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ORIGINAL PAPER

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POPULATION GENETICS ANALYSIS OF *Garlic virus A*, *Garlic virus B*, *Garlic virus C* AND *Garlic virus X*

Maria Bereda, Elżbieta Paduch-Cichal✉

Department of Plant Pathology, Warsaw University of Life Sciences, Nowoursynowska 159, 02-776 Warsaw, Poland

ABSTRACT

Garlic virus A (GarV-A), *Garlic virus B* (GarV-B), *Garlic virus C* (GarV-C) and *Garlic virus X* (GarV-X) are members of the genus *Allexivirus* in the family *Alphaflexiviridae*. In this study, we collected 10, 30, 10 and 14 isolates of GarV-A, GarV-B, GarV-C and GarV-X, respectively, from different parts of Poland. All sequences of coat protein (CP) and nucleic-acid binding protein (NABP) regions of *Allexivirus* isolates available in GenBank were also included in this study. The nucleotide and amino acid sequences identities within each population differed substantially depending on the region of the genome and virus species. The results of selection pressure analysis showed that populations of each *Allexivirus* underwent negative selection, but the extent of the negative selection varied. It was also concluded that the GarV-A and GarV-C populations underwent a decrease in population size or balancing selection, while the GarV-B and GarV-X populations underwent an increase in population size. It was concluded that both populations of GarV-X evolved independently in each respective area, in contrast to populations of GarV-A, GarV-B and GarV-C.

Key words: allexiviruses, recombination, sequences similarities, phylogenetic analysis

INTRODUCTION

RNA viruses have high variation potential, because they generate a large population with high mutation and recombination rates [Domingo and Holland 1997, Drake and Holland 1999, Uzcategui et al. 2001]. To date, almost all studies on the mechanism of recombination in RNA viruses have supported a copy-choice model [Simon-Loriere and Holmes 2011]. Under this model, hybrid RNAs are formed when the viral RNA-dependent RNA polymerase (RdRp) complex, which lacks proofreading activity, switches from one RNA molecule to another. The fast and flexible evolution of RNA viruses creates populations, which are all different and which adapt rapidly to new hosts [Ohshima et al. 2010]. Also, this leads to the concept of RNA viruses being variants with little divergence of nucleotide sequences [Eigen and Biebricher1988].

Estimation of the variability of genetic structure and diversity of the plant virus population is important for better understanding the plant virus evolution and virus-plant interaction [Garcia-Arenal et al. 2003]. It is known that understanding the molecular variation of viruses is necessary to design knowledge-based strategies for their control.

The genus *Allexivirus* comprises eight species of viruses, which, by 2015, were thought to infect only members of the *Allium* genus [King et al. 2012]. However, Fidan et al. [2015] detected *Garlic virus D* in *Urginea maritime*. Further studies involving larger number of species of potential host plants could

✉ elzbieta_paduch_cichal@sggw.pl

provide new data on allexiviruses epidemiology and evolution. Allexiviruses were first detected in shallot in Russia [Vishnichenko et al. 1993]. They are now known to occur in various parts of the world.

Virions of the genus *Allexivirus* are non-enveloped, highly flexuous and filamentous, about 12 nm in diameter and about 800 nm in length. The genomic ssRNA is about 9.0 kb in length, containing six open reading frames (ORFs). ORF1 is subjected to direct translation to polymerase with the weight of 195 kDa, ORF2 and ORF3 encode two proteins (26 and 11 kDa) associated with the transport of viruses, and ORF4 encodes a protein (42 kDa), which possibly participates in the process of assembling an entire protein particle. *Via* the sgRNA, ORF5 encodes a capsid protein (28 kDa) and ORF6 encodes a protein (15 kDa) with an unspecified function containing domains of a zinc finger type binding DNA [Adams et al. 2004].

Many researchers have described the variability among the *Allexivirus* populations and the phylogenetic relationship between virus isolates [Chen and Chen 2002, Wylie et al. 2012, Mohammed et al. 2013, Oliveira et al. 2014, Wylie et al. 2014]. However, literature data contain only details about natural populations of GarV-D with their genetic diversity and recombination breakpoint analysis [Bereda et al. 2015]. In our previous research, no recombination events were found in CP sequences of GarV-D, whilst it was proven that NABP could undergo the diversifying selection inside a conservative zinc finger motif. The ratio of non-synonymous to synonymous polymorphic sites indicated that mostly purifying selection has acted within the analyzed genes of GarV-D.

In this study, we collected isolates of GarV-A, GarV-B, GarV-C and GarV-X and investigated their population genetics by recombination, phylogenetic, selection pressure and genetic differentiation analyses.

MATERIALS AND METHODS

Virus samples, RNA extraction, RT-PCR and sequence analysis. From 2012 to 2013, a total of 321 garlic samples were collected randomly from 26 fields located in five geographical districts of Poland: northern (Pomorskie province), east-central (Mazowieckie and Łódzkie provinces), west-central (Wielkopolskie province), southern (Małopolskie and Śląskie provinces) and south-western (Dolnośląskie and Opolskie provinces). Detection of viruses was first performed by DAS-ELISA in garlic leaf and bulb tissue with specific antibodies against GarV-A, GarV-B and GarV-C obtained from Leibniz Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The ELISA--positive samples were lyophilized and stored to further analysis.

Total RNA was extracted from positive samples using the silica capture (SC) method described originally by Boom et al. [1990] and adapted to the diagnosis of plant viruses by Malinowski [1997] and quantified by spectrophotometric measurement. RNA extracts were subjected to translation and amplification by reverse transcription-polymerase chain reaction (RT-PCR) using the Transcriptor One-Step RT-PCR Kit (Roche Applied Science, Germany). RT-PCR with total RNA and appropriate primers (Tab. 1) was used to confirm DAS-ELISA results and to detect and identify isolates of GarV-X in garlic plants. Now, for each virus species, a specific primer pair was designed by the authors from consensus sequences available in the GenBank sequence database and synthesized for amplifying the region including complete CP and NABP genes (Tab. 2). After RT-PCR, amplicons of the expected size were ligated to the pCRTM4--TOPO vector in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Two clones of each isolate were sequenced in both directions with universal T3 and T7 primers. The nucleotide sequences were determined using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were assembled using DNA Baser Sequence Assembler ver. 4 (HeracleBioSoft, Romania). Sequence alignments were constructed in MEGA ver. 7 [Kumar et al. 2016]. Sequences of other isolates used in this study, originating from different parts of the world, were retrieved from GenBank. Accession numbers for each published sequence of the viruses are provided in the phylogenetic trees (Fig. 1).

