



Phylogenetic and diversity analyses revealed that leek yellow stripe virus population consists of three types: S, L, and N

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Abstract

Phylogenetic and evolutionary analyses were performed on the P1 and CP genes of global isolates to clarify the phylogrouping of leek yellow stripe virus (LYSV, genus *Potyvirus*), a pathogen affecting *Allium* spp. worldwide, into different types based on genetic variation and host species. The constructed phylogenetic trees divided the isolates into three major groups: S, L, and N. Low nucleotide (nt) and amino acid (aa) percent identities among the three groups were observed on full ORF (75.4–99.0 and 79.1–99.0), P1 (59.1–98.3 and 36.8–98.3), and CP (76.6–100 and 75.7–100) coding regions. The dN/dS values of P1 and CP confirmed that both genes are under strong negative (purifying) selection pressure. Neutrality tests on Eastern Asian isolates suggested that the ancestors of current LYSV isolates evolved with garlic while they were in Asia before spreading to other world regions through garlic propagative materials. Genetic differentiation and gene flow analysis showed extremely frequent gene flow from S group to L and N groups, and these phylogroups differentiated from each other over time. Host differences, inconsistent serological test results, substantial nt and aa variation, and phylogenetic and diversity analyses in this study supported previous reports that LYSV can be separated into three major evolutionary lineages: S, L, and N types.

Keywords Diversity analysis · Phylogenetic analysis · Molecular identification · Population study · Selection pressure

Introduction

Allium spp. cultivations are affected by viruses from several genera [1–3]. Among these viruses, leek yellow stripe virus (LYSV, genus *Potyvirus*) is of particular interest because it

infects leek (*Allium ampeloprasum* L.), garlic (*Allium sativum* L.), and onion (*Allium cepa* L.) worldwide [4–7]. This virus' genome sequence is 10,131 nucleotide (nt) long with a single open reading frame (ORF) of 9,456 nt and encodes a polyprotein of 3,152 amino acids (aa), which is typical of *Potyvirus* [8].

Important information on population structures, new host adaptations, and evolutionary mechanisms of RNA viruses can be understood through molecular evolution studies [9,

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10]. In *Potyvirus*, the coat protein (CP) gene is commonly examined in phylogenetic and population studies since it is the most conserved region in the genome [11, 12]. In addition to the CP gene, the P1 gene which has the most variable nt sequences among *Potyvirus* genes is known to contribute key data for evolutionary analysis [13, 14].

The phylogeny of LYSV is divided into the following six groups according to the diversity of P1 gene: N, U, Sp, L, O, and S [15]. However, a recent report simplified this phylogeny into two major types: S and N [16]. The L group is restricted to isolates from naturally infected leeks only; however, this group is combined with the N group (isolates from garlic) into the N type due to the lack of available data on L isolate sequence [16]. Unfortunately, these phylogroups based on the P1 gene are not widely applied because molecular characterization works on LYSV mostly target the CP gene. *Allium* viruses could provide excellent data on the evolution of viruses spreading mainly through propagative materials; however, only a few evolutionary studies on these species have been conducted [17]. A small study involving 12 fully sequenced isolates found that selection pressures are higher on P1, CP, and P3 than on other coding regions of the LYSV genome [8]; nevertheless, this report still could not provide a complete picture of the virus evolution. In the present research, the nt sequences of P1 and CP genes of leek and garlic isolates were collected from several provinces of Turkey and other isolates from diverse regions of the world and different *Allium* spp. already registered in NCBI GenBank were analyzed to obtain insights into the global genomic diversity, evolution, and population structure of LYSV. The possibilities to separate the L group into a distinct type and to use the CP gene as the base for future LYSV phylogrouping were also investigated.

Materials and methods

RT-PCR and sequencing

Molecular examination against LYSV using RT-PCR was conducted on 10 leek and 3 garlic samples collected from Aydın, İzmir, Osmaniye, Bursa, Kastamonu, Çanakkale, and Düzce provinces of Turkey in 2007–2008 to increase the number of available nt sequences of L isolates for population analysis. The samples were positive for LYSV as tested by DAS-ELISA with standard manufacturer protocols (Bioreba, Switzerland) shortly after collection. They were then kept in desiccated leaf tissue over silica gel at 4 °C for long-term storage.

