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Molecular characterisation of a complex mixture of viruses in garlic with mosaic symptoms in China

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Summary. Degenerate primers were used to detect and amplify cDNA of viruses of the genera *Carlavirus*, *Allexivirus* and *Potyvirus* from garlic plants with mosaic symptoms growing in Zhejiang province, China. Plants contained a complex mixture of viruses and strains. Three distinct stains of *Garlic latent virus* were detected; the most frequent one was completely sequenced and partial sequences were obtained for the other two. The complete sequence (8363 nt) was 76.4% identical to a Korean isolate. Two allexiviruses were detected and completely sequenced. One (8319 nt) was identified as *Garlic virus X* and comparisons showed that a published Korean isolate (which had 90.2% identical nucleotides) had an N-terminal deletion in the serine-rich ORF4. The other isolate (8451 nt), tentatively named *Garlic virus E*, appeared to be a new member of the genus. Phylogenetic analyses of the different viral proteins and distinctive conserved sequence motifs within the genus are discussed. This is the first report of allexiviruses from China. Using potyvirus primers, three distinct isolates of *Onion yellow dwarf virus* and one of *Leek yellow stripe virus* were detected and the 3′-terminal sequences of their genomes were determined. In a coat protein phylogenetic analysis, the new isolates were most closely related to other published isolates from Japan and China.

Introduction

Virus diseases of garlic (*Allium sativum* L.) are widespread throughout the world, causing serious losses in crop yields and deterioration of quality. They accumulate in bulbs because of the vegetative propagation of the crop. If the viruses are to be controlled effectively, it is first necessary to establish their identity and to determine their molecular characteristics.

Various filamentous viruses associated with mosaic symptoms have been reported from garlic, often in complex mixtures. These include at least three members of the genus *Potyvirus*, (*Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), *Shallot yellow stripe virus* (SYSV)) two or more members of the genus *Carlavirus* (*Garlic common latent virus* (GCLV), *Garlic latent virus* (GLV) = Shallot latent virus (SLV) [18]) and various members of the recently established genus *Allexivirus* [2, 10, 13, 14, 16–20, 22, 23]. These viruses all have a single component of single-stranded, positive sense, RNA with a 3′-poly A tail but differ in genome organisation. The potyviruses and carlaviruses are transmitted by aphids and the allexiviruses are believed to be mite-transmitted.

Garlic is an economically important crop in China and mosaic disease causes severe loss of yield and poor quality. Local scientists tend to assume that garlic potyvirus and garlic latent carlavirus are the two major viruses responsible but no detailed study has so far been reported.

In this paper, we report the nucleotide sequences of cDNAs of the major viruses detected in garlic plants showing mosaic or streak symptoms at a site in Zhejiang province, China. The correct names and classification of these viruses is also discussed.

Materials and methods

Garlic leaves with typical mosaic symptoms were collected from fields in Taiping cottage, Yuhang, Zhejiang province in April 2000 and stored at $-80\,^{\circ}$C until used. Viruses were purified from the infected leaves and viral RNAs were extracted using published methods [13]. First-strand cDNA was synthesised using Expand reverse transcriptase (Roche) according to the manufacturer's instructions and a specially designed initial primer named M4T (5′-GTT TTC CCA GTC ACG AC $(T)_{15}$-3′). The PCR reaction used an Expand^{TM} Long Template PCR system (Roche) according to the manufacturer's protocols. Sequences of viral cDNAs were amplified by RACE methods. Fragments were separated by electrophoresis through 1% (w/v) agarose gels and purified using the QIAGEN Gel Extraction Kit (Qiagen). Amplification of the 3′-terminus of viral RNA used the first-strand cDNAs produced as described above and the primer M4 (5′-GTT TTC CCA GTC ACG AC-3′) in combination with one of three primers designed from consensus sequences of the virus genera of interest. These were Sprimer (5′-GGX AAY AAY AGY GGX CAZ CC-3′), pCar1 (5′-ATG CCX CTX AXX CCX CC-3′; X = A, T, G or C) and pGV-3t (5′-TGG XCX TGC TAC CAC AAX GG-3′) for the potyviruses, carlaviruses and allexiviruses, respectively (X = A, G, C or T; Y = T or C; Z = A or G). The 5′-parts of the genomes of the viruses were obtained using a 5′-RACE method similar to that described by Diao et al. [4], but using ZHM1 (5′-CTC TTC CCC TCC CTC CTC-3′; modified: 5′-terminus phosphorylation and 3′-terminus amination) as ligation primer and ZHM2 (5′-GAG GAG GGA GGG GAA GAG-3′) as corresponding PCR primer. PCR fragments were cloned into the pGEM-T vector (Promega) and were sequenced in both directions by the ABI PRISM 377 DNA Sequencer, using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Sequence analysis was done by using programs from the University of Wisconsin Genetics Computer Group (GCG) package [8]. Phylogenetic analyses were done using programs in PHYLIP version 3.572c [6]. Genetic distances between pairs of amino acid sequences were calculated using PROTDIST (Kimura formula). Phylogenetic trees were constructed by a distance method (NEIGHBOR) using the original data set and 100 bootstrap data sets generated by the program SEQBOOT from the

