Phylogenetic analysis of allexiviruses identified on garlic from Australia

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Abstract Partial genome sequences with identity to members of the genus *Allexivirus* were described in Australia from *Allium sativum* L. (garlic). Phylogenetic analysis of coat protein and nucleotide binding protein gene sequences with those of 82 other allexivirus isolates confirmed that the Australian isolates were members of *Garlic virus A*, *Garlic virus B*, *Garlic virus C*, and *Garlic virus D*.

Keywords Garlic · Virus incursion · Horticulture · Next-generation sequencing

International trade and the vegetative means of propagating garlic (*Allium sativum* L.) favours dissemination of viruses and their accumulation in bulbs. Complex mixtures of viruses of the genera *Potyvirus*, family *Potyviridae*; *Carla-virus*, family *Betaflexiviridae*; and *Allexivirus*, family *Alphaflexiviridae*, occur frequently in *Allium sativum* L. (garlic) wherever it is grown, causing serious damage to crop yields and quality. The potyviruses onion yellow dwarf virus (OYDV) and leek yellow stripe virus (LYSV), and carlaviruses shallot latent virus (SLV) and garlic common latent virus (GCLV), were described on garlic from Australia (Sward 1990; M. Saqib, personal communication), but only recently were sequences representing allexiviruses identified there (Wylie et al. 2011). Here we compare the partial genome sequences of Australian allexivirus isolates with sequences of those from elsewhere in the world,

speculate on their geographical origin, and discuss possible implications of the incursion into Australia.

Isolation of viral RNA from a single garlic plant purchased from a hardware chain store in Perth, Australia, and determination of nucleotide (nt) sequences using a massively parallel sequencing approach, was described previously (Wylie et al. 2011). Along with other garlic viruses, four distinct nt sequences with identity to allexiviruses were obtained. The partial genomes obtained were from 2,880–7,708 nts in length, representing approximately 35–92% of complete genome sequences. Allexivirus isolate sequences used in this study were from Asia, Europe, South America, and Australasia (Table 1). The majority sequences used were from a previous study on isolates from China (Chen et al. 2004), and these represented a region of ~723–770 nucleotides (nt) of the 3' region of the coat protein (CP) gene and the 5' region of the nucleotide-binding protein (NTBP). All 86 allexivirus sequences on GenBank were globally aligned using Geneious Pro v5.3 (Drummond et al. 2011) to give an alignment of 723 nt. A tree was constructed in MEGA 5 (Tamura et al. 2011) using the Neighbor-Joining method and the Maximum Composite Likelihood substitution model. Data were bootstrapped with 1,000 resamplings to assess the robustness of the lineages in the trees. The homologous region of an isolate of *Pepino mosaic virus* (family *Alphaflexiviridae*, genus *Potexvirus*) was provided as the outgroup.

The allexivirus sequences from Australia grouped with members of four allexivirus species: *Garlic virus A*, *Garlic virus B*, *Garlic virus C*, and *Garlic virus D* (Fig. 1). Australian isolate GarVA-Bate1 shared greatest nt identity to Chinese GarVA isolates SS and LZ (85.7%) from Guangdong and Guangxi, respectively, and least identity (76.8%) with isolate CFH from Mongolia. When the available sequence of GarVA-Bate1 (4,363 nt representing the partial replicase module, triple gene block (TGB) 1 and 2, 40 kDa

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Table 1 Allexvirus sequences used