Measuring genetic diversity and phylogenetic analysis. Sequence similarity and identity analysis was performed in BioEdit [Hall 1999]. To measure the genetic diversity of the *Allexivirus* populations, we calculated the average genetic distance with MEGA7 [Kumar et al. 2016]. Sequences of CP and NABP

Table 1. Primer sequences used for detection and identification of GarV-A, GarV-B, GarV-C and GarV-X

Virus	Primer sequence (5′ to 3′)	Product size (bp)	Position on viral genome
GarV-A	ATGTCGAATCCAACTCAGTCG AGACCATGTTGGTGGCGCG	441	7611-7631 8090-8072
GarV-B	TGACGGGCAAACAGCAGAATAA ATATAGCTTAGCGGGTCCTTC	574	7518-7537 8131-8111
GarV-C	TTGCTACCACAATGGTTCCTC TACTGGCACGAGTTGGGAAT	673	7443-7463 8155-8136
GarV-X	GCGGTAATATCTGACACGCTCCA ACGTTAGCTTCACTGGGGTAGAATAT	386	521-543 954-929

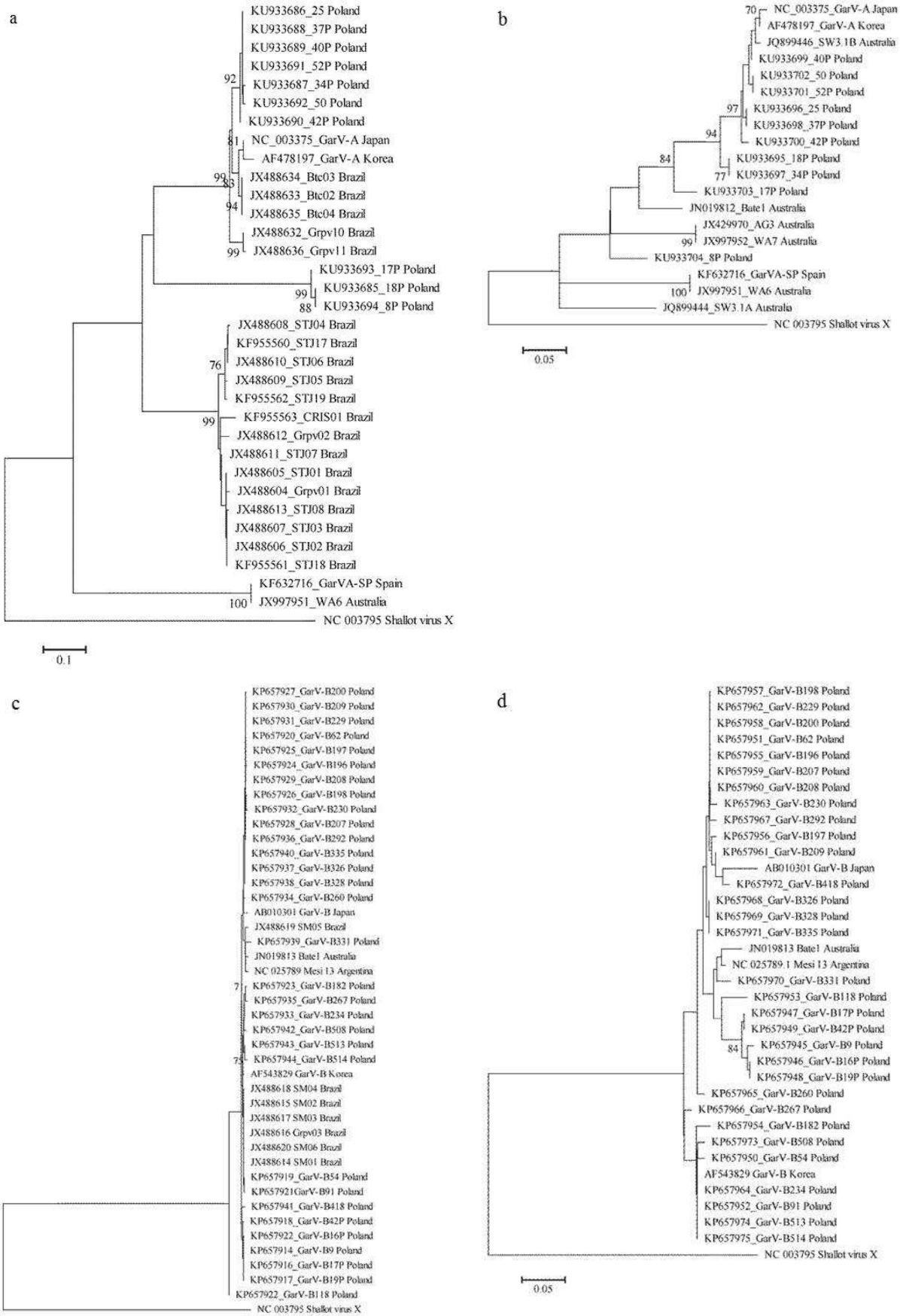
Table 2. Primer sequences used for amplifying the CP and NABP genes of GarV-A, GarV-B, GarV-C and GarV-X

Virus	Primer sequence	Product size (bp)	Position on viral genome
GarV-A	TGTCTCGCGCTCCTACATCAGAA TCTGGGGACAATAGTTGTTGCAAGGT	1330	7280-7302 8657-8632
GarV-B	TTGTGTTAAGTTTGGAYTTGGGTTGA TGATATCAACAGCATGGGTGTCTT	1216	7024-7049 8288-8265
GarV-C	AGTGATTTGSAMCCATAYCAAGC TAGTAATATCAACAAGCATGGGTGT	1557	6756-6778 8359-8335
GarV-X	ATCAGAGAYGARGTACTATGTTAAGT TTGTCCATGTCCAGAGCCCT	1195	6807-6832 8046-8027

regions were used to construct phylogenetic trees for the four allexiviruses. *Shallot virus X* was used as an outgroup. Phylogenetic trees were constructed with MEGA7 [Kumar et al. 2016] using the maximum-likelihood (ML) and neighbor joining (NJ) methods with 1000 bootstrap replications.

Recombination analysis and estimation of selection pressure. Putative recombination break point analysis was carried out using recombination detection methods implemented in RDP3 [Martin 2009]. Furthermore, GARD (genetic algorithm recombination detection) and SBP (single breakpoint recombination) implemented in the Datamonkey web server were used to determine whether recombination events are present in the studied sequences [Kosakovsky Pond and Frost 2005, Kosakovsky Pond et al. 2006]. The selection pressure was estimated by the dN/dS ratio. The value of dN represents the average number of non-synonymous substitutions per non-synonymous site, and dS is the average number of synonymous substitutions per synonymous site. Tests for selection were conducted using the SLAC (single--likelihood ancestor counting), FEL (fixed effects likelihood), IFEL (internal fixed effects likelihood) and REL (random effects likelihood) methods in the Datamonkey web server [Kosakovsky Pond et al. 2006].