original set. In all cases the consensus tree was generated by the program CONSENSE. The nucleotide sequences obtained were deposited with the DDBJ/GenBank/EMBL databases with the accession numbers AJ292223-AJ292231 (see Tables 1 and 2; Fig. 3).

Results and discussion

Carlaviruses

A total of 26 clones of the 3′-terminal virus sequences amplified by primers pCar1/M4 were partially sequenced. 22 sequences had >99.5% identical nucleotides and were designated isolate YH1. Three other partial sequences (YH2) were different to YH1 but identical to each other and there was one other distinctive sequence (YH3). Obviously YH1 was the dominant isolate amongst these samples.

The complete sequence of YH1 (AJ292226) was determined. The linear, single stranded, positive sense RNA was 8363 nt long (excluding the poly-A tail) and had the expected six open reading frames (ORFs) which, by comparison with other published sequences, are believed to produce the six virus proteins. These are, from 5′- to 3′-, the RNA replicase (218.2 kDa), the three triple gene block (TGB) proteins (26.0, 12.0 and 7.1 kDa), the coat protein (CP; 32.8 kDa) and a nucleic acid binding protein (NABP; 11.1 kDa) (Fig. 1). The partial sequences of isolates YH2 (AJ292227: 1810 nt excluding the poly-A tail; 78.8% nucleotides identical to isolate YH1) and YH3 (AJ292228: 2978 nt excluding the poly-A tail; 78.3% nucleotides identical to isolate YH1 and 79.8% identical to isolate YH2) were also determined. The partial sequence of YH3 contained the five smaller ORFs but only the extreme C-terminus of the replicase and YH2 was without both ORF1 and ORF2. The predicted proteins were very similar in size to those of isolate YH1 but in isolate YH3 an AUG codon at position 2556–2558 could theoretically generate a larger NABP of 12.6 kDa, although comparisons with other isolates suggested that the protein was more likely to be initiated at 2589–2591. Comparisons of the translation context of the AUG codons to determine the likelihood of initiation was not very informative, but suggested that the eukaryotic consensus sequences previously described [12] probably do not apply to plant viruses.

Pairwise (GAP) sequence comparisons showed that these carlavirus isolates from China had between 70.1 and 81.2% nucleotides identical to the 16 published isolates of GLV and SLV. There were only 45.8 to 49.7% nucleotides identical to the five published isolates of GCLV. Species demarcation criteria given in the 7th ICTV report [24] are that the CP core region of different carlavirus species have less than 68% identical amino acids, while strains of the same virus have 75–90% identical amino acids. Comparisons were made between the core region CP amino acid sequences of all the available GLV/SLV accessions and the three new Chinese sequences which showed that all belonged to the single species GLV (90–100% identical amino acids; data not shown).

The sequence databases contain one complete sequence of GLV (Z68502: Korean isolate), which has 76.4% nucleotides identical to GLV-YH1. Comparisons between the ORFs of this sequence and those of the three new Chinese ones (Table 1) show that the CP is generally less variable than the other genes.

