand. However, the California isolates clustered by themselves, but this subclade did not have any bootstrap support. Four haplotypes were found in this clade from the isolates identified in this study; three consisting of singletons and the other containing three isolates.

Clade C (100%) contained isolates from the two Italian bulbil cultivars and from California 'Elephant' garlic samples. Three haplotypes were found in this clade with the largest consisting of ten isolates with this same haplotype identified from all three hosts. Two haplotypes were singletons from California 'Elephant' garlic and the other consisted of two isolates (379 and 401) from the Italian cultivar 'Rosso di Sulmona'. All of the isolates were most closely related to two additional viruses, one from The United Arab

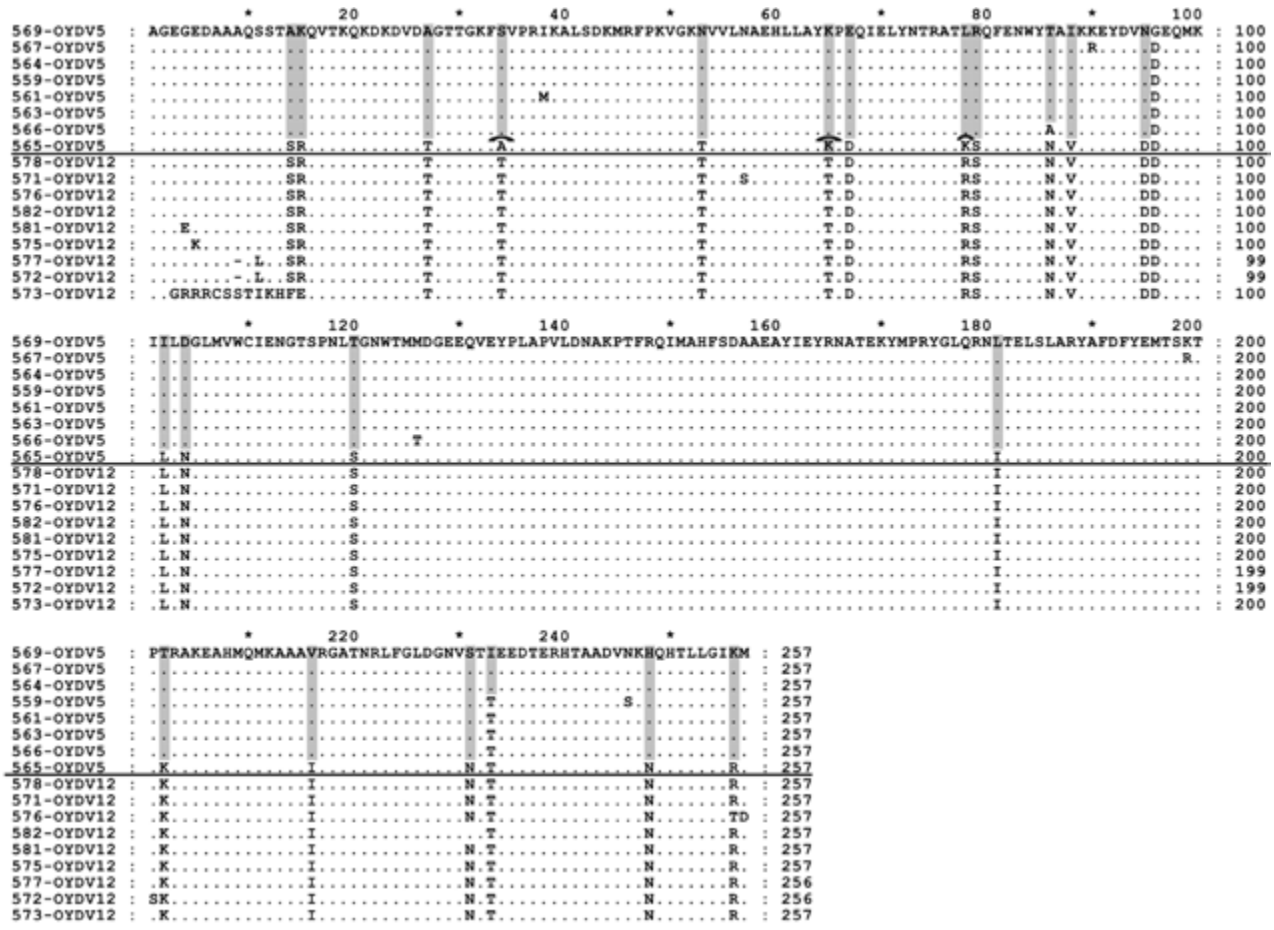

Figure 3. Amino acid sequence of the coat protein of *Onion yellow dwarf virus* (OYDV) isolates from Argentinian garlic (OYDV5), and *Chenopodium quinoa* (OYDV12) mechanically inoculated with crude extract of the Argentinian garlic. Twenty two major mutated sites are highlighted. The three mutations distinguishing the 565-OYDV5 clone from the garlic and *C. quinoa* isolates are capped. 559-582: Serial numbering of OYDV cDNA clones. Sequences have been deposited in GenBank under accession numbers GQ475372-88.