Population demographic analysis. The software package DnaSP version 5 [Librado and Rozas 2009] was used to perform Tajima's D [Tajima 1989] and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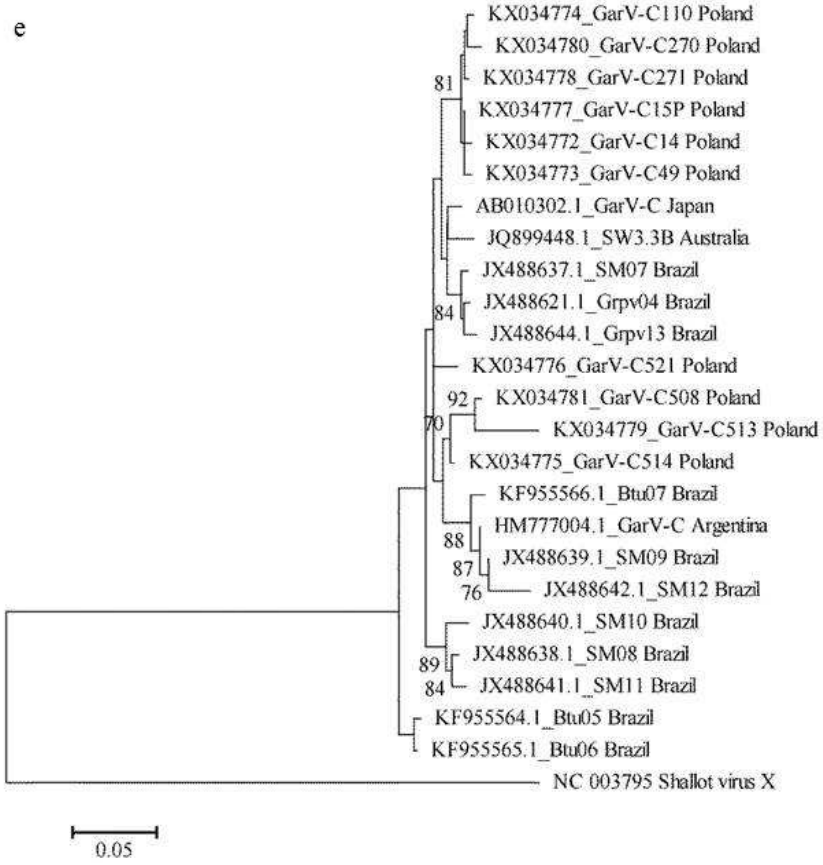
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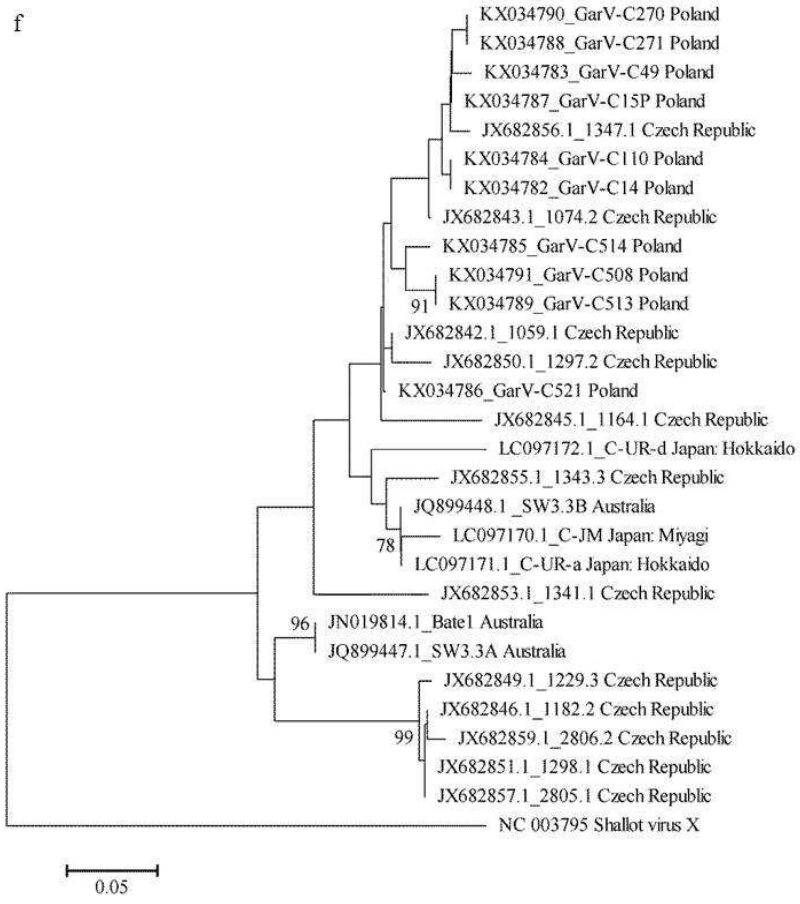
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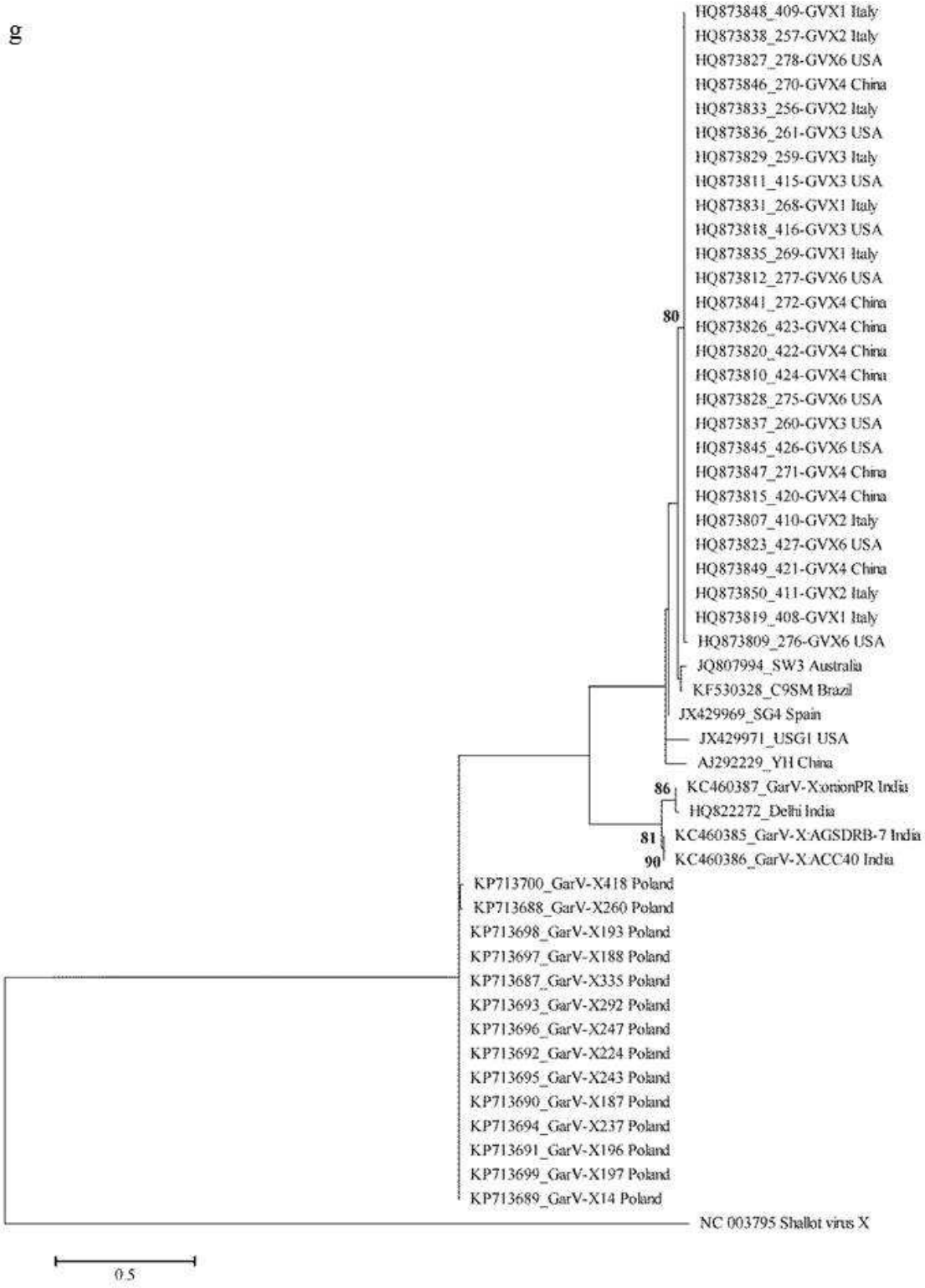
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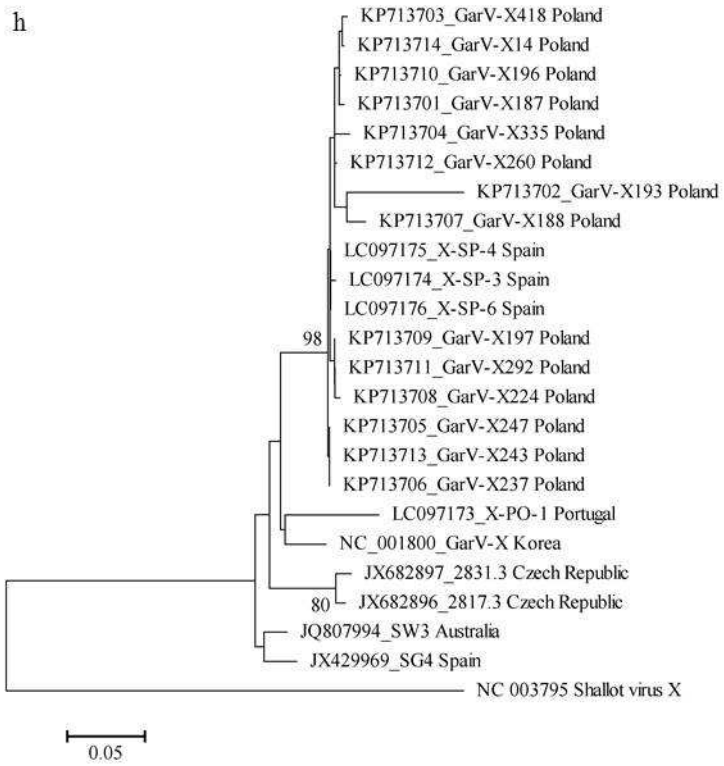