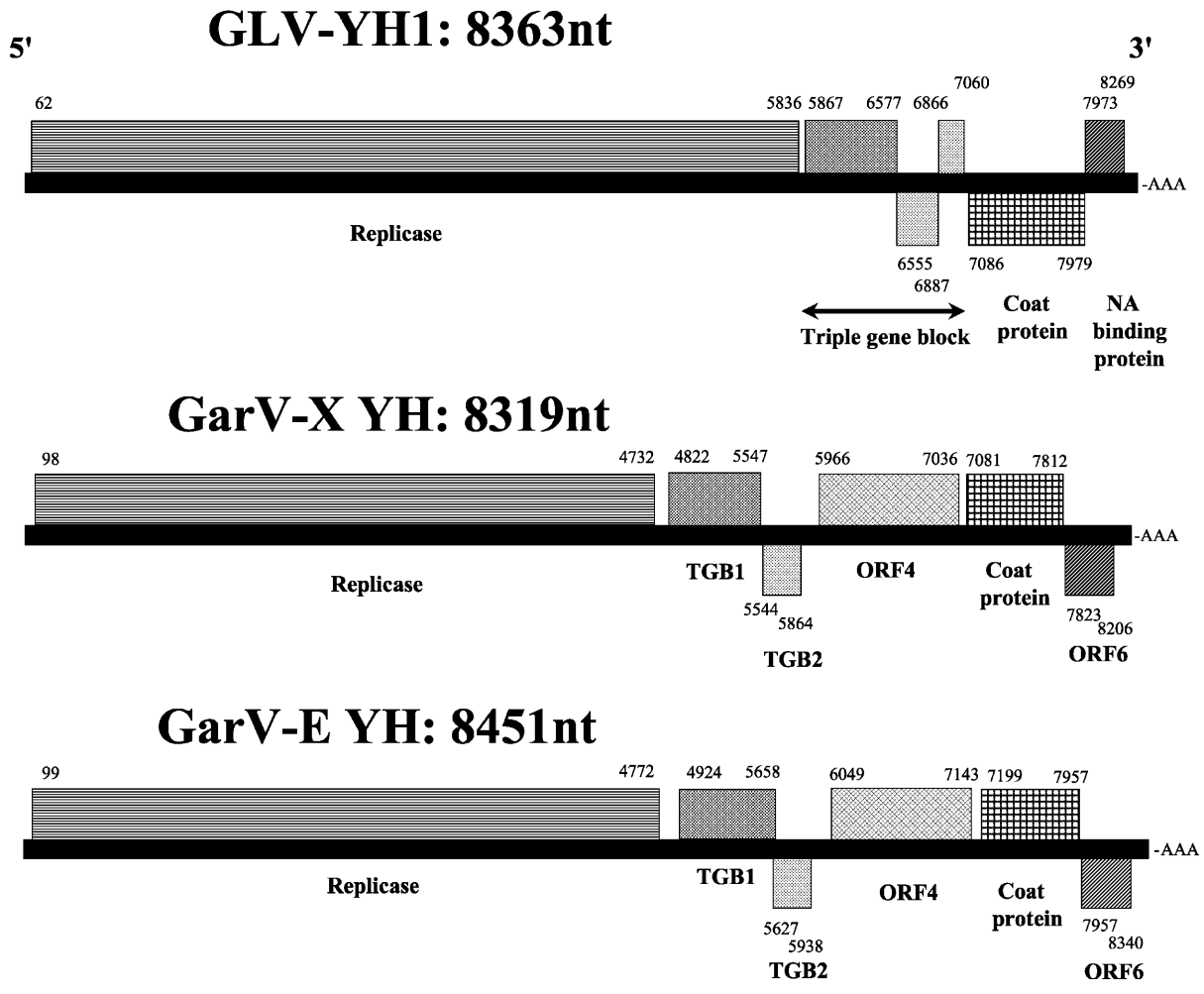

Fig. 1. Genome organisation of *Garlic latent virus* (GLV), *Garlic virus X* (GarV-X) and *Garlic virus E* (GarV-E) as determined from Chinese garlic plants

Allexiviruses

A total of 19 clones of the 3′-terminal virus sequences amplified by primers pGV-3t/M4 were partially sequenced. These were of two types: 14 sequences (>98% identical nucleotides) showed substantial similarity to *Garlic virus X* (GarV-X) in a BLAST search while the other 5 clones (>98% identical nucleotides) were more distantly related to published Allexivirus sequences and have been provisionally named Garlic virus E (GarV-E). The complete sequences of both viruses were determined.