Emirates (GenBank: AB000839) and the other from Indonesia (GenBank: AB000841). However, these two isolates clustered by themselves in a subclade at the 90% bootstrap support level.

The minimum similarity of the coat protein nucleic and amino acid sequences of the detected LYSV in comparison to GenBank deposits was 76 and 79% respectively (LYSV from this study, GenBank: GQ475411-18 and LYSV from Japan, GenBank: AB194638), falling within the demarcation limits for the genus (Fauquet *et al*., 2005). The amino acid alignment revealed a total of 60 variable sites. The majority (45/60 or 75%) were located in the N-terminal region while the core and C-terminal regions appeared conserved.

The nucleotide and haplotype diversity of the LYSV bulb isolates were significantly greater (P<0.001) than the bulbil isolates. The LYSV number of polymorphic sites and haplotypes were not significantly different for both types of propagation material (Table 3).

Garlic virus X (GVX)

GVX phylogenetic analysis was based on 65 coat protein sequences. In total, 444 characters were analyzed which had 38 parsimony-uninformative and 140 parsimony-informative characters. Figure 5 shows one of the three parsimony trees found with a length of 358, CI = 0.603, and RI = 0.777. A single well supported

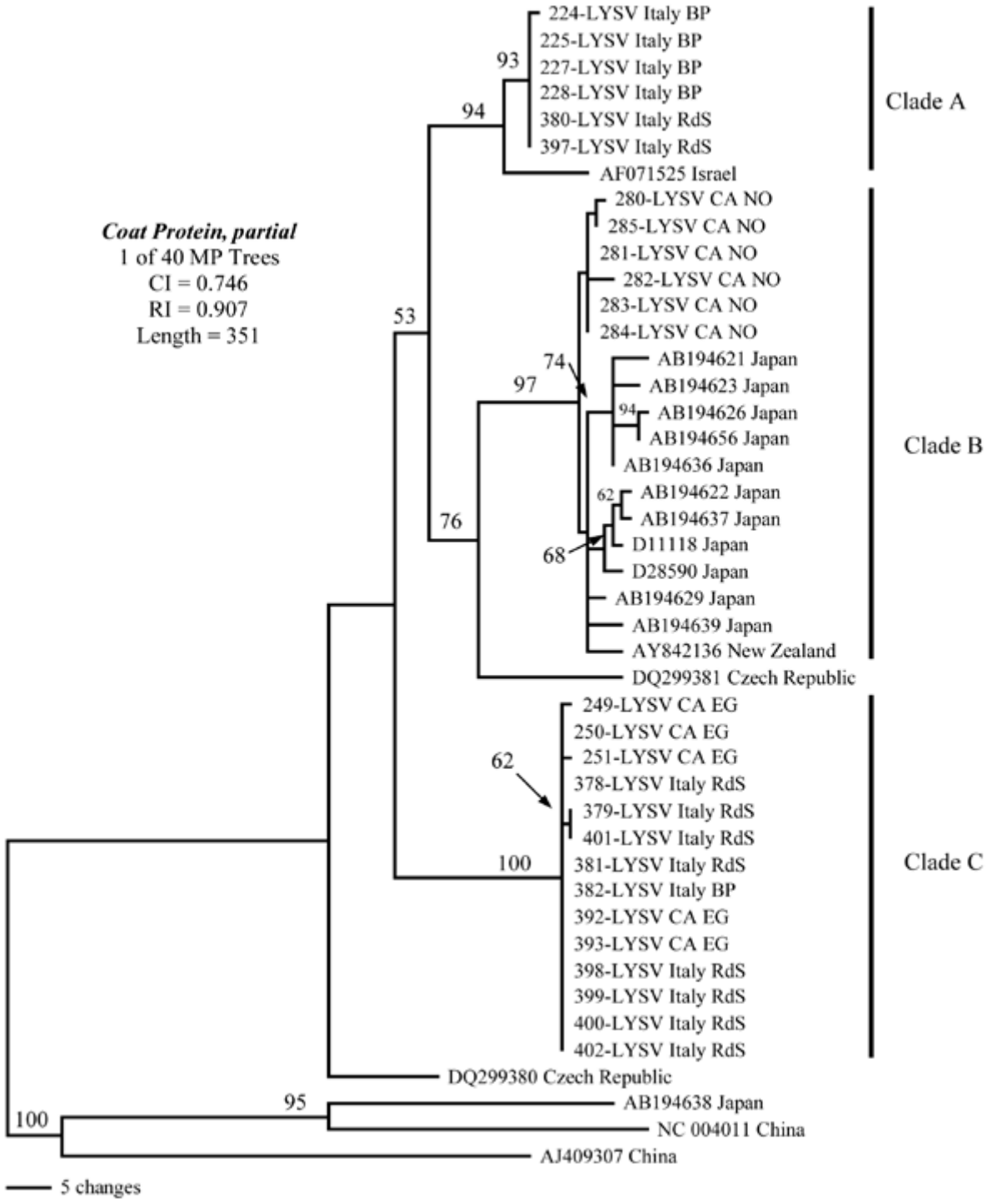

Figure 4. Maximum parsimony analyses of the coat protein gene for *Leek yellow stripe virus* (LYSV) isolates obtained in this study, as well as sequences from GenBank. Bootstrap support of 50% and above is indicated above nodes based on 1,000 replicates. 224-402: Serial numbering of cDNA LYSV clones, BP: 'Bianco Piacentino', RdS: 'Rosso di Sulmona', CA: California, NO: Non-organic, and EG: 'Elephant' garlic. Sequences have been deposited in GenBank under accession numbers HQ873735- 60.