Fig. 1. Maximum likelihood phylogenetic trees of amino acid sequences of coat proteins and nucleic acid binding proteins of isolates of GarV-A (a, b), GarV-B (c, d), GarV-C (e, f) and GarV-X (g, h). The percentages of replicate maximum likelihood trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The corresponding sequence of S*hallot virus X* (ShVX) was used as an out-group

Fu and Li's D and F [Fu and Li 1993] statistical tests and to estimate the haplotype diversity. Tajima's D is based on the difference between the number of segregating sites and the average number of nucleotide differences. Fu and Li's D test is based on the difference between the numbers of singletons and the total numbers of mutations. Fu and Li's F test is based on the difference between the numbers of singletons and the average number of nt differences between each pair of sequences. Haplotype diversity was calculated based on the frequency and number of haplotypes in the populations.

RESULTS

Results obtained by DAS-ELISA and RT-PCR assays showed that the incidence of GarV-B and GarV-X was 64 and 59%, respectively, and these were the most abundant viruses in all sampled regions of Poland. GarV-A and GarV-C were detected and identified in five geographical districts of Poland in 41 and 26% of samples, respectively. From positive samples, 10, 31, 10 and 14 isolates were chosen from GarV-A, GarV-B, GarV-C and GarV-X populations for further investigations.

***Garlic virus A*.** In all ten GarV-A isolates, the length of the CP gene was found to be 759 nucleotides (nts). The length of the NABP gene was 393-396 nts depending on the virus isolate. CP and NABP genes display considerable variability among isolates (Tab. 3). The sequence diversity analysis confirmed that GarV-A has very high diversity in both CP and NABP regions (Tab. 4).

The results of recombination analysis showed no evidence of recombination events in 33 sequences analyzed. Further analysis of CP using the GARD and SBP methodologies did not show any recombination events in the analyzed gene. Tests for positive selection using SLAC, FEL, IFEL and REL confirmed these results. These algorithms did not indicate any codons under positive selection and revealed 29 common negatively selected sites.

Subsequently, when the recombination detection methods were used with default settings and a Bonferroni corrected P-value cut-off of 0.05, evidence of recombination events were detected in 3 of the 19 sequences analyzed. Table 5 lists the possible break point positions and their putative parental sequences along with methods of detection of potential recombinant sequences and associated P values. The SBP algorithm revealed evidence of recombination at position 210 in alignment of NABP sequences. The GARD algorithm was able to find a possible recombination site within GarV-A capsid sequences at position 144. The results from both experiments strongly suggest that the recombination event was located around positions 144-210.

The SLAC model revealed 11 sites under purifying selection, IFEL detected 28 codons under negative selection, FEL suggested 44 codons under negative selection, and the REL model indicated 48 sites. Moreover, two models, FEL and IFEL, indicated one common site (codon 41) under positive selection. Also, the SLAC, FEL, IFEL and REL models indicated one common site (codon 129) under positive selection (Tab. 6). Changes in the nucleotide sequences were observed in isolates WA7, WA6, SW3.1A, Bate1 and AG3 from Australia and GarVA-SP from Spain. The sequences of these isolates had deletions, including 1 (Bate1), 2 (WA6, GarVA-SP), or 3 (WA7, SW3.1A) nts. These changes concerned a fragment immediately upstream from codon 129.

We estimated the nt polymorphism of each population using the CP and NABP gene sequences distinguishing the Polish population from the GarV-A population and from other countries across the world using Tajima's D and Fu and Li's D and F statistical tests in order to discriminate between demographic expansion and contraction (Tab. 7). A negative result in these statistical tests indicates that the population is in a state of increase, while a positive value indicates a decrease in the population size or that there is balancing selection. All values were positive in the CP region in the GarV-A populations. On the other hand, the values were negative in the NABP region in the Polish population and Fu & Li's F and Tajima's D values in all the GenBank isolate populations.

Phylogenetic analysis of the CP amino acid sequences of GarV-A isolates comprising the Polish isolates collected in this study and those downloaded from GenBank showed that they were split into four distinct clusters. The first was an extensive group encompassing accessions reported in Poland along with sequences from Brazil, Korea and Japan. Three

Table 3. Sequence identities (%) of the nucleotides and amino acids of CP and NABP regions of GarV-A, GarV-B, GarV-C and GarV-X

Virus/Isolates	Polish		All (Polish + GenBank)	
	CP	NABP	CP	NABP
GarV-A	84-100 nt/95-100 aa	84-100 nt/85-100 aa	76-100 nt/85-100 aa	74-100 nt/76-100 aa
GarV-B	87-100 nt/93-100 aa	90-100 nt/91-100 aa	87-100 nt/93-100 aa	90-100 nt/91-100 aa
GarV-C	88-100 nt/89-100 aa	88-100 nt/90-100 aa	82-100 nt/83-100 aa	78-100 nt/83-100 aa
GarV-X	97-100 nt/98-100 aa	93-100 nt/91-100 aa	80-100 nt/89-100 aa	87-100 nt/aa

Table 4. Nucleotide distances of all *Allexivirus* populations

Virus	Genomic region used for analysis	Isolates	Average nucleotide distance	Standard errors
GarV-A	CP	Polish (*n = 10*)	0.073	0.005
		other (*n = 24*)	0.092	0.005
		all (*n = 34*)	0.107	0.006
	NABP	Polish (*n = 10*)	0.059	0.007
		other (*n = 9*)	0.171	0.011
		all (*n = 19*)	0.125	0.009
GarV-B	CP	Polish (*n = 31*)	0.086	0.006
		other (*n = 11*)	0.064	0.006
		all (*n = 42*)	0.089	0.006
	NABP	Polish (*n = 31*)	0.052	0.006
		other (*n = 4*)	0.066	0.009
		all (*n = 35*)	0.056	0.007
GarV-C	CP	Polish (*n = 10*)	0.063	0.006
		other (*n = 14*)	0.085	0.006
		all (*n = 24*)	0.086	0.005
	NABP	Polish (*n = 10*)	0.059	0.007
		other (*n = 18*)	0.140	0.012
		all (*n = 28*)	0.126	0.010
GarV-X	CP	Polish (*n = 14*)	0.007	0.002
		other (*n = 36*)	0.053	0.004
		all (*n = 50*)	0.068	0.005
	NABP	Polish (*n = 14*)	0.019	0.004
		other (*n = 3*)	0.049	0.009
		all (*n = 17*)	0.035	0.004

isolates from Poland have been separated, and other isolates from Brazil formed a separate group. Isolates from Australia and Spain were clearly separated from other isolates (Fig. 1a).