Garlic virus X

The genome of the Yuhang (YH) isolate, accession AJ292229, was 8319 nucleotides long (excluding the poly-A tail) with the expected six ORFs that are typical of the genus (Fig. 1). Over the entire sequence, there were 90.2%

Table 1. Percentage amino acid identities (top right) or similarities (bottom left) between the three Chinese *Garlic latent virus* isolates, YH1 (AJ292226), YH2 (AJ292227) and YH3 (AJ292228), and the Korean isolate (Z68502) in each open reading frame

	YH1	YH2	YH3	Z68502	YH1	YH2	YH3	Z68502
	Replicase				**Triple Gene Block protein 1**			
YH1	*	–	–	85.3	*	–	78.1	75.0
YH2	–	*	–	–	–	*	–	76.7
YH3	–	–	*	–	84.1	–	*	73.7
Z68502	93.2	–	–	*	79.7	80.6	78.0	*
	Triple Gene Block protein 2				**Triple Gene Block protein 3**			
YH1	*	77.5	80.2	79.3	*	66.2	73.8	70.8
YH2	82.0	*	80.2	78.4	80.0	*	84.6	73.8
YH3	90.1	88.3	*	89.2	84.6	84.6	*	76.9
Z68502	87.4	85.6	93.7	*	80.0	80.0	86.2	*
	Coat protein				**Nucleic-acid binding protein**			
YH1	*	90.6	93.0	90.8	*	87.9	84.8	83.8
YH2	93.6	*	94.0	90.8	90.9	*	86.9	83.8
YH3	94.6	96.6	*	93.6	86.9	87.9	*	90.9
Z68502	92.5	92.9	93.9	*	85.9	86.9	91.9	*

Comparisons that were not available are marked (–)

nucleotides identical to the Korean isolate of GarV-X (U89243; [16]). Amino acid identities in the different ORFs were between 85.5 and 95.3% (Table 2). Most of the genome features were very similar to those previously reported for GarV-X and are therefore not repeated here. However, the YH isolate was *c*. 200 nt longer than the Korean isolate at the 5′-end of ORF4 and comparisons with other members of the genus suggests that the YH isolate represents the full length, while the Korean isolate had a deletion in this region.

Garlic virus E

The GarV-E genome, accession AJ292230, was 8451 nts long (excluding the polyA tail) and its organisation was similar to that of the other allexiviruses but with only 62.8–64.8% nucleotides identical to them. ORF1 encodes a 176 kDa replicase of 1557 residues, ORFs 2 and 3 resemble the first two TGB proteins of carlaviruses and potexviruses, with sizes of 27 kDa (244 residues) and 11 kDa (103 residues), respectively and ORF4 encodes a 40 kDa protein of 364 residues. ORF5 would encode a 35 kDa CP (316 residues) if translated from the first AUG at position 7007–7009, but alignment with other allexiviruses suggests that it is more likely to be translated from an AUG at position 7199–7201 to encode a 28 kDa polypeptide of 252 residues. ORF6 encodes a 15 kDa NABP of 127 residues.

Table 2. Comparisons of nucleotide (complete sequence, 5′- and 3′- untranslated regions [UTRs]) and amino acid sequences (open reading frames [ORFs]) amongst the complete allexivirus sequences (Garlic viruses A, C, E and X; *Shallot virus X*)

		GarV-C AB010302	GarV-E AJ292230	GarV-X U89243	GarV-X AJ292229	ShV-X M97264	GarV-C AB010302	GarV-E AJ292230	GarV-X U89243	GarV-X AJ292229	ShV-X M97264
		Complete sequence					**5′-UTR**				
GarV-A	AB010300	61.0	64.8	61.1	60.4	64.7	60.2	70.1	64.6	65.6	79.8
GarV-C	AB010302		64.4	65.8	65.2	62.4		83.7	86.6	84.5	63.2
GarV-E	AJ292230			63.8	63.4	62.8			77.3	76.3	63.8
GarV-X	U89243				90.2	61.2				96.9	56.2
GarV-X	AJ292229					61.1					57.3
		Replicase					**Triple Gene Block protein 1**				
GarV-A	AB010300	70.1	69.0	69.6	69.3	74.5	50.8	58.0	56.6	57.2	62.8
GarV-C	AB010302		77.4	80.3	81.7	67.7		55.8	61.1	61.6	57.3
GarV-E	AJ292230			78.1	78.5	67.6			54.5	55.4	66.2
GarV-X	U89243				93.4	67.2				91.1	59.1
GarV-X	AJ292229					66.9					61.3
		Triple Gene Block protein 2					**ORF4**				
GarV-A	AB010300	68.3	81.7	56.7	55.8	62.5	40.8	55.6	42.3	36.8	44.9
GarV-C	AB010302		67.3	66.4	64.4	60.6		39.6	37.2	35.3	37.7
GarV-E	AJ292230			59.6	58.7	59.6			42.0	40.3	47.1
GarV-X	U89243				91.6	53.8				85.5	37.5
GarV-X	AJ292229					51.9					32.6
		Coat protein					**ORF6**				
GarV-A	AB010300	63.4	79.7	67.4	69.6	64.5	52.7	64.1	54.7	55.5	61.2
GarV-C	AB010302		66.3	69.7	72.7	61.3		52.0	68.8	68.8	61.2
GarV-E	AJ292230			69.3	69.9	67.1			55.1	55.1	68.8
GarV-X	U89243				93.0	65.0				95.3	63.3
GarV-X	AJ292229					66.3					64.1
		3′-UTR									
GarV-A	AB010300	83.5	93.3	75.7	82.5	84.5					
GarV-C	AB010302		83.5	85.8	89.9	82.6					
GarV-E	AJ292230			75.5	80.7	86.4					
GarV-X	U89243				88.7	77.4					
GarV-X	AJ292229					85.3					