Total RNA was isolated from the samples using the CTAB method [18]. Complementary DNAs (cDNAs) were synthesized from the obtained total RNAs with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

F-5'ATCTCAACACAACCTTATGCAA3' plus R-5'GCACAC AACGACCGTCCAATCT3' [8], and F-5'TCACTGCAT ATGCGCACCAT3' plus R-5'GCACCATACAGTGAATTG AG3' [19] primer pairs were applied in the 1000 bp of partial P1 and 1020 bp of partial NIb + complete CP genes amplification, respectively, using 2X Emerald Master Mix (Takara, Japan) and MJ Mini Thermal Cycler (Biorad, USA).

Successfully amplified RT-PCR products were submitted to a commercial biotech company (BM Labosis, Ankara, Turkey) for bidirectional sequencing by the Sanger method. The obtained raw sequence data were assembled in CLC Main Workbench V.20 (<https://digitalinsights.qiagen.com/>) and then deposited into NCBI GenBank.

LYSV isolate selection and recombination analysis of CP gene

The nt sequence of P1 gene is 1290 nt long (encoding a protein of 430 aa) and covers positions 83–1372 nt, and that of CP gene is 867 nt long (encoding a protein of 289 aa) and covers positions 8879–9745 in the LYSV genome (RefSeq: LYSV-MG [KP258216]). However, large variations exist in nt and aa lengths due to indel mutations in some isolates.

Fourteen and thirty-five isolates with complete and partial nt sequences of P1 (for a total of forty-nine isolates), respectively, and eighty-one isolates with complete nt sequences of CP were obtained from NCBI GenBank. The sequences were aligned to novel Turkish isolates length and trimmed accordingly using ClustalW with default parameters in MEGA X [20] to create P1 and CP alignments. Recombination signals on each P1 or CP sequence alignment were scanned by Recombination Detection Program (RDP v.4.56). A credible recombination event should be supported by at least five of RDP, GENECONV, BootScan, MaxChi, ChiMaera, SiScan, and 3Seq algorithms with Bonferroni-corrected *P* value of <0.05 [21].

Analysis of phylogenetic and identity percentage of P1 and CP genes

Phylogenetic trees based on each P1 and CP nt sequence were constructed in MEGA X using the maximum likelihood statistical method. The best nt substitution model for both alignments was determined using the lowest Bayesian Information Criterion scores to be Tamura-Nei [22] and Gamma distributed with Invariant sites (TN93 + G + I). The statistical significance of isolate clusters was tested with 1000 bootstrap replicates. The furcation of major lineages was visualized using the neighbor-net method implemented in Splitstree4 freeware (version 4.17.1, built 28 Jun 2021) [23]. Percentages of nt and aa identity among phylogroups in P1, CP, and full ORF were estimated using Sequence Demarcation Tool v1.2 [24].

Amino acid variation in P1 and CP

Fourteen and thirty-nine isolates with complete P1 and CP sequences, respectively, were aligned with eleven novel Turkish isolates. Differences in the aa sequence of P1 and CP were examined using BioEdit v.7.2.5 [25].

Mean evolutionary distance and diversity

The mean evolutionary distance of the P1 and CP genes of LYSV within and between the phylogroups was calculated using the Tamura–Nei parameter [22] in MEGA X. Estimates of standard error (SE) were obtained by a bootstrap procedure of 1000 replicates.

Analysis of genetic diversity and polymorphism, neutral selection, and gene flow and genetic differentiation among populations

Genetic diversity and polymorphism were analyzed using DnaSP v.6.12.03 [26]. A gene is under positive (diversifying), neutral, or negative (purifying) selection when the dN/dS (ω ratio) is > 1 , $= 1$, or < 1 , respectively [26]. Three “neutrality tests” (Fu and Li’s F^* , Fu and Li’s D^* , and Tajima’s D) were carried out in DnaSP v.6.12.03 (default window length of 100 sites and step size of 25 sites; without out-group) [26]. Independent K_S^* , K_{ST}^* , Z^* , Snn, and F_{ST} test values [27, 28], which determine genetic differentiation in each P1 or CP population, were calculated by DnaSP v.6.12.03. K_{ST}^* is near zero when genetic differentiation does not exist (null hypothesis) [29]. A small Z^* indicates minimal genetic differentiation among populations [27]. Snn describes the range from exact same population (value of 0.5) (null hypothesis) to distinctly differentiated population (value of 1) [28]. The null hypothesis in K_S^* , K_{ST}^* , Z^* , and Snn is rejected by a significant P value [27–29]. F_{ST} ranges from the exact same population (value of 0) to fully distinct populations (value of 1) [27, 29]. F_{ST} (fixation index) > 0.33 usually indicates large gene flow and genetic differentiation between tested populations [30].