Phylogenetic analysis of the NABP amino acid sequences of GarV-A isolates split some of the isolates in the groups with a common ancestor. The first group formed isolates from Japan, Korea, Australia and Poland. The second and third groups included isolates from Poland. Also some groups included isolates from Australia, and Spain and Australia (Fig. 1b).

***Garlic virus B*.** The length of the nucleotide sequence of the 31 Polish GarV-B isolates was 735 nt in the CP and 384 nt in the NABP regions. The diversity of the CP and NABP nt and aa sequences of GarV-B isolates from Poland was equivalent to the diversity of all GarV-B isolates currently present in GenBank (Tab. 3). Sequence diversity analysis showed that the GarV-B population had high diversity in the CP and NABP regions (Tab. 4).

Based on the CP sequences of the GarV-B coat protein region, one recombinant from Poland (GarV-B418) was selected among the virus isolates (Tab. 5). SBP inferred a breakpoint at nucleotide 440, while GARD indicated the location of the breakpoint at nucleotide 276. The SLAC model revealed 73 sites under purifying selection, IFEL detected 96 codons under negative selection, and FEL and REL suggested 131 codons under negative selection. Furthermore, the dN/dS values of the CP gene were less than 1 (0.127), which means that the GarV-B population underwent negative selection.

Based on the NABP gene sequences, more than one recombinant sequence was detected among the GarV-B isolates. MaxChi and Chimaera algorithms found that the isolates GarV-B118, GarV-B16P, GarV-

Table 5. Breakpoint analysis using RDP3 of GarV-A, GarV-B, GarV-C and GarV-X CP and NABP sequences and their putative parental sequences

Recombinant isolate	Break points[1]		Major parent	Minor parent	Recombination P-value
	BN	EN			
GarV-A / NABP[2]					
17P	60	381	42P	Bate1	6.832×10^{-3}
34P	79	381	42P	Bate1	8.472×10^{-4}
18P	201	382	42P	Bate1	3.381×10^{-5}
GarV-B / CP					
GarV-B418	483	719	GarV-B9	GarV-B_Japonia	1.840×10^{-2}
GarV-B / NABP					
GarV-B118	104	271	GarV-B54	GarV-B_Japonia	>1.0
GarV-B16P	101	369	GarV-B54	GarV-B_Japonia	>1.0
GarV-B17P	101	367	GarV-B54	GarV-B_Japonia	>1.0
GarV-B19P	101	367	GarV-B54	GarV-B_Japonia	>1.0
GarV-B9P	104	271	GarV-B54	GarV-B_Japonia	>1.0
GarV-C / CP					
GarV-C513	732	197	GarV-C514	Unknown	6.742×10^{-5}
GarV-C / NABP					
GarV-C508	359	7	GarV-C513	Unknown	1.717×10^{-3}
GarV-X / NABP					
GarV-X193	279	340	GarV-X335	Unknown	7.795×10^{-5}

[1]Break points consist of the beginning nucleotide (BN) and the ending nucleotide (EN) of recombination fragment detected in the break point analysis

[2]Analysed virus species and genome fragment

Table 6. Positively selected sites in CP and NABP genes estimated by SLAC, FEL, IFEL and REL models

Virus	Partitions	Model	Codon	Base/amino acid changes	Normalized dN-dS	P-value/Posterior probability	Bayes factors
GarV-A	NABP	SLAC	129	CGT/Arg to TGT/Cys CGT/Arg to TTT/Phe TGT/Cys to TAT/Tyr TGT/Cys to TTA/Leu	10.8089	0.0769615	–
		FEL	41	GCT/Ala to TCT/Ser GCT/Ala to TTT/Phe ACT/Thr to GCT/Ala	2.65584	0.0545477	–
			129	CGT/Arg to TGT/Cys CGT/Arg to TTT/Phe TGT/Cys to TAT/Tyr TGT/Cys to TTA/Leu	6.15201	0.0442097	–
		IFEL	41	GCT/Ala to TCT/Ser GCT/Ala to TTT/Phe ACT/Thr to GCT/Ala	4.75297	0.0263979	–
			129	CGT/Arg to TTT/Phe CGT/Arg to TGT/Cys TGT/Cys to TAT/Tyr TGT/Cys to TTA/Leu	3.1531	0.0909411	–
		REL	129	CGT/Arg to TGT/Cys CGT/Arg to TTT/Phe TGT/Cys to TAT/Tyr TGT/Cys to TTA/Leu	1.28029	0.999889	22983.3
GarV-B	NABP	FEL	105 118	AAC/Asn to ACC/Thr GAC/Asp to AAC/Asn	5.89094 5.14017	0.0515903 0.0652836	–
		REL	105 118	AAC/Asn to ACC/Thr GAC/Asp to AAC/Asn	0.382109 0.37979	0.938123 0.937022	82.2026 80.6705
GarV-C	CP	FEL IFEL	146	TTT/Phe to TCT/Ser TTT/Phe to CAT/His TTT/Phe to TAT/Tyr	2.23955 4.66103	0.0929437 0.028243	– –
		REL	146	TTT/Phe to TCT/Ser TTT/Phe to CAT/His TTT/Phe to TAT/Tyr	−0.0890785	0.684162	51.0022
	NABP	FEL IFEL	18	GCT/Ala to ACT/Thr GCT/Ala to TCT/Ser ACT/Thr to TTA/Leu	1.88439 1.56118	0.0284682 0.0685398	– –
		REL	18	GCT/Ala to ACT/Thr GCT/Ala to TCT/Ser ACT/Thr to TTA/Leu	1.02375	0.999548	10711.5

– no data

B17P, GarV-B19P and GarV-B9P were recombinants of GarV-B54 and GarV-B_Japan, as major and minor parents, respectively. However, SBP and GARD algorithms did not show any recombination events in the analyzed gene. Further analysis with SLAC, IFEL, FEL and REL models revealed 15, 21, 28 and 9 sites under purifying selection, respectively. Besides codons under negative selection, FEL and REL algorithms indicated two common sites under positive selection (Tab. 6). The dN/dS value of the NABP gene was less than 1 (0.220), which means that the GarV-B population underwent negative selection.

The neutrality test showed that the GarV-B population was increasing, because all values were negative in the CP and NABP regions (Tab. 7).

The results of phylogenetic analysis performed on CP gene sequences of GarV-B isolates showed that they formed a unique group, regardless of geographical origin (Fig. 1c).

Phylogenetic analysis based on sequences of the NABP of Polish isolates and isolates from the rest of the world showed that they formed two main groups. Polish isolates are in both and in all subgroups where a foreign isolate is always present (Fig. 1d).

Garlic virus C. The longest nucleotide sequence, 780 nt long, was obtained for the CP gene of Polish GarV-C isolates. The NABP region of these isolates was 387 nt in length. The nucleotide sequence identity of the CP and NABP region between the Polish isolates and isolates from the rest of the world was low, which was confirmed by sequence diversity analysis (Tab. 3, Tab. 4). Values obtained for sequences of the NABP gene were very high, which means that global sequences of the GarV-C isolate population are strongly divergent.