Pairwise (GAP) comparisons between amino acid sequences of the different proteins of the available complete allexivirus sequences (Table 2) shows that the differences between GarV-E and the other members of the genus are similar to those between the other distinct species. A more comprehensive comparison using all available CP amino acid sequences (Table 3) shows that GarV-E had 63.9–79.8% amino acids identical with other allexiviruses. Species demarcation criteria given in the 7th ICTV report [24] for the genus *Allexivirus* suggest that distinct species have <90% identical amino acids in the CP, and <90% identical nts in the 3′-UTR. We therefore conclude that GarV-E should be considered as a separate member of the genus. Phylogenetic trees based on alignments of the different protein amino acid sequences showed that GarV-E usually grouped with GarV-D and GarV-A, whereas GarV-X was closer to GarV-B and to some extent GarV-C. The most informative trees are shown in Fig. 2. The results also show that accession L38892 (named Japanese garlic virus in the sequence file but listed as *Garlic mite-borne filamentous virus* in the ICTV 7th report) is actually an isolate of GarV-D. Accession D49443 (named Garlic mite-borne virus in the sequence file but listed as *Garlic virus B* in the ICTV 7th report) is actually GarV-C. Accession X98991 (named *Garlic mite-borne filamentous virus* in the sequence file) is possibly an isolate of GarV-A.

General characteristics of the allexivirus proteins

ORF1 encodes a 170–195 kDa polypeptide which is thought to be a virus-specific RNA replicase. A conserved motif $SGx_3Tx_3NTx_{18-37}GDD$, which is the proposed active site of the RNA-dependant RNA polymerase was found at amino acid positions 1307–1342 and 1319–1354 in GarV-X and GarV-E respectively, and the NTP binding motif GxxGxGKS, which assumed to be involved in helicase activity, was found at 748–755 and 760–767. A leucine zipper pattern L-X_6-L-X_6-L-X_6-L, which has not previously been described, was found at 159–180 in both GarV-X and GarV-E and can also be found in other members of the genus. ORF2 encodes a 25–28 kDa protein which has similarities to the TGB1 found in all carla- and potexviruses. Although the two proteins have little other similarity, an NTP binding motif similar to that in ORF1 has been identified (at 37–44 and 30–37 in GarV-X and GarV-E respectively), suggesting that the protein may function as an NTP-dependent helicase. This motif has also been reported to be in the TGB1 of furo-, carla-, potex- and hordeiviruses [26]. The protein is leucine-rich (ca. 12%) and can possibly form amphipathic α-helixes which are thought to bind DNAs or RNAs. ORF3 encodes an 11–12 kDa TGB2 protein homologous to those of other allexi-, carla- and potexviruses. Secondary structure analysis using a range of software including [9, 15, 21, 25], showed that TGB2 of all allexi-, carla- and potexviruses included a helix-coil-helix structure, forming two transmembrane (TM) regions with N- and basic C-termini exposed inside (data not shown). By analogy with other viruses, the TGB proteins may have a role in cell-to-cell movement [1, 2, 3, 5, 11]. The 32–43 kDa ORF4 of allexiviruses is quite distinct from the third ORF in the triple gene block of carla- and potexviruses.