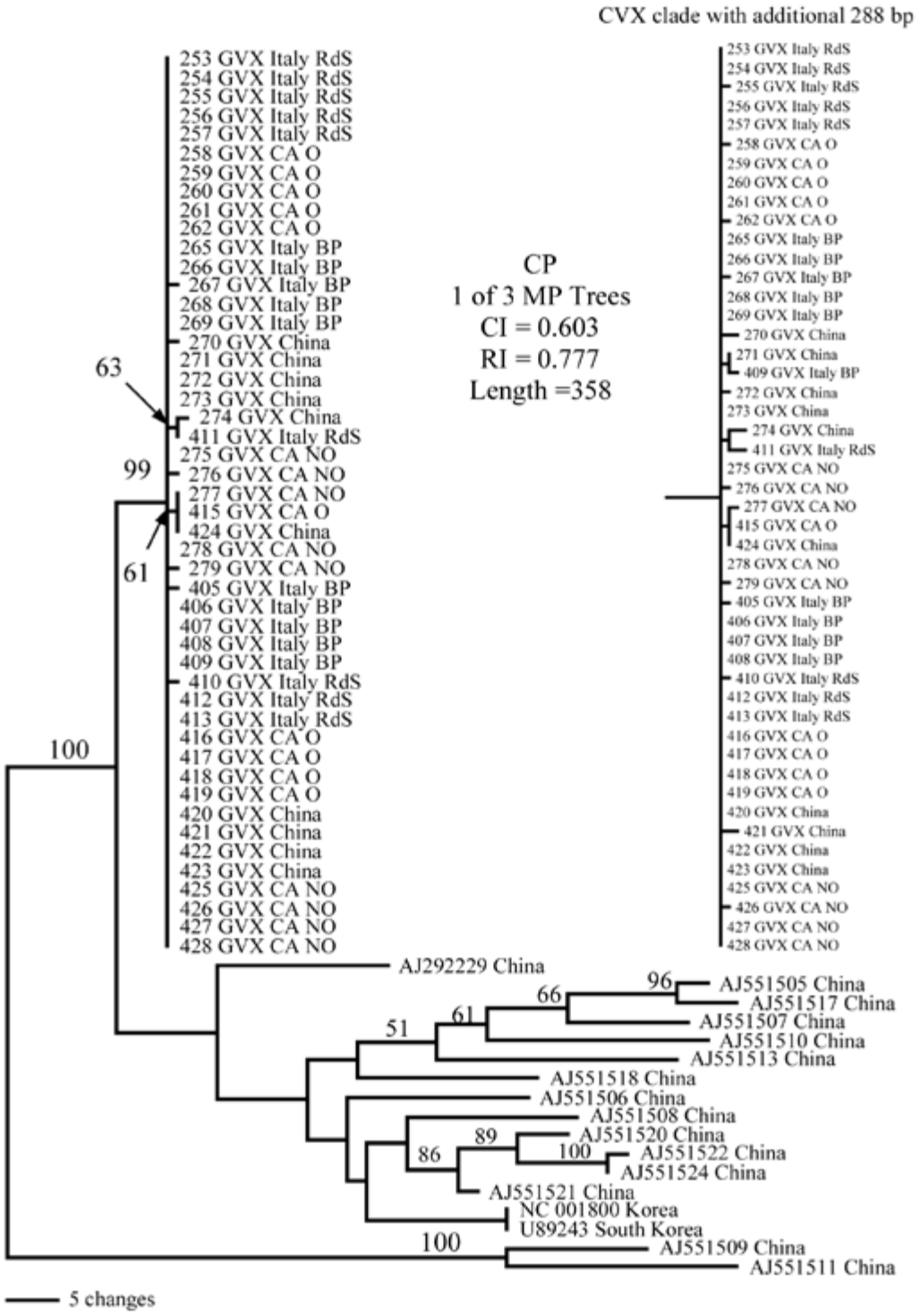

Figure 5. Maximum parsimony analyses of the coat protein gene for *Garlic virus X* isolates obtained in this study as well as sequences from GenBank. Bootstrap support of 50% and above is indicated above nodes based on 1,000 replicates. 253-428: Serial numbering of GVX cDNA clones, BP: 'Bianco Piacentino', RdS: 'Rosso di Sulmona', CA: California, O: Organic, and NO: Non-organic. Sequences have been deposited in GenBank under accession numbers HQ873807-51 and GQ475424-26.