Results

RT-PCR and sequencing

Partial P1 sequences were obtained from 10 leek and 1 garlic isolates (Acc no. OL944631-OL944641), and complete CP sequences were obtained from 8 leek and 3 garlic isolates

(Acc no. OL944620-OL944630) (Supplementary material 1-Table 1).

Recombination analysis of the nt sequences of CP gene

Only the results of the recombination analysis of CP gene sequences were reported because the analysis of the highly variable P1 gene sequences did not generate reliable results. Recombinant signals in the CP gene of four isolates were detected by at least five statistical methods and supported by Bonferroni-corrected P value of < 0.05 . Recombinant events in isolate AG1 (JX429967) occurred in two different regions. Among novel Turkish isolates, LYSV-18 (OL944630) was determined to be recombinant (Table 1). These isolates with multiple parents were omitted from population analysis to produce accurate results.

Analysis of phylogenetic and identity percentage of P1 and CP genes

The constructed phylogenetic trees suggested that evolutionary processes occurred simultaneously and in the same direction throughout the LYSV genome because the P1 and CP trees consistently clustered all the isolates into three major lineages (S, L, and N). In addition, each isolate that provided the nt sequences of both genes was clustered in the identical group in both trees. In P1, the S-type lineage was further divided into two subtypes (named SP1a and SP1b) based on nt changes and deletions in the compared sequences. In CP, the S-type was separated into 10 subtypes (named SCPa–SCPj) (Fig. 1). In line with the results of recombinant analysis, LYSV-18 was positioned in S lineage in P1 and in L lineage in CP trees. Congruence between the two genes was further confirmed by SplitsTree network analysis, which trifurcated P1 and CP split trees into S, L, and N-types (Fig. 2).

Comparative analysis revealed a large variation in nt and aa sequence identities of P1, not only among S, L, and N but also within the S-type. For CP, the nt and aa identity percentages among S-type isolates were lower than those among N-type isolates. S-type isolates also had higher nt and aa differences than N-type isolates. Among the 92 complete CP sequences, the lowest nt identity percentage found among S-type and N-type isolates (76.6%) was close to the limit for *Potyvirus* species demarcation (76%), and the lowest aa identity percentage (75.7%) was well below this limit (80%) (Table 2). Further comparison on 16 complete ORF sequences determined that the lowest nt identity percentages for S-type versus N-type and L-type versus N-type isolates were also lower than the limits for *Potyvirus* species demarcation (Table 2).

Table 1 Recombination analysis of LYSV CP gene nucleotide sequences

No	Recombinant isolate (origin)	Parents: major (origin)/minor (origin)	Breakpoints ^a		RDP-implemented method ^b (<i>P</i> -value)
			begin	end	
1	OL944630 (LYSV-18) (Turkey)	OL944622 (LYSV-9) (Turkey)/unknown	8868	9460	B (2.845×10^{-12}) M (5.908×10^{-5}) C (2.018×10^{-2}) S (1.335×10^{-13}) 3S (7.991×10^{-12})
2	JX429967 (Australia)	AB194639 (Japan)/JQ899450 (Australia)	8712	8834	G (4.336×10^{-9}) B (5.846×10^{-2}) M (6.832×10^{-5}) C (6.486×10^{-5}) S (2.688×10^{-7}) 3S (7.905×10^{-8})
3	JX429967 (Australia)	D11118 (Japan)/GU373816 (China)	8884	8984	R (2.091×10^{-10}) G (3.706×10^{-9}) M (2.207×10^{-5}) C (1.959×10^{-5}) 3S (7.047×10^{-8})
4	MK336976 (Ethiopia)	MK336975 (Ethiopia)/unknown	9099	9546	R (3.876×10^{-2}) M (1.276×10^{-8}) C (1.547×10^{-5}) S (1.207×10^{-16}) 3S (5.869×10^{-03})
5	MT232838 (Iran)	AB194641 (China)/KF597283 (Mexico)	8702	8914	G (2.413×10^{-9}) M (1.243×10^{-8}) C (6.423×10^{-7}) S (8.943×10^{-16}) 3S (2.936×10^{-6})