Virus ^a	Isolate name (if given)	Location	Length of sequence. Nucleotides	Host species	GenBank accession
GarMbFV	–	Argentina	762	<i>Allium sativum</i>	X98991
GarMbFV	–	Korea	723	<i>A. sativum</i>	AY390254
GarMbMV	GM88	Japan	2,518	<i>A. sativum</i>	D49443
GarVA	–	Japan	8,660 ^c	<i>A. sativum</i>	AB010300
GarVA	–	Korea	1,573	<i>A. sativum</i>	AF478197
GarVA	Bate1	Australia, WA ^b	4,363	<i>A. sativum</i>	JN019812
GarVA	BT	China: Mongolia	755	<i>A. sativum</i>	AJ551468
GarVA	CFH	China: Mongolia	755	<i>A. sativum</i>	AJ551467
GarVA	CFL	China: Mongolia	755	<i>A. sativum</i>	AJ551469
GarVA	CS1	China: Hunan	755	<i>A. sativum</i>	AJ551470
GarVA	CS2	China: Hunan	755	<i>A. sativum</i>	AJ551471
GarVA	HK	China: Hainan	755	<i>A. sativum</i>	AJ551472
GarVA	LA	China: Anhui	755	<i>A. sativum</i>	AJ551473
GarVA	LZ	China: Guangxi	755	<i>A. sativum</i>	AJ551474
GarVA	SS	China: Gungdong	755	<i>A. sativum</i>	AJ551475
GarVA	WH	China: Hubei	755	<i>A. sativum</i>	AJ551476
GarVA	YY1	China: Jiangxi	755	<i>A. sativum</i>	AJ551477
GarVA	YY2	China: Jiangxi	755	<i>A. sativum</i>	AJ551478
GarVB	–	Japan	5,077	<i>A. sativum</i>	AB010301
GarVB	–	Korea	1,373	<i>A. sativum</i>	AF543829
GarVB	Bate1	Australia, WA ^b	4,923	<i>A. sativum</i>	JN019813
GarVB	TS1-1	China: Gansu	772	<i>A. sativum</i>	AJ551479
GarVB	TS1-2	China: Gansu	772	<i>A. sativum</i>	AJ551480
GarVB	TS2	China: Gansu	772	<i>A. sativum</i>	AJ551481
GarVB	YC	China: Ningxia Hui	772	<i>A. sativum</i>	AJ551482
GarVC	–	Japan	8,405 ^c	<i>A. sativum</i>	AB010302
GarVC	–	Argentina	777	<i>A. sativum</i>	HM777004
GarVC	Bate1	Australia, WA ^b	7,708	<i>A. sativum</i>	JN019814
GarVC	GarV-6	Spain	771	<i>A. sativum</i>	HQ724845
GarVC	GarV-9	Spain	771	<i>A. sativum</i>	HQ724848
GarVD	–	Japan	4,497	<i>A. sativum</i>	AB010303
GarVD	–	Korea	1,544	<i>A. sativum</i>	AF519572
GarVD	AB	China: Sichuan	755	<i>A. sativum</i>	AJ551483
GarVD	Bate1	Australia, WA ^b	2,280	<i>A. sativum</i>	JN019815
GarVD	CFH	China: Mongolia	755	<i>A. sativum</i>	AJ551484
GarVD	CFL	China: Mongolia	755	<i>A. sativum</i>	AJ551485
GarVD	DL	China: Yunnan	755	<i>A. sativum</i>	AJ551486
GarVD	GarV-1	Spain	755	<i>A. sativum</i>	HQ724840
GarVD	GarV-2	Spain	755	<i>A. sativum</i>	HQ724841
GarVD	GarV-3	Spain	755	<i>A. sativum</i>	HQ724842
GarVD	GarV-4	Spain	756	<i>A. sativum</i>	HQ724843
GarVD	GarV-5	Spain	755	<i>A. sativum</i>	HQ724844
GarVD	GarV-7	Spain	755	<i>A. sativum</i>	HQ724846
GarVD	GarV-8	Spain	755	<i>A. sativum</i>	HQ724847
GarVD	HK	China: Hainan	755	<i>A. sativum</i>	AJ551487
GarVD	LA	China: Anhui	755	<i>A. sativum</i>	AJ551488
GarVD	LZ	China: Guangxi	755	<i>A. sativum</i>	AJ551489
GarVD	SS	China: Guangdong	755	<i>A. sativum</i>	AJ551490
GarVD	TM	China: Jilin	755	<i>A. sativum</i>	AJ551491
GarVD	WH	China: Hubei	752	<i>A. sativum</i>	AJ551492
GarVD	XA	China: Shanxi	755	<i>A. sativum</i>	AJ551493

Table 1 (continued)