(99%) clade was detected that contained all of the GVX isolates identified in this study which were isolated from Italian cultivars Rosso di Sulmona and Bianco Piacentino (bulbils) and from bulb samples from China and organic and non-organic samples from California.

Figure 5 is based on a 444 character dataset from all of the sequences used in the analysis. Initially, the dataset was trimmed to 444 bp because all of the AJ5515XX sequences downloaded from GenBank did not possess an additional 288 bp of data that was sequenced from the isolates identified in this study. Therefore, the analysis was redone on the larger data set (adding the 288 bp) so that haplotypes could be resolved since polymorphisms were found within this 288 bp region. This clade is superimposed on Figure 5 to the right. A total of 19 haplotypes were found, with 17 consisting of singletons. One haplotype contained the isolates 415 and 424 from a California organic sample and from a Chinese sample, respectively. The last haplotype consisted of 29 isolates, and this haplotype was isolated from all of the hosts in which the GVX viruses were found. The GVX isolates identified in this study were not closely related to any of the GVX sequences found in GenBank which were from China (GenBank: AJ292229 and AJ5515XX), Korea (GenBank: NC001800) and South Korea (GenBank: U89243).

The minimum similarity of the coat protein nucleic and amino acid sequences of the detected GVX in comparison to GenBank deposits was 90% and 79% respectively (GVX from this study, GenBank: GQ475424-26 and GVX from South Korea, GenBank: U89243), falling within the demarcation limits for the genus (Fauquet *et al.*, 2005). The amino acid alignment revealed a total of 17 variable sites. The majority (11/17 or 65%) of the variable sites was located in the N-terminal region. However, 15–20 amino acid residues for both N- (start) and C- (end) terminal regions appeared conserved. The core of the protein acquired a small number of mutations, but approximately 80 residues in the area appeared conserved.

The nucleotide and haplotype diversity of the GVX bulb isolates were significantly greater ($P<0.001$) than the bulbil isolates. The GVX number of polymorphic sites and haplotypes were not significantly different for both types of propagation material (Table 3).

Garlic common latent virus (GCLV)

GCLV phylogenetic analysis was based on 23 coat protein sequences. In total, 965 characters were analyzed which had 157 parsimony-uninformative and 138 parsimony-informative characters. Figure 6 shows the single most parsimonious tree found with a length of 433, CI = to 0.801 and RI = 0.780. A single clade was found with high bootstrap support (100%) that included all of the GCLV viruses identified in this study, which were all isolated from bulbs from China or from California organic bulb samples. A total of 14 haplotypes were found with 12 consisting of singletons. One haplotype consisted of the isolates 336 and 341 from China and California organic samples, respectively, and the other consisted of isolates 368 and 370 from California organic garlic and isolate 334 from China. The closest GCLV sequence in GenBank to the isolates identified in this study was from Japan (GenBank: AB004566) but the sequence was highly divergent. The other GCLV sequences from various countries that were identified in GenBank were also highly divergent from the GCLV isolates found in this study (Figure 6).

The minimum similarity of the coat protein nucleic and amino acid sequences of the detected GCLV in comparison to GenBank deposits was 93% and 92% respectively (GCLV from this study, GenBank: GQ475419-23 and GCLV from Germany GenBank: X81138), falling within the demarcation limits for the genus (Fauquet *et al.*, 2005). The amino acid sequence analysis revealed a total of 28 variable sites for GCLV. All these sites were distributed within 110 residues from the two terminal regions of the protein. The majority (18/28 or 64%) was located in the N-terminal region while the core was conserved.

Discussion

A variety of different garlic tissues, such as meristem tips, shoot apices, stem discs, cloves, and bulbils, have been used and evaluated as sources of specific plant material for the production of virus-free propagation material (Ramírez-Malagón *et al.*, 2006). The use of inflorescence-produced bulbils in therapeutic protocols has been controversial. It has been reported that there is no difference in virus content between bulbs and bulbils, and therefore there is no advantage on using bulbils over cloves for the production of virus-free propagation material (Verbeek *et al.*, 1995). However, virus elimination and growth habits of *in vitro* culture of bulbils were superior to bulbs and comparable with that of meristem tips (Ebi *et al.*, 2000; Shiboleth *et al.*, 2001). The present study