^aNucleotide position according to RefSeq NC_004011 sequence

^bR RDP; G GENECOV; B BootScan; M MaxChi; C Chimaera; S Siscan; 3S 3 Seq

Amino acid variation in P1 and CP

The main aa changes in P1 among three LYSV types occurred throughout the coding sequence. SP1a isolates experienced large nt deletion at positions 320–523 (RefSeq: KP258216), resulting in the deletion of 68 aa residues (Supplementary material 2-Fig. 1). The nt and aa sequences of SP1b, SP1c, L, and S isolates in this position remained intact, but SP1b and SP1c had higher nt and aa identities with SP1a than L and S. In addition, L and N isolates experienced nt deletion at positions 128–142, resulting in the deletion of five aa residues. Meanwhile, the sequences of SP1a, SP1b, and SP1c in this region were complete. Therefore, SP1b and SP1c isolates could be an intermediate form in the evolution of the P1 gene of three LYSV types.

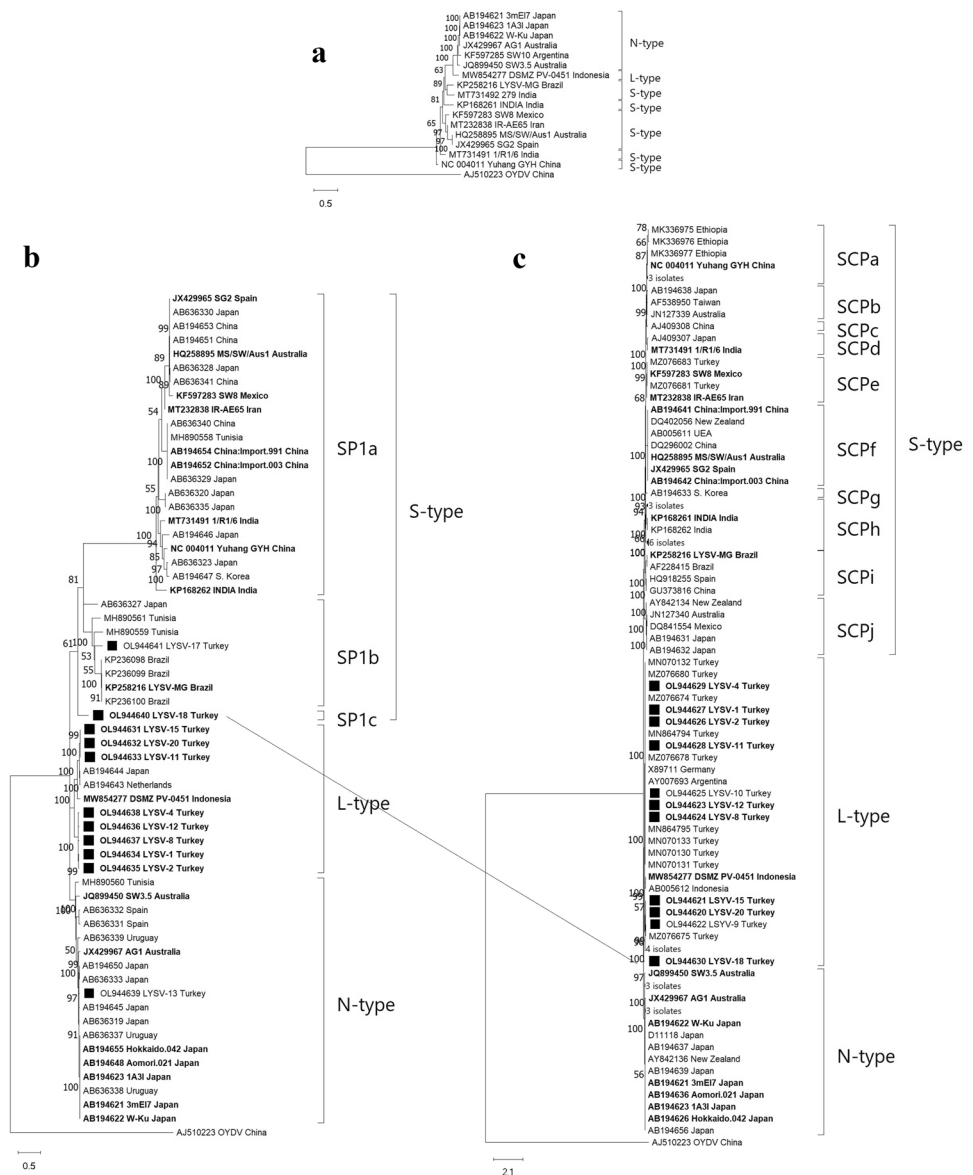
The aa changes in CP among three LYSV types mostly occurred on the 5' end half of the coding sequence, and the remaining half toward the 3' end was highly conserved. SCPa, SCPe, and SCPH isolates experienced deletion at