Virus ^a	Isolate name (if given)	Location	Length of sequence. Nucleotides	Host species	GenBank accession
GarVD	XX	China: Henan	755	<i>A. sativum</i>	AJ551494
GarVD	YY1	China: Jiangxi	755	<i>A. sativum</i>	AJ551495
GarVD	YY2	China: Jiangxi	755	<i>A. sativum</i>	AJ551496
GarVD	ZHaZ	China: Fujian	755	<i>A. sativum</i>	AJ551497
GarVE	CFH	China: Mongolia	755	<i>A. sativum</i>	AJ551498
GarVE	CS2	China: Hunan	752	<i>A. sativum</i>	AJ551499
GarVE	TS1	China: Gansu	752	<i>A. sativum</i>	AJ551500
GarVE	TS2	China: Gansu	752	<i>A. sativum</i>	AJ551501
GarVE	YC	China: Ningxi Hui	752	<i>A. sativum</i>	AJ551502
GarVE	YH	China: Zhejiang	8,451 ^c	<i>A. sativum</i>	AJ292230
GarVX	267-GVX1	Italy	732	<i>A. sativum</i>	GQ475424
GarVX	405-GVX1	Italy	732	<i>A. sativum</i>	GQ475425
GarVX	407-GVX1	Italy	732	<i>A. sativum</i>	GQ475426
GarVX	AB1	China: Sichuan	767	<i>A. sativum</i>	AJ551503
GarVX	AB2	China: Sichuan	765	<i>A. sativum</i>	AJ551504
GarVX	BD	China: Hebei	767	<i>A. sativum</i>	AJ551505
GarVX	BT	China: Mongolia	766	<i>A. sativum</i>	AJ551506
GarVX	CQ	China: Sichuan	767	<i>A. sativum</i>	AJ551507
GarVX	CS1-1	China: Hunan	765	<i>A. sativum</i>	AJ551508
GarVX	CS1-2	China: Hunan	769	<i>A. sativum</i>	AJ551509
GarVX	CS2-1	China: Hunan	769	<i>A. sativum</i>	AJ551511
GarVX	CS2-1	China: Hunan	741	<i>A. sativum</i>	AJ551510
GarVX	Delhi	India: Delhi	732	<i>A. sativum</i>	HQ822272
GarVX	DL	China: Yunnan	767	<i>A. sativum</i>	AJ551512
GarVX	GY	China: Guizhou	767	<i>A. sativum</i>	AJ551513
GarVX	JX	China: Shandong	765	<i>A. sativum</i>	AJ551514
GarVX	Korea	Korea	8,106 ^c	<i>A. sativum</i>	U89243
GarVX	LA	China: Anhui	767	<i>A. sativum</i>	AJ551515
GarVX	LZ	China: Guangxi	765	<i>A. sativum</i>	AJ551516
GarVX	NJ	China: Jiangsu	767	<i>A. sativum</i>	AJ551517
GarVX	TM	China: Jilin	766	<i>A. sativum</i>	AJ551518
GarVX	TP	China: Xinjiang	767	<i>A. sativum</i>	AJ551519
GarVX	TS2	China: Gansu	766	<i>A. sativum</i>	AJ551521
GarVX	XA	China: Shanxi	766	<i>A. sativum</i>	AJ551522
GarVX	XX	China: Henan	767	<i>A. sativum</i>	AJ551523
GarVX	YC	China: Ningxia Hui	766	<i>A. sativum</i>	AJ551524
GarVX	YH	China: Zhejiang	8,451 ^c	<i>A. sativum</i>	AJ292229
GarVX	ZhaZ	China: Fujian	767	<i>A. sativum</i>	AJ551525
GarVX	ZheZ	China: Henan	767	<i>A. sativum</i>	AJ551526
JGV	UK	UK	2,756	<i>A. sativum</i>	L38892
PepMV	P22	Poland	6,412 ^c	<i>Solanum lycopersicum</i>	HQ650560
ShMbLV	IPO-DLO	Netherlands	912	<i>Allium cepa</i> var. <i>aggregatum</i>	EU835196
ShVX	—	Russia	8,832 ^c	<i>A. cepa</i> var. <i>aggregatum</i>	M97264
ShVX	1995	Russia	2,452	<i>A. cepa</i> var. <i>aggregatum</i>	L76292
ShVX	Dindugal	India	1,222	<i>A. cepa</i> var. <i>aggregatum</i>	GQ268322
ShVX	NZ	New Zealand	912	<i>A. cepa</i> var. <i>aggregatum</i>	EU835197

^a Names given as in original publication or GenBank record. GarMbFV, *Garlic mite-borne filamentous virus*; GarMbMV, *Garlic mite-borne mosaic virus*; GarVA, *Garlic virus A*; GarVB, *Garlic virus B*; GarVC, *Garlic virus C*; GarVD, *Garlic virus D*; GarVE, *Garlic virus E*; GarVX, *Garlic virus X*; JGV, *Japanese garlic virus*; PepMV, *Pepino mosaic virus*; ShMbLV, *Shallot mite-borne latent virus*; ShVX, *Shallot virus X*

^b WA, Western Australia

^c Listed as complete genome sequence

Fig. 1 Phylogenetic tree of the nucleotide (nt) sequences representing the 3' region of the coat protein gene and 5' region of the nucleotide binding protein gene of 86 allelixivirus isolates, including four from Australia (boxed). Species groups are shown. Virus names are shown as in original publication or GenBank record with GenBank accession codes. GarMbFV, *Garlic mite-borne filamentous virus*; GarMbMV, *Garlic mite-borne mosaic virus*; GarVA, *Garlic virus A*; GarVB, *Garlic virus B*; GarVC, *Garlic virus C*; GarVD, *Garlic virus D*; GarVE, *Garlic virus E*; GarVX, *Garlic virus X*; JGV, *Japanese garlic virus*; ShMblV, *Shallot mite-borne latent virus*; ShVX, *Shallot virus X*. The sequence of PepMV, (*Pepino mosaic virus*, family *Alphaflexiviridae*, genus *Potexvirus*) is provided as the outgroup. The robustness of the tree was assessed by bootstrapping with 1,000 replications. Bootstrap values > 50% are shown. The tree was drawn to scale, with evolutionary distance used to infer the branch length in nt substitutions per site