Table 3. Comparisons (% identical amino acids) between the coat protein amino acid sequences of allexiviruses (*Garlic mite-borne filamentous virus*; Garlic viruses A, B, C, D, E and X; *Shallot virus X*) and potato virus X (PVX)

	GarV-A (AB010300)	GarV-B (AB010301)	GarV-C (AB010302)	GarV-C[a] (D49443)	GarV-D (AB010303)	GarV-D[b] (L38892)	GarV-E (AJ292230)	GarV-X (U89243)	GarV-X (AJ292229)	ShV-X (L76292)	ShV-X (M97264)	PVX (D00344)
GarMbFV (X98991)	90.8	65.8	65.3	65.7	76.9	76.5	79.8	66.2	66.2	66.4	65.2	29.1
GarV-A (AB010300)		68.9	63.4	63.9	79.2	78.4	79.7	67.4	69.6	65.3	64.5	29.3
GarV-B (AB010301)			69.5	70.0	63.6	64.0	66.9	84.8	86.5	66.0	64.8	29.7
GarV-C (AB010302)				98.1	67.1	67.9	66.3	69.7	72.7	62.5	61.3	32.1
GarV-C[1] (D49443)					67.1	67.9	66.7	69.7	73.1	61.6	62.1	32.6
GarV-D (AB010303)						97.6	78.9	63.5	64.4	68.5	67.3	27.8
GarV-D[2] (L38892)							78.9	63.9	65.3	68.9	67.7	28.2
GarV-E (AJ292230)								69.3	69.9	67.9	67.1	29.1
GarV-X (U89243)									93.0	66.3	65.0	27.8
GarV-X (AJ292229)										67.5	66.3	30.4
ShV-X (L76292)											95.8	28.1
ShV-X (M97264)												28.1