Among GarV-C isolates, one recombinant was detected, based on alignment of CP gene sequences. When analyzing CP sequences of 24 GarV-C isolates, GarV-C513 from Poland was identified as a recombinant of proposed parents listed in Tab. 5. Subsequently, analysis of putative recombination events using SBP and GARD algorithms found

Table 7. Neutrality test and haplotype diversity of GarV-A, GarV-B, GarV-C and GarV-X populations

Virus	Gene	Isolates	Fu & Li's D	Fu & Li's F	Tajima's D	Number of haplotype	Haplotype diversity
GarV-A	CP	all	1.63611*	1.34703	0.18967	23	0.971 (0.00021)
		PL	1.42401*	1.58893	1.35093	7	1.42401(0.00598)
	NABP	all	0.01803	–0.16118	–0.50441	17	0.988(0.00044)
		PL	–0.36053	–0.39717	–0.32450	9	0.978(0.00292)
GarV-B	CP	all	–0.69536	–0.55276	–0.05903	40	0.997 (0.00004)
		PL	–0.61330	–0.46069	0.04879	29	0.994 (0.00012)
	NABP	all	–0.57348	–0.55839	–0.27467	29	0.983 (0.00017)
		PL	–0.75688	–0.65345	–0.14593	25	0.987 (0.00026)
GarV-C	CP	all	–0.12512	–0.23725	–0.36599	24	1.000 (0.00014)
		PL	–0.52300	–0.59329	–0.53024	10	1.000 (0.00200)
	NABP	all	0.19218	0.19303	0.10493	25	0.992 (0.00014)
		PL	–0.41950	–0.48659	–0.46410	9	0.978 (0.00292)
GarV-X	CP	all	–0.21565	–0.39790	–0.52370	32	0.911 (0.00133)
		PL	–1.92031	–2.07062	–1.49680	11	0.967 (0.00134)
	NABP	all	–1.72567	–1.95319	–1.58589	15	0.985 (0.00063)
		PL	–2.07613	–2.20277	–1.50040	12	0.978 (0.00119)

Numbers in parentheses are the standard errors
* $P < 0.05$

evidence of a recombination event around position 219-265 in the sequence alignment of the CP gene. The SLAC model revealed 29 negatively selected sites, IFEL detected 32 codons under negative selection, FEL found 39 codons under negative selection and REL indicated 29 sites. The dN/dS value determined by SLAC analysis was 0.158, which means that the GarV-C population underwent negative selection. However, the FEL, IFEL and REL models indicated one common site (codon 146) under positive selection (Tab. 6). Changes in the codon were observed in the first and the second position or in the second position. The presence of non-synonymous substitutions was detected in seven isolates, including three isolates from Poland, three from Brazil and one from Argentina.

Based on the sequences of the NABP gene, one recombinant from Poland (GarV-C508) was detected using GENECONV and Chimaera, with GarV-C513 as a major parent. However, SBP and GARD did not detect evidence of recombination, while the FEL, IFEL and REL models indicated one common site under positive selection (codon 18). Non-synonymous substitutions were detected in one sequence of Polish and Japanese isolates, in all three sequences of isolates from Australia and nine sequences of Czech isolates.

Results obtained for the CP and NABP regions of GarV-C isolates indicated that the Polish population was increasing, because Tajima's D, and Fu and Li's D and F values were negative. When analyzing the CP sequences of all isolates, the results were consistent with those obtained for Polish isolates and indicated that the GarV-C population was increasing. However, when the analysis was based on NABP sequences of all virus isolates, the values obtained for all three tests were positive, which means that the GarV-C population had undergone a decrease in size or balancing selection (Tab. 7).

Analysis of the phylogenetic relationship between the GarV-C isolates, based on amino acid sequences of the coat protein, revealed that most of the isolates from Poland and Brazil were grouped by origin. However, isolates from Japan and Argentina have a close common ancestor with, respectively, isolates from Australia and isolates from Brazil (Fig. 1e).

Phylogenetic analysis with amino acid sequences of NABP of GarV-C isolates identified that Czech isolates are closely related to Polish isolates and equally related to the isolates from Japan and Australia (Fig. 1f).

Garlic virus X. The length of nucleotide sequences of 14 Polish GarV-X isolates and isolates from other countries was 732 nt in the CP and 384 nt in the NABP regions. Comparison of the sequences of the CP gene of Polish isolates and isolates from other countries indicated very high diversity in the global GarV-X population (Tab. 3). Sequence diversity analysis confirmed the results obtained for identities (Tab. 4).

RDP analysis did not reveal any recombination breakpoint in CP gene sequences. Analysis with SBP and GARD algorithms did not indicate any recombination sites within the sequences of the CP gene of the GarV-X isolates. Also, the test performed to investigate the type of selection did not find that any of the codons are under positive selection. The REL algorithm revealed that no rates with dN > dS were inferred for these sequences, suggesting that all sites were under purifying selection. SLAC, FEL and IFEL, on the other hand, found 31 common sites under negative selection, with the value of dN/dS = 0.110.

Based on NABP sequences, three methods – GENECONV, BootScan and 3SEQ – implemented in RDP3 detected the common recombinant GarV-X193 with the proposed parental sequence of the GarV-X335 isolate. The SBP algorithm detected evidence of a recombination event at position 261, whereas GARD did not indicate putative recombination in the gene analyzed. Also, all algorithms – SLAC, FEL, IFEL and REL – indicated negatively selected sites only.

The values in three statistical tests were negative in the CP and NABP regions in both GarV-X populations, which means that the size of the virus population was increasing (Tab. 7).

Phylogenetic analysis based on the CP sequences of GarV-X isolates showed that 14 Polish isolates were separated from the isolates from other countries, which formed a large group including two separate subgroups. The first subgroup included all isolates originating from China, Italy, the USA, Australia, Brazil and Spain, and the second subgroup contained isolates from India (including one isolate from onion) (Fig. 1g).

Phylogenetic analysis based on the NABP sequences of GarV-X isolates showed that isolates from Spain (X-SP-4, X-SP-3, X-SP-6) are closely

related to isolates from Poland. Another isolate from Spain (SG4) is closely related to the Australian isolate. This tree also revealed a close correlation between isolates from Portugal and Korea (Fig. 1h).

DISCUSSION

The genetic diversity and structure of *Garlic virus D* (GarV-D) has been investigated previously [Bereda et al. 2015]. In the present study, we investigated GarV-A, GarV-B, GarV-C and GarV-X population genetics. Among the isolates originating from Poland, the most similar within each species were those of GarV-X and GarV-D. Based on all available isolates, the most similar population consisted of GarV-B isolates. All GarV-A isolates (from this study and downloaded from GenBank) shared only 76−100% aa identity. These values are lower than the species delimitation point of 93% identity over the complete CP gene aa sequence defined by Chen et al. [2004].

Similarly, several studies also reported that strong genetic variability could be identified in the CP region of GarV-A and other *Allexivirus* isolates [Mohammed et al. 2013, Oliveira et al. 2014, Wylie et al. 2014].