protein gene, CP, NTBP, and 3' untranslated region (3' UTR)) was compared with the complete genome sequence of the Japanese isolate (8,660 nt, AB010300) they shared 83.7% identity over the entire homologous region, and their CP genes shared 85.1% identity. Australian isolate GarVB-Bate1 shared greatest identity (91.7%) with the Korean isolate, and least (90.0%) with isolate TS1-2 from China. When the available sequence of GarVB-Bate1 (4,923 nt, representing the partial replicase module, TGB1, TGB2, 40 kDa protein gene, CP, NTBP, and 3'UTR) was compared with the available sequence of the Japanese isolate (5,077 nt, AB010301), they shared 88.8% identity over the homologous region, and their CP genes shared identity of 85.1%. Isolates in the GarVB group aligned closely with those of GarVX (Fig. 1) and when the available sequence of isolate GarVB-Bate1 was compared to complete genomes of GarVX isolate YH from China (8,451 nt, AJ292229) and one from Korea (8,106 nt, U89243), it shared 70.9% and 68.9% identity with them, respectively. GarVC-Bate1 shared greatest identity (81.5%) with the GarVC isolate from Japan, and least (79.5%) with the isolate from Argentina. When the available sequence of GarVC-Bate1 (7,708 nt, representing the partial replicase module, TGB1, TGB2, 40 kDa protein gene, CP, NTBP, and 3'UTR), representing approximately 92% of its genome, was compared with the complete genome sequence of the Japanese isolate (8,405 nt, AB010302), they shared only 70.0% identity, while their complete CP genes shared 77.3%. Australian isolate GarVD-Bate1 shared greatest identity (93.8%) with a group of isolates from Spain (GarV-4, GarV-5, GarV-7, GarV-8), and least (84.9%) with isolate LA from China. When the available sequence of GarVD-Bate1 (2,880 nt, representing the partial TGB1, TGB2, 40 kDa protein gene, CP, NTBP, and 3'UTR) was compared with the partial genome sequence of the Japanese isolate of GarVD (4,494 nt, AB010303), they shared 87.6% identity over the homologous region, while their CP genes shared 89.8% identity.

This study confirmed the taxonomic placement of four allelixivirus isolates identified on garlic from Australia as members of GarVA, GarVB, GarVC, and GarVC. Overall,

sequences of the partial CP and NTBP gene region of Australian isolates shared greater than 76.8% identity with those of known members of the species. This figure is above the species delimitation point of 72% identity over the complete CP gene nt sequence proposed by Adams et al.

(2004). Unfortunately complete CP sequences were not available for most isolates listed on GenBank. When the complete CP gene sequences of the new isolates were compared to complete CP genes from Japanese isolates there was greater than 77% nt identity in each case, which confirmed identity of each new isolate at the species level.

GarVC-Bate1 was the most divergent isolate with only 70.0% nt identity over its 90%-complete genome sequence with the complete genome sequence of the isolate from Japan. Surprisingly, the large study of allexiviruses in China failed to detect GarVC in any of the 28 plants from 24 provinces that they tested (Chen et al. 2004), and no GarVC sequences from China are currently available on GenBank (Table 1). Conversely, GarVX was present in most samples tested in the Chinese study (Chen et al. 2004), but was absent in the Australian sample. The presence of GarVC and the absence of GarVX in the single garlic sample tested from Australia suggest that the origin of the new isolates may not be China. Similarly, the genetic distance of the Australian isolates from those from Japan suggests there is not the origin. Unfortunately, the data does not present a clear alternative origin. Despite this evidence, it is probable that China is the ultimate source of the Australian allexivirus isolates. This is because approximately 85% of fresh garlic consumed in Australia is imported from China (Hickey 2006) as viable bulbs, and the remainder is either imported from the Americas and Spain, or grown locally. Growing imported garlic cloves in home gardens and in nurseries for sale to home gardeners is a common practice that will establish any viruses infecting them in Australia. It is likely therefore that allexiviruses are already established throughout populated Australia and analysis of more garlic plants there should confirm this. The garlic industry in Australia is small, and growers are generally aware of the virus risks

associated with growing untested propagules (Sward and Brennan 1994), so it is unlikely that incursion into Australia by allexivirus isolates will have a great economic impact. Allexiviruses are mite-borne and have narrow host ranges, being restricted to alliums. As there are no indigenous members of the *Alliaceae* in Australia they are unlikely to have an impact on the native flora.

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