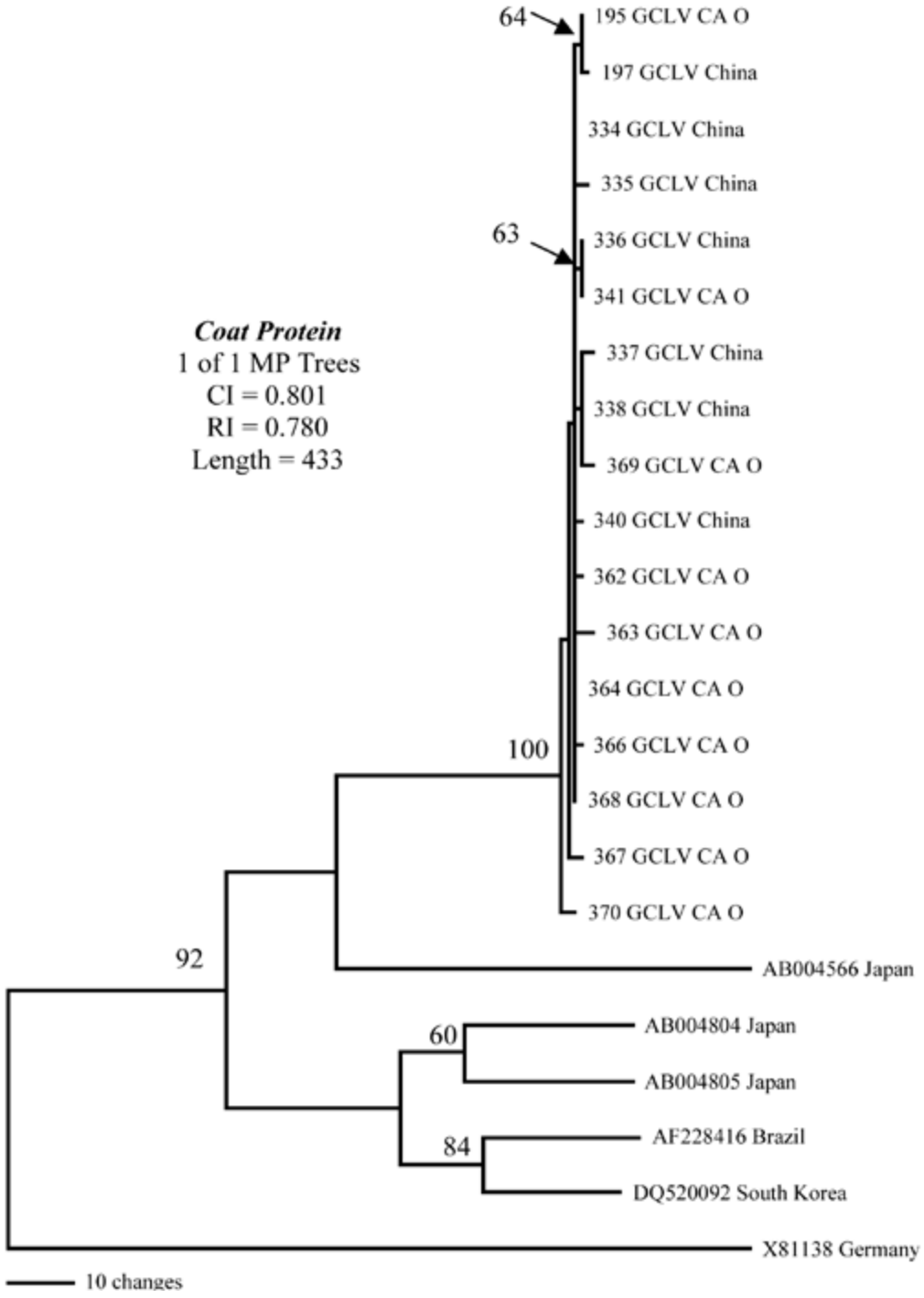

Figure 6. Maximum parsimony analyses of the coat protein gene for *Garlic common latent virus* (GCLV) isolates obtained in this study as well as sequences from GenBank. Bootstrap support of 50% and above is indicated above nodes based on 1,000 replicates. 195-370: Serial numbering of GCLV cDNA clones, CA: California, and O: Organic. Sequences have been deposited in GenBank under accession numbers HQ873852-63 and GQ475419-23.

revealed mixed infections at levels of 86% for OYDV, 57% for LYSV, 71% for GVX, and 29% for GCLV, for either the bulbil or bulb propagative tissues. This indicates that the inflorescence-produced bulbils carry virus infections similarly to bulbs, which have been repeatedly reported to be infected with multiple allium viruses (Takaichi *et al.*, 1998; Pappu *et al.*, 2008). Even though it is possible that the laboratory manipulations (i.e. surface sterilization and agar germination) impacted negatively the rate of bulbil germination, it appeared that the triple virus infection severely restricted the initial bulbil growth and subsequent garlic development, making the direct use of bulbils as propagative material an even less attractive option.

The phylogenetic and genetic analysis of the detected viruses in the two types of propagative material presented evidence of potential host tissue selection, depending upon the virus type and/or geography. For example, sequences of OYDV from clade A and clade C were only found in bulbils from Italy. In contrast, the OYDV sequences in clade E were detected both in bulbils from Italy and bulbs from China and California. A similar situation was also found for LYSV, in that one clade (A) was specific only to isolates found in Italian bulbils whereas LYSV isolates from clade C were found in bulbils from Italy as well as in bulbs from California. The type of the garlic propagative material also affected the population dynamics of the coat protein gene of all the detected viruses. With the exception of the OYDV haplotype diversity, both nucleotide and haplotype diversity (measurements independent of the sample size and applicable to haploid organisms) indicated that the virus populations in the bulbs were more diverse than those in the bulbils.