The nucleotide changes in the sequences of CP and NABP genes of GarV-B and GarV-X isolates were only substitutions. Plant RNA and DNA viruses can produce a huge number of indel (insertion and deletion) mutants during genome replication [Eigen 1996]. In some circumstances, lethal mutants (e.g. deletion mutants) will constitute the majority of the viral population. These viruses are shorter than the wild type virus, and they replicate much faster [Moutailler et al. 2011]. In alignment of nucleotide sequences of the CP gene of GarV-A isolates, on the other hand, one deletion of 3 nucleotides (codon 47) was observed. However, in subsequent analyses this codon was not found to be under positive selection or to be a site of putative recombination. Also, in alignment of nucleotide sequences of the NABP gene of GarV-A isolates, deletions were found in 5 of 19 sequences of virus isolates. A deletion including 1-3 nts was located at the end of the NABP coding region. This deletion may correlate with codon 129, which in one isolate (Bate1) includes a deletion of 1 nt and undergoes positive selection. Two sequences of the CP gene of GarV-C isolates were shorter by 60 nts than other virus isolates.

Therefore, to study the genome organization of allexiviruses, it will be better to obtain and study the whole-genome sequences for *Allexivirus* isolates collected from many different areas of the world.

We performed phylogenetic analysis to investigate the possible relationship between the origin and genetic diversity of the *Allexivirus* isolates. Phylogenetic analysis of GarV-A and GarV-B isolates demonstrated that Polish isolates in the largest group were closely related to the isolates from Japan, Korea, Brazil and Australia. Also, the phylogenetic tree of GarV-C isolates based on CP sequences showed that Polish isolates were closely related to the isolates from Japan, Australia, Brazil and Argentina. In contrast, the phylogenetic analysis of the GarV-C NABP gene suggested that most Polish isolates are related to the isolates from the Czech Republic rather than to the isolates from Asia or Australia. Majority of the prior studies have indicated a lack of genetic differentiation and frequent gene flow of the genus *Allexivirus* among various countries or several areas in one country [Chen et al. 2004, Melo-Filho et al. 2004, Wylie et al. 2012, Wylie et al. 2014]. Our results showed that only GarV-X populations of isolates originating from Poland and isolates from other countries were genetically differentiated. Polish isolates were clearly separated on the phylogenetic tree based on CP sequences, but on the tree constructed on the basis of NABP gene sequences were placed in one group with isolates from Spain. Generally, populations of allexiviruses were mixed with isolates from different parts of the world, sometimes very distant parts of the world, such as isolates of GarV-A from Korea, Japan and Brazil or the isolate GarV-B418 from Poland and isolates from Japan, which formed one phylogenetic group. Interestingly, the GarV-B418 isolate was indicated as a recombinant of the isolate from Japan.

Based on the presented results, we conclude that the membership of isolates in the phylogenetic groups is not dependent on their origin. Similar results have been observed by other authors analyzing other plant viruses such as *Tomato spotted wilt virus* or *Sugarcane mosaic virus* [Lian et al. 2013, Moradi et al. 2017]. We suspect the reason for this phenomenon may be the exchange of garlic bulbs between countries and

high availability of reproductive material. Some garlic producers have imported bulbs for consumption used for cultivation. A lot of imported garlic offered on Polish market is often supplied illegally. This may result in the introduction of a new species or new virus isolates, which could pose a real threat to native plants in the future. Thus, intensive development of trade in plant material, especially reproductive material, which contributes significantly to the spread of plant pathogens, should be conducted while maintaining phytosanitary measures.

Recombination analysis of *Allexivirus* isolates identified a total of 12 recombination sequences, among GarV-A, GarV-C and GarV-X NABP sequences and GarV-B CP and NABP sequences. All recombination events were detected among Polish isolates. Major parents of detected recombin ants originated from Poland. We suspect that many newly introduced isolates could recombine with local or other introduced isolates in order to adapt to new environmental conditions. Garlic cultivars do not produce seeds; thus it is only vegetative propagated. Vegetative means of propagating garlic favor dissemination of viruses and their accumulation in bulbs. Therefore, detection of recombinant sequences among Polish isolates with parental sequences from Poland was expected.

Single-stranded RNA viruses use recombination to adapt the isolate to new environmental conditions. To date, recombination events have been detected in various species of the genus *Potyvirus* [Ohshima et al. 2002, Desbiez and Lecoq 2004, Chare and Holmes 2005, Seo et al. 2009, Olarte Castillo et al. 2011, Feng et al.2014, Bereda et al. 2015a].

Results of selection pressure analysis showed that populations of each *Allexivirus* species underwent the negative selection. This is reflected in the dN/dS values, which were below 1. Interestingly, higher dN/dS values were obtained for the NABP gene of the *Allexivirus* isolates. Bereda et al. [2015] also reported that the dN/dS value of the NABP gene was higher. Also, returning to studies carried out in 2015, we found that in the NABP gene of GarV-D all four models indicated one common site (codon 41) under positive selection [Bereda et al. 2015]. In this study, FEL and IFEL algorithms also revealed that codon 41 in the GarV-A NABP gene is under positive selection. This codon is part of a zinc-finger motif, a characteristic domain of allexiviruses. Further investigation should be based on this amino acid change and its effect on protein function, especially considering that the nucleic acid-binding protein of another virus (*Grapevine virus A*) is able to suppress RNA silencing [Zhou et al. 2006].

These analyses support the conclusion that the majority of mutations occurring in the analyzed sequences are deleterious. Our results support the hypothesis of the domination of purifying selection in the case of plant viruses [García-Arenal et al. 2001].

Moreover, previous finding was supported by the neutrality test for the CP and NABP genes. The values of three statistical tests performed using the CP and NABP genes of the GarV-B and GarV-X populations were negative, while those of the GarV-A population were negative in the NABP gene and positive in the CP gene. On the other hand, values of the GarV-C isolates were reversed, i.e. based on the CP gene, the value obtained was negative, and based on the NABP gene, it was positive. Overall, it was concluded that the GarV-A and GarV-C populations underwent a decrease in population size or balancing selection, while the GarV-B and GarV-X populations underwent an increase in the population size. Also, previous studies assessing the scale of garlic infection by viruses showed that GarV-B and GarV-X were the most common [Chodorska et al. 2014].

The allexiviruses have been detected relatively recently and are not a well-researched area. Therefore, we did not find any previous data on genetic populations of these viruses. All previous research was concerned with virus detection and phylogenetic analysis of collected isolates. The results obtained in this study suggest domination of purifying selection in *Allexivirus* evolution. Nevertheless, to carry out a complete analysis concerning the genetic diversity, origin, and mechanism of genetic exchange for allexiviruses, investigations based on the whole genome of each species are necessary.

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REFERENCES

Adams, M.J., Antoniw, J.F., Bar-Joseph, M., Brunt, A.A., Candresse, T., Foster, G.D., Martelli, G.P., Milne, R.G., Fauquet, C.M. (2004). The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Arch. Virol., 149, 1045–1060.

Bereda, M., Kalinowska, E., Paduch-Cichal, E., Szyndel, M.S. (2015). Low genetic diversity of a natural population of *Garlic virus D* from Poland. Eur. J. Plant Pathol., 142, 411–417.

Bereda, M., Paduch-Cichal, E., Kalinowska, E., Szyndel, M.S. (2015a). Genetic diversity and evidence of recombination in the coat protein gene of *Onion yellow dwarf virus.* Eur. J. Plant Pathol., 142, 377–387.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-Van Dillen, P.M.E., van der Nordaa, J., (1990). Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol., 28, 495–503.

Chare, E.R., Holmes, E.C. (2005). A phylogenetic survey of recombination frequency in plant RNA viruses. Arch. Virol., 151, 933–946.