positions 9005–9007 nt (RefSeq: KP258216), resulting in single aa residue deletion at position 43 of the CP sequence. Deletion was also observed at positions 8987–8995 of the nt sequences of SCPj isolates, resulting in three aa residue deletions at positions 37–39 (Supplementary material 2- Fig. 2). DAG motif, which is important in *Potyvirus* transmission by aphids, was conserved in the CP of all tested isolates except for no. AJ409307, in which DAG was changed to DSG (Supplementary material 2- Fig. 2).

Mean evolutionary distance and diversity

The overall evolutionary distance of P1 was 0.590 ± 0.050 , and the values within main lineages were 0.570 ± 0.050 (S-type), 0.190 ± 0.020 (L type), and 0.090 ± 0.010 (N type). The evolutionary distance between phylogroups was 0.798 ± 0.070 (S/L type), 0.766 ± 0.068 (S/N type), and 0.452 ± 0.045 (L/N type). The overall mean evolutionary distance of CP was 0.230 ± 0.011 , and the values within

Fig. 1 Phylogenetic tree constructed by Maximum Likelihood test of nucleotide sequences of LYSV Complete ORF **a**, P1 gene **b**, and CP gene **c**. Isolates printed in bold were used in both P1 and CP comparisons. Novel Turkish isolates were indicated with black squares. Bootstrap values on each branch were supported by 1000 replicates; only values greater than 50% were shown. An onion yellow dwarf virus (OYDV, *Potyvirus*) isolate was used as out-group. The bars are distance scales that shows the genetic variation (distance) among isolates



main lineages were 0.230 ± 0.020 (S-type), 0.070 ± 0.010 (L-type), and 0.050 ± 0.008 (N-type). In addition, the evolutionary distance between the main phylogroups was 0.277 ± 0.019 (S/L type), 0.296 ± 0.0122 (S/N type), and 0.205 ± 0.019 (L/N type).

Genetic diversity and evolutionary selection pressure on the P1 and CP genes of LYSV

In general, π values were lower among the phylogroups and continental populations in the CP gene region compared with those in the P1 gene region. In P1, the highest π among the phylogroups and continent populations was 0.2883 (S-type) and 0.3614 (Africa), respectively, and the lowest was 0.0905 (N type) and 0.2684 (America). H_d values, which provide important information for predicting evolutionary processes,

reached 1.000 for all phylogroups and continental populations. Haplotype values were obtained in descending order for S (31), N (18), and L (11) groups. Meanwhile, the values of S and η parameters were the highest in other Asia and East Asia populations (Table 3). These results indicated high genetic diversities among global LYSV populations, with Asia as the diversity center.

In the analysis of CP gene, π ranged from 0.0446 (N type) to 0.1706 (East Asia). The highest π for phylogroup and continental populations was 0.1671 (S-type) and 0.1706 (East Asia), respectively. Although the number of isolates in the N group was quite large, the S, η , k, and π values were extremely low, suggesting scarce variation among isolates. For H_d , high values varying between 0.0998 and 1.000 were obtained for all phylogroups and continental populations (Table 3).