The phylogenetic relationships identified in our study indicated that only a part of the OYDV and LYSV isolates were related to the type of germplasm and the geographical origin, which is in agreement with previous reports (Chen *et al.*, 2002). In addition, both the phylogenetic and genetic variability findings fall within one of the fundamental selection mechanisms for plant virus and subvirus RNA pathogens. The differentiation and adaptation of natural virus populations according to host plant, the heterogeneous distribution of virus variants from a single virus population at different locations within the same host, and the severe selection bottlenecks on virus populations during events of systemic infection and invasion of young organs, have been well documented for plant viruses (Magome *et al.*, 1999; Garcia-Arenal *et al.*, 2001; French and Stenger, 2003; Sacristan *et al.*, 2003; Li and Roossinck, 2004; Jridi *et al.*, 2006). The bottlenecks of systemic infection and colonization of different tissues (i.e. sieve elements or companion cells) and organs (i.e. photosynthetic or and sink tissues) may result in a very small number of virus variants that arrive and finally invade the newly formed bulbils at the top of the inflorescence. This process generates a founder effect that may explain, at least partially, the reduced genetic variability identified in the virus populations of garlic plants grown from bulbils (Jridi *et al.*, 2006).

Coupled with potential host tissue and/or geographic specificity, it was also found that some of the viruses had more phylogeographic signal than others, and some were phylogenetically much more diverse than others. For example, isolates of OYDV from clades A and C were only found in samples from Italy and isolates from clades B and D were only found in samples from California and Argentina, respectively. In contrast, clade E isolates were identified from California, China, Italy, United Arab Emirates, and Indonesia, but the latter two isolates were also supported by a 90% bootstrap value suggesting they are phylogenetically more divergent than the other isolates identified in this study. A similar situation was observed for LYSV; clades A and a subclade of B were unique to Italy or California, whereas isolates from clade C were found in both geographic locations.

The effects of geographic origin on the phylogenetic and/or genetic relationships of the potyviruses have been variable. Correlation of coat protein gene variability and geographical regions has been reported for LYSV and *Papaya ringspot potyvirus* (Bateson *et al.*, 1994; Takaki *et al.*, 2005). On the other hand, coat protein sequence variation for some garlic isolates of the OYDV had no or partial correlation with geographic localization (Tsuneyoshi *et al.*, 1998b; Chen *et al.*, 2002). Our results captured all the aspects of the geographic origin effects on allium potyvirus evolution, that according to previous observations can be independent but similar through a unique process dependent on the garlic host (Tsuneyoshi *et al.*, 1998b).

OYDV and LYSV were both found to be phylogenetically diverse, with virus isolates falling into five and three clades, respectively. However, most of the virus sequences were unique, based on BLAST

searches coupled with closely related sequences included into the analysis. For OYDV, no closely related sequences were found that fell into clades A, B, and C, and only two deposited sequences fell into clade E, but they were unique based on high bootstrap support. Similarly for LYSV, do deposited sequences were identified with affinities to clades A and C but isolates from Japan and New Zealand fell within clade B (97% support), but the sequences were variable and some additional signal was found within this clade.

In contrast to OYDV and LYSV, less diversity was found in GVX and GCLV, in which all recovered viruses fell into a single well-supported clade for each virus. Isolates from the same clade of GVX found in this study were from Italy, California, and China, and GCLV was from California and China. Previous reports on the phylogenetic relationships of allexi- and carla-viruses are in agreement with the presented phylogenetic relationships. These studies suggested that there is little overall relationship between sequences and geographical origin, while no apparent sequence variations were related to the host plant species (Tsuneyoshi *et al.*, 1998a; Chen *et al.*, 2004). Since these two viruses have distinct biological features (e.g. aphid vs. mite transmission) their "cosmopolitan nature" suggests that they have mostly been distributed with vegetatively propagated planting material.

Novel as well as previously reported mutational patterns in the amino acid sequence of the coat proteins were identified for the detected viruses. The sequence variability of the N-terminal region of OYDV and LYSV is in agreement with previous reports for potyviruses (Bateson *et al.*, 1994; Moury *et al.*, 2002; Takaki *et al.*, 2005), similar to that of GCLV as typical carlavirus (Wegener *et al.*, 2006; Singh *et al.*, 2007). However, the data for GCLV indicated a variable C-terminal region as well as a highly conserved core region (0 out of the 28 variable sites). In agreement with previous reports for allexiviruses, the mutations detected in the GVX coat protein were clustered in the N-terminal regional, while the core region was conserved (Lu *et al.*, 2008). However, the data from the present study indicate that conserved amino acid sequences exist both in the core region of the protein and at the C-terminal region as well. The novel coat protein mutational patterns reported here did not affect the efficacy of the ELISA test utilized for OYDV and GCLV.