[a]Named Garlic mite-borne virus
[b]Named Japanese garlic virus

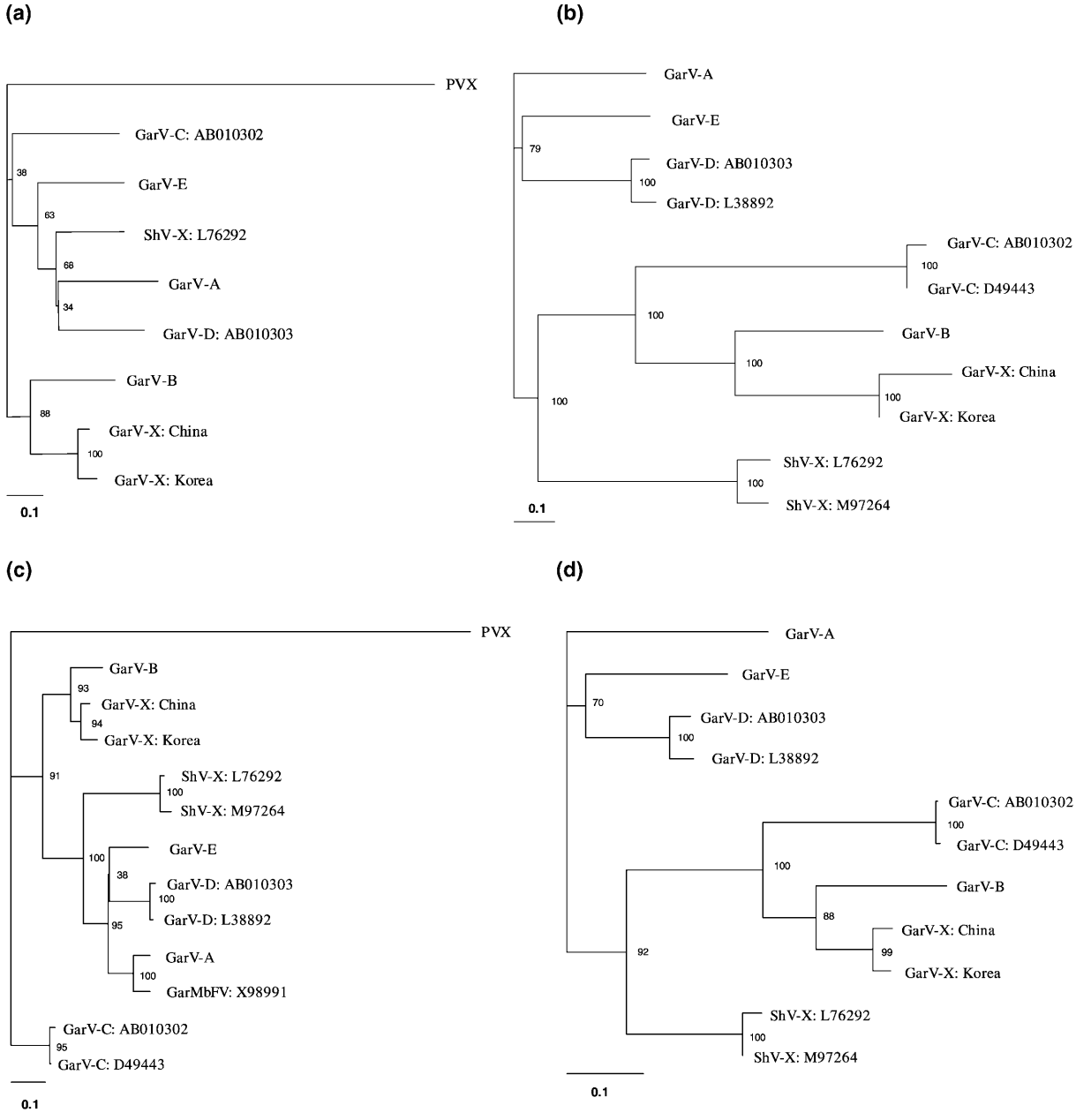

Fig. 2. Phylogenetic trees of the amino acid sequences of **a** the Triple Gene Block protein 1, ORF2 **b** the serine-rich protein, ORF4, **c** the coat protein, ORF5 and **d** the nucleic acid binding protein, ORF6, of members of the genus *Allexivirus* (Garlic viruses A, B, C, D, E and X; *Shallot virus X*) obtained using NEIGHBOR analysis. The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base. Trees **a** and **c** are rooted with the outgroup *Potato virus X* (PVX, accession no. D00344)

This protein, which seems to be the hallmark of allexiviruses, is extremely rich in serine (ca. 11%) and threonine (ca. 11%), and contains about 30 phosphorylation sites that may be recognized by kinase and phosphatase. If phosphorylated, the protein would carry lots of negative electric charges. It is the most variable of the virus proteins (Table 2). The 26–29 kDa CP (ORF5) is highly conserved, except at its N-terminus. ORF6 encodes a leucine-rich 14–15 kDa nucleic acid binding protein with similar putative zinc binding consensus, which suggests that it may be involved in regulation of RNA replication.

Potyviruses

After PCR amplification of cDNA, using Sprimer/M4 two specific bands of *c.* 2100 and 1800 bp were obtained. After cloning and partial sequencing, 12 independent clones of the larger band were all identified as an isolate of LYSV (named as LYSV-GYH). The 1800 bp band represented OYDV isolates; a total of 33 clones were partially sequenced which showed that there were three variations, named OYDV-YH1 (15/33 clones), YH2 (10/33) and YH3 (8/33). SYSV was not detected.

In the CP region, the new OYDV isolates had 80–98% identical amino acids when compared with all (14) other OYDV isolates, while LYSV-GYH (AJ292225) shared 78–92% identical amino acids with the 9 other LYSV isolates. LYSV and OYDV isolates are particularly variable, especially at the N-terminus of the CP. A phylogenetic tree was prepared using the CP amino acid sequences of all LYSV, OYDV and SYSV sequences (Fig. 3). The new sequences were most closely related to other isolates from China and Japan although OYDV-YH3 (AJ292224) was quite distinct from the YH1 (AJ292231) and YH2 (AJ292223) isolates.

General comments

In many cases, infection with one virus will protect plants from infection by a closely related virus (cross protection, see [7]). The effects are complex but clearly do not prevent infection of garlic plants by different isolates of the same virus (e.g. OYDV, LYSV or GLV) or closely related members of the same genus (e.g. GarV-X and GarV-E). In the study reported here RT-PCR amplification and sequencing of total plant RNA extracted from a single leaf confirmed their presence in the same plant. Virtually all samples examined contained mixtures of OYDV, LYSV and GLV isolates. The allexiviruses were present at lower concentrations but were also widespread.