Chen, J., Chen, J. (2002). Genome organization and phylogenetic tree analysis of *Garlic virus E,* a new member of genus *Allexivirus*. Chin. Sci. Bull., 47, 33–37.

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

Chodorska, M., Paduch-Cichal, E., Kalinowska, E., Szyndel, M.S. (2014). Assessment of allexiviruses infection in garlic plants in Poland. Acta Sci. Pol. Hortorum Cultus, 13(2), 179–186.

Desbiez, C., Lecoq, H. (2004). The nucleotide sequence of *Watermelon mosaic virus* (WMV, *Potyvirus*) reveals interspecific recombination between two related potyviruses in the 5′ part of the genome. Arch. Virol., 149, 1619–1632.

Domingo, E., Holland, J.J. (1997). RNA virus mutations and fitness for survival. Annu. Rev. Microbiol., 51, 151–178.

Drake, J.W., Holland, J.J. (1999). Mutation rates among RNA viruses. Proc. Natl. Acad. Sci., 96, 13910–13913.

Eigen, M., Biebricher, C.K. (1988). Sequence space and quasispecies distribution: In: RNA genetics, Domingo, E., Holland, J.J., Ahlquist, P. (ed.), vol. 3. CRC Press, Boca Raton, 211–245.

Eigen, M. (1996). On the nature of virus quasispecies. Trends Microbiol., 4, 216–218.

Feng, X., Poplawsky, A.R., Nikolaeva, O.V., Myers, J.R., Karasev, A.V. (2014). Recombinants of *Bean common mosaic virus* (BCMV) and genetic determinants of BCMV involved in overcoming resistance in common bean. Phytopathology, 104, 786–793.

Fidan, H., Çağlar, B.K., Baloğlu, S., Yılmaz, M.A. (2015). *Urginea maritime* (L.) is a new host of *Allexivirus* group on onion and garlic plants in Turkey. Acta Hortic., 1002, 309–312.

Fu, Y.X., Li, W.H. (1993). Statistical tests of neutrality of mutations. Genetics, 133, 693–709.

García-Arenal, F., Fraile, M., Malpica, J.M. (2001). Variability and genetic structure of plant virus populations. Annu. Rev. Phytopathol., 39, 157–186.

García-Arenal, F., Fraile, A., Malpica, J.M. (2003). Variation and evolution of plant virus populations. Int. Microbiol., 6, 225–232.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser., 41, 95–98.

King, A.M., Adams, M.J., Lefkowitz, E.J., Carstens, E.B. (2012). Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier.

Kosakovsky Pond, S.L., Frost, S.D.W. (2005). Datamonkey: rapid detection of selective pressures on individual sites of codon alignments. Bioinformatics, 21, 2531–2533.

Kosakovsky Pond, S.L., Posada, D., Gravenor, M.B., Woelk, C.H., Frost, S.D.W. (2006). Automated phylogenetic detection of recombination using a genetic algorithm. Mol. Biol. Evol., 23, 1891–1901.

Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol., 33(7), 1870–1874.

Lian, S., Lee, J., Cho, W., Yu, J., Kim, M., Choi, H., Kim, K. (2013). Phylogenetic and recombination analysis of *tomato spotted wilt virus.* PloS ONE, 8(5), e63380.

Librado, P., Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451–1452.

Malinowski, T. (1997). Silica capture-reverse transcription-polymerase chain reaction (SC-RT-PCR): application for the detection of several plant viruses. In: Diagnosis and identification of plant pathogens, proceedings of 4th international EFPP symposium Bonn, 9–12 September 1996, Dehne, H.W., Adam, G., Diekmann, M., Rahm, J., Mauler-Machnik, A., van Halteren, P. (eds.). Kluwer Academic Publishers, Budapest, 445–448 pp.

Martin, D.P. (2009). Recombination detection and analysis using RDP3. Methods Mol. Biol., 537, 185–205.

Melo-Filho, P.A., Nagata, T., Dusi, A.N., Buso, J.A., Torres, A.C., Eiras, M., Resende, R.O. (2004). Detection of three *Allexivirus* species infecting garlic in Brazil. Pesq. Agropec. Bras., 39, 375–340.

Mohammed, H.S., Zicca, S., Manglli, A., Mohamed, M.E., El Siddig, M.A.R., El Hussein, A.A., Tomassoli, L. (2013). Occurrence and phylogenetic analysis of *Potyviruses*, *Carlaviruses* and *Allexiviruses* in garlic in Sudan. J. Phytopathol, 161, 642–650.

Moradi, Z., Nazifi, E., Mehrvar, M. (2017). Occurrence and evolutionary analysis of coat protein gene sequences of Iranian isolates of *Sugarcane mosaic virus*. Plant Path. J., 33, 296–306.

Moutailler, S., Roche, B., Thiberge, J.M., Caro, V., Rougeon, F., Failloux, A.B. (2011). Host alternation is necessary to maintain the genome stability of Rift valley fever virus. PLoS Negl. Trop. Dis., 5, e1156.

Ohshima, K., Yamaguchi, Y., Hirota, R., Hamamoto, T., Tomimura, K., Tan, Z., et al. (2002). Molecular evolution of Turnip mosaic virus: evidence of host adaptation, genetic recombination and geographical spread. J. Gen. Virol., 83, 1511–1521.

Ohshima, K., Akaishi, S., Kajiyama, H., Koga, R., Gibbs, A.J. (2010). Evolutionary trajectory of turnip mosaic virus populations adapting to a new host. J. Gen. Virol., 91, 788–801.

Olarte Castillo, X.A., Ferminb, G., Tabimaa, J., Rojasb, Y., Tennantc, P.F., Fuchs, M., et al. (2011). Phylogeography and molecular epidemiology of Papaya ringspot virus. Virus Res., 159, 132–140.

Oliveira, M.L., De Marchi, D.R., Mituti, T., Pavan, M.A., Krause-Sakate, R. (2014). Identification and sequence analysis of five allexiviruses species infecting garlic crops in Brazil. Trop. Plant. Pathol., 39, 483–489.

Seo, J.K., Ohshima, K., Lee, H.G., So, M., Choi, H.S., Lee, S.H., Sohn, S.H., Kim, K.H. (2009). Molecular variability and genetic structure of the population of *Soybean mosaic virus* based on the analysis of complete genome sequences. Virology, 393, 91–103.

Simon-Loriere, E., Holmes, E.C. (2011). Why do RNA viruses recombine? Nat. Rev. Microbiol., 9, 617–626.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123, 585–595.

Uzcategui, N.Y., Camacho, D., Comach, G., Cuello de Uzcategui, R., Holmes, E.C., Gould, E.A. (2001). Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. J. Gen. Virol., 82, 2945–2953.

Vishnichenko, V.K., Konareva, T.N., Zavriev, S.K. (1993). A new filamentous virus in shallot. Plant Pathol., 42, 12–126.

Wylie, S.J., Li, H., Jones, M.G.K. (2012). Phylogenetic analysis of allexiviruses identified on garlic from Australia. Australas. Plant Dis. Notes, 7, 23–27.

Wylie, S.J., Li, H., Saqib, M., Jones, M.G.K. (2014). The global trade in fresh produce and the vagility of plant viruses: a case study in garlic. PloS ONE, 9, e105044.

Zhou, Z.S., Dell'Orco, M., Saldarelli, P., Turturo, C., Minafra, A., Martelli, G.P. (2006). Identification of an RNA silencing suppressor in the genome of *Grapevine virus A*. J. Gen. Virol., 87, 2387–2395.