Chenopodium quinoa has been reported as a non-susceptible host for OYDV (Bos *et al.*, 1978; Marys *et al.*, 1994; Büchen-Osmond, 2006). A single report (Bos 1976), however, indicated that local lesions were obtained with some OYDV isolates in *C. quinoa*. In the present study, the OYDV coat protein gene sequence of a *C. quinoa* isolate was identified. The comparison of the coat protein of the OYDV-Chenopodium with the original OYDV-garlic inoculum revealed 21 amino acid mutations, that may play an important role for chenopodium infectivity. More importantly, however, the OYDV-garlic population contained a haplotype that shared all but three amino acid substitutions of the coat protein of the OYDV-Chenopodium. Without disregarding the potential role of other virus genes these findings provide a first insight to a "coat protein signature" that may play a critical role for the infectivity of OYDV to *C. quinoa*. Additional studies are required to investigate the association of the identified OYDV coat protein signature with infectivity to other *Chenopodium* species such as *C. amaranticolor* or *C. murale*.

In conclusion, the results of this study indicated that the virus status of garlic bulbils is not different from that of bulbs. Furthermore, while it is possible that our analyses were biased due the limited number of geographic locations, bulbil isolates, or viral sequences analyzed, our results clearly highlight the distinct responses of poty-, carla- and allexi- viruses to the selection pressures of tissue specialization and/or geographic origin, as this may be related to alternative hosts, environmental conditions, and/or vector transmission.

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Literature cited

Bateson M.F., J. Henderson, W. Chaleeprom, A.J. Gibbs and J.L. Dale,1994. Papaya Ringspot Potyvirus - Isolate Variability and the Origin of Prsv Type-P (Australia). *Journal of General Virology* 75, 3547–3553.

Bos L., 1976. Onion yellow dwarf virus. *CMI/AAB Descriptions of Plant Viruses* 158, 4.

Bos L., N. Huijberts, H. Huttinga and D.Z. Maat, 1978. Leek Yellow Stripe Virus and Its Relationships to Onion Yellow Dwarf Virus - Characterization, Ecology and Possible Control. *Netherlands Journal of Plant Pathology* 84, 185–204.

Büchen-Osmond C., 2006. Index to ICTVdB virus descriptions. In: *ICTVdB - The Universal Virus Database, version 4* (C. Büchen-Osmond ed.) ICTVdB Management, Mailman School of Public Health, Columbia University, New York, NY, USA.

Cafrune E.E., M.C. Perotto and V.C. Conci, 2006. Effect of two Allexivirus isolates on garlic yield. *Plant Disease* 90, 898–904.

Chen J., J.P. Chen and M.J. Adams, 2002. Characterisation of some carla- and potyviruses from bulb crops in China. *Archives of Virology* 147, 419-428.

Chen J., H.Y. Zheng, J.F. Antoniw, M.J. Adams, J.P. Chen and L. Lin, 2004. Detection and classification of allexiviruses from garlic in China. *Archives of Virology* 149, 435–445.

Conci V.C., A. Canavelli, P. Lunello, J. Di Rienzo, S.F. Nome, G. Zumelzu and R. Italia, 2003. Yield losses associated with virus-infected garlic plants during five successive years. *Plant Disease* 87, 1411–1415.

Dovas C.I., E. Hatziloukas, R. Salomon, E. Barg, Y. Shiboleth and N.I. Katis, 2001. Incidence of viruses infecting *Allium* spp. in Greece. *European Journal of Plant Pathology* 107, 677–684.

Ebi M., N. Kasai and K. Masuda, 2000. Small inflorescence bulbils are best for micropropagation and virus elimination in garlic. *Hortscience* 35, 735–737.

Etoh T. and P. Simon, 2002. Diversity, Fertility and Seed Production of Garlic. In: *Allium crop science: recent advances,* (H.D. Rabinowitch and L. Currah). CABI International, New York, USA, 101–117.

FAO, 2009. FAOSTAT, FAO Statistics Division. FAO, Rome, Italy. Rome: FAO.

Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (ed), 2005. *Virus Taxonomy, Classification and Nomenclature of Viruses*, VIII Report of the International Committee on the Taxonomy of Viruses. Elsevier/Academic Press, London, UK.

French R. and D.C. Stenger, 2003. Evolution of Wheat streak mosaic virus: Dynamics of Population Growth Within Plants May Explain Limited Variation. *Annual Review of Phytopathology* 41, 199–214.

Garcia-Arenal F., A. Fraile and J.M. Malpica, 2001. Variability and genetic structure of plant virus populations. *Annual Review of Phytopathology* 39, 157–186.

Hall T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. 95–98.

Jridi C., J.F. Martin, V. Marie-Jeanne, G. Labonne and S. Blanc, 2006. Distinct Viral Populations Differentiate and Evolve Independently in a Single Perennial Host Plant. *Journal of Virology* 80, 2349–2357.

Li H. and M.J. Roossinck, 2004. Genetic Bottlenecks Reduce Population Variation in an Experimental RNA Virus Population. *Journal of Virology* 78, 10582–10587.