Two thousand years ago, garlic from the Mediterranean region came into China via the “Silk Road”. Later, it was transported to Korea and Japan and is now widely planted in Asia. The garlic plants used in this study were from a typical farmhouse cultivar of southern China and produced small white bulbs which the farmers save for planting again the next year. These plants are unlikely to be closely related to Korean and Japanese cultivars but may have had a common ancestor. The viruses detected are all believed to be restricted to some *Allium* spp., and are not known to have any wild host species in Asia. It seems

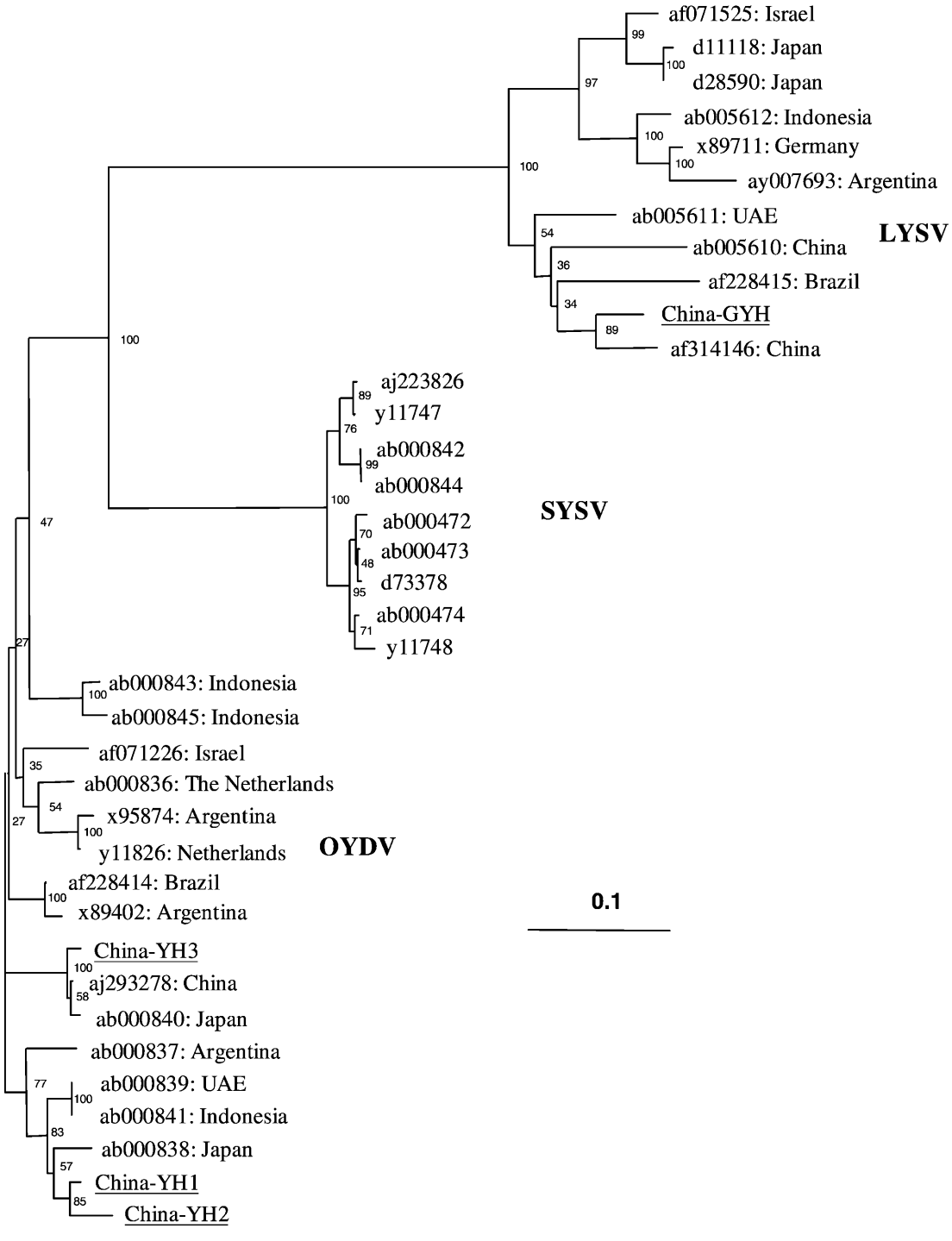

Fig. 3. Phylogenetic tree of the amino acid sequences of the coat proteins of *Leek yellow stripe virus* (LYSV), *Shallot yellow stripe virus* (SYSV) and *Onion yellow dwarf virus* (OYDV) isolates obtained using NEIGHBOR analysis. The values at the forks indicate the number of times out of 100 trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base

probable, therefore, that the viruses arrived in Asia with the host plants and that the substantial diversity that occurs amongst these species is a consequence of repeated vegetative propagation. This diversity is particularly seen amongst GLV isolates, but is also evident in the other genera. Our results are the first report of allexiviruses infecting garlic plants in China.

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