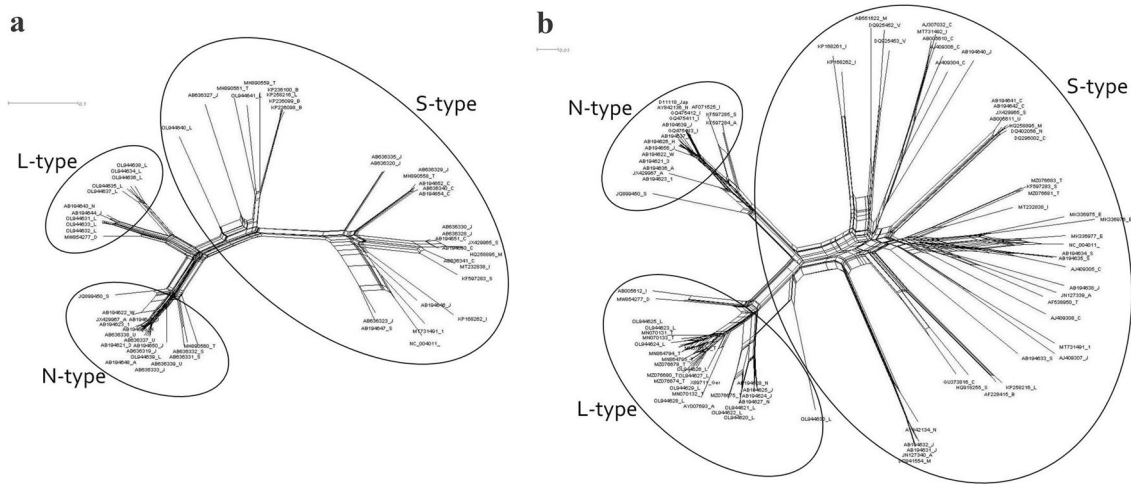


Fig. 2 Network visualization of leek yellow stripe virus (LYSV) isolates based on the **a** P1 and **b** CP genes analysis using the neighbor-net method in SplitsTree software. Both split trees revealed trifurcation of LYSV evolution into S, L, and N-types

Table 2 Identity percentage of nucleotide and amino acid sequences of different genomic regions among three LYSV types

Genomic region	Comparison	Identity (%)		Nucleotide identity threshold (%) for species discrimination [11]
		nt	aa	
Complete ORF (<i>n</i> = 16)	All	75.4–99.0	79.1–99.0	76.0
	S-type	89.7–98.6	93.3–98.6	
	L-type	100	100	
	N-type	77.8–99.0	84.3–99.0	
	S vs L	81.7–81.8	88.5–89.2	
	S vs N	75.6–78.2	79.3–83.9	
	L vs N	75.4–78.4	79.1–83.4	
Complete P1 (<i>n</i> = 14)	All	59.1–98.3	36.8–98.3	58.0
	S-type	67.8–98.4	58.1–98.3	
	L-type	100	100	
	N-type	86.1–98.3	81.6–98.1	
	S vs L	59.6–71.9	38.1–58.3	
	S vs N	59.1–71.4	36.8–53.9	
	L vs N	77.3–78.3	69.5–73.6	
Complete CP (<i>n</i> = 92)	All	76.6–100	75.7–100	76.0
	S-type	76.9–99.5	77.5–100	
	L-type	85.8–100	90.0–100	
	N-type	90.2–99.9	92.0–100	
	S vs L	77.3–83.8	75.7–84.6	
	S vs N	76.6–84.7	79.2–86.7	
	L vs N	82.9–85.9	86.2–91.7	

The demographic parameter π value for ORF was 0.1833 for S-type and 0.0699 for N type. Furthermore, the dN/dS ratios between phylogenetic groups and continental populations ranged from 0.3019 to 0.4358 for the P1 gene

and from 0.0884 to 0.1768 for the CP gene. In addition, the ω of ORF was 0.1237 (Table 3). All dN/dS results showed that the CP, P1, and ORF regions were under highly negative selection pressure.

Table 3 Summary of the genetic diversity and polymorphism analyses of partial P1 and complete CP gene sequences of LYSV from different populations