Lu Y.W., J. Chen, H.Y. Zheng, M.J. Adams and J.P. Chen, 2008. Serological relationships among the over-expressed coat proteins of allexiviruses. *Journal of Phytopathology (Berlin)* 156, 251–255.

Maddison D. and W. Maddison, 2001. MacClade, Version 4.03. Sutherland, MA: Sinauer Associates Inc.

Magome H., N. Yoshikawa and T. Takahashi, 1999. Single-strand conformation polymorphism analysis of apple stem grooving capillovirus sequence variants. *Phytopathology* 89, 136–140.

Marys E., O. Carballo and M.L. Izaguirre-Mayoral, 1994. Isolation and Characterization of Viruses Present in Four Clones of Garlic (Allium sativum) in Venezuela. *Journal of Phytopathology (Berlin)* 142, 227–234.

Moury B., C. Morel, E. Johansen and M. Jacquemond, 2002. Evidence for diversifying selection in Potato virus Y and in the coat protein of other potyviruses. *Journal of General Virology* 83, 2563–2573.

Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York, USA 512 pp.

Nicholas K. and H. Nicholas Jr, 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. *Distributed by the author,* 14.

Pappu H., B. Hellier and F. Dugan, 2008. Evaluation of the National Plant Germoplasm System's Garlic Collection for Seven Viruses. *Plant Health Progress,* doi:10.1094/PHP-2008-0919-01-RS.

Pooler M.R. and P.W. Simon, 1994. True Seed Production in Garlic. *Sexual Plant Reproduction* 7, 282–286.

Ramírez-Malagón R., L. Pérez-Moreno, A. Borodanenko, G.J. Salinas-González and N. Ochoa-Alejo, 2006. Differential organ infection studies, potyvirus elimination, and field performance of virus-free garlic plants produced by tissue culture. *Plant Cell, Tissue and Organ Culture* 86, 103–110.

Rozas J. and R. Rozas, 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15, 174–175.

Sacristan S., J.M. Malpica, A. Fraile and F. Garcia-Arenal, 2003. Estimation of Population Bottlenecks during Systemic Movement of Tobacco Mosaic Virus in Tobacco Plants. *Journal of Virology* 77, 9906–9911.

Sambrook J. and D.W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*.Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA..

Shiboleth Y., A. Gal-On, M. Koch, H. Rabinowitch and R. Salomon, 2001. Molecular characterisation of Onion yellow dwarf virus (OYDV) infecting garlic (*Allium sativum* L.) in Israel: Thermotherapy inhibits virus elimination by meristem tip culture. *Annals of Applied Biology* 138, 187–195.

Singh L., V. Hallan, N. Jabeen, A.K. Singh, R. Ram, D.P. Martin and A.A. Zaidi, 2007. Coat protein gene diversity among Chrysanthemum virus B isolates from India. *Archives of Virology,* 152, 405–413.

Swofford D., 2002. PAUP*. *Phylogenetic analysis using parsimony (* and other methods)*. Version 4b10. Sinauer, Sunderland, Massachusetts, USA.

Takaichi M., M. Yamamoto, T. Nagakubo and K. Oeda, 1998. Four garlic viruses identified by reverse transcription-polymerase chain reaction and their regional distribution in northern Japan. *Plant Disease* 82, 694–698.

Takaki F., T. Sano, K. Yamashita, T. Fujita, K. Ueda and T. Kato, 2005. Complete nucleotide sequences of attenuated and severe isolates of Leek yellow stripe virus from garlic in northern Japan: Identification of three distinct virus types in garlic and leek world-wide. *Archives of Virology* 150, 1135–1149.

Thompson J., T. Gibson, F. Plewniak, F. Jeanmougin and D. Higgins, 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* 25, 4876.

Tsuneyoshi T., T. Matsumi, T.C. Deng, I. Sako and S. Sumi, 1998a. Differentiation of Allium carlaviruses isolated from different parts of the world based on the viral coat protein sequence. *Archives of Virology* 143, 1093–1107.

Tsuneyoshi T., T. Matsumi, K.T. Natsuaki and S. Sumi, 1998b. Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in Allium plants. *Archives of Virology* 143, 97–113.

Verbeek M., P. Van Dijk and P.M.A. Van Well, 1995. Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meristem-tip culture. *European Journal of Plant Pathology* 101, 231–239.

Walkey D.G.A., 1989. Agronomic Evaluation of Virus-Free and Virus-Infected Garlic (*Allium-Sativum* L). *Journal of Horticultural Science* 64, 53–60.

Wegener L., Z. Punja, R. Martin, M. Bernardy and L. MacDonald, 2006. Epidemiology and identification of strains of Blueberry scorch virus on highbush blueberry in British Columbia, Canada. *Canadian Journal of Plant Pathology* 28, 250–262.

Zimmermann S., S. Schillberg, Y. Liao and R. Fisher, 1998. Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in shape *Nicotiana tabacum*. *Molecular Breeding* 4, 369–379.

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