Population	N	h	Hd	S	η	k	π	dS	dN	ω
P1										
All	60	60	1.000	377	703	151.099	0.3243	0.6192	0.2444	0.3947
S- type	31	31	1.000	355	610	135.213	0.2883	0.5931	0.2056	0.3467
L-type	11	11	1.000	257	281	101.291	0.1373	0.2938	0.0959	0.3264
N-type	18	18	1.000	282	316	65.229	0.0905	0.1984	0.0599	0.3019
Regions¹										
East Asia	26	26	1.000	354	565	149.458	0.2815	0.5373	0.2115	0.3936
South-East Asia	1	nd	nd	nd	nd	nd	nd	nd	nd	nd
Other Asia	14	14	1.000	416	624	169.088	0.2759	0.5015	0.2148	0.4283
Oceania	3	3	1.000	239	256	165.000	0.2692	0.4869	0.2096	0.4305
Europe	4	4	1.000	261	316	157.167	0.2943	0.5296	0.2308	0.4358
Africa	4	4	1.000	275	345	169.500	0.3614	0.6868	0.2701	0.3933
America	8	8	1.000	289	347	142.500	0.2684	0.4815	0.2081	0.4321
CP										
All	88	88	1.000	458	743	141.218	0.1652	0.4737	0.0745	0.1573
S- type	43	43	1.000	421	642	142.823	0.1671	0.5026	0.0693	0.1379
L-type	28	27	0.998	232	275	54.995	0.0634	0.2127	0.0188	0.0884
N-type	17	17	1.000	175	184	38.647	0.0446	0.1411	0.0165	0.1169
Regions¹										
East Asia	31	31	1.000	391	573	145.839	0.1706	0.4916	0.0769	0.1564
South-East Asia	5	5	1.000	255	276	140.400	0.1625	0.4491	0.0794	0.1768
Other Asia	28	27	0.998	370	497	104.910	0.1214	0.3523	0.0529	0.1502
Oceania	8	8	1.000	308	392	144.222	0.1681	0.4799	0.0766	0.1596
Europe	8	8	1.000	277	324	122.714	0.1415	0.3878	0.0682	0.1759
Africa	2	2	1.000	118	122	80.000	0.0926	0.2862	0.0374	0.1307
America	6	6	1.000	263	311	137.000	0.1602	0.4788	0.0682	0.1424
ORF										
All	16	16	1.000	4387	6148	1830.617	0.1939	0.6054	0.0749	0.1237
S-type	9	9	1.000	3896	4962	1730.000	0.1833	0.6042	0.0614	0.1016
L-type	1	nd	nd	nd	nd	nd	nd	nd	nd	nd
N-type	6	6	1.000	1665	1736	674.867	0.0699	0.2231	0.0258	0.1156

¹East Asia: China, Japan, South Korea, Taiwan; South-East Asia: Indonesia, Myanmar, Vietnam; Other Asia: India, Iran, Israel, Turkey, UEA; Europe: Spain, Germany, Netherlands, Italy; America: Argentina, Brazil, Mexico, Uruguay; Oceania: Australia, New Zealand; Africa: Tunisia, Ethiopia

N number of isolates, *h* number of haplotypes, *Hd* haplotype diversity, *S* number of variable sites, η total number of mutations, *k* average number of nucleotide differences between sequences, π : nucleotide diversity (per site), *dN* non-synonymous nucleotide diversity, *dS* synonymous nucleotide diversity, ω dN/dS

nd not determined, at least two data sets are needed for calculation

Neutral selection analysis

Fu and Li's F^* & D^* assigned statistically nonsignificant negative or positive values to the P1 and CP of all populations. The results demonstrated that different LYSV populations are undergoing either balancing selection or bottlenecks. Negative values with statistical significance were only predicted by Tajima's D test for Europe and Africa populations in P1 (Table 4). Meanwhile, the positive values assigned to East Asia and America populations indicated that these species emerged from an excess of intermediate frequency alleles and can evolve out of

population balancing selection, bottlenecks, or structures (Table 4).

Gene flow and genetic differentiation among populations

Analysis of the gene flow and genetic differentiation of LYSV populations revealed that all phylogroups (S, L, and N types) in P1, CP, and ORF comparisons had K_S^* and K_{ST}^* values that were statistically significant and greater than 0. The K_S^* and K_{ST}^* results indicated statistically significant genetic differences between lineages at P1, CP, and ORF

Table 4 Summary of demography test statistics between LYSV populations, based on partial P1 and complete CP gene sequences comparison

Gene region	Tajima's <i>D</i>	Fu and Li <i>D</i> *	Fu and Li <i>F</i> *
P1			
All	0.00816 ns	0.18989 ns	0.14101 ns
S-type	− 0.44472 ns	− 0.12996 ns	− 0.28121 ns
L-type	0.26997 ns	− 0.19852 ns	− 0.08836 ns
N-type	− 1.23510 ns	− 1.07809 ns	− 1.30772 ns
Regions¹			
East Asia	0.03763 ns	0.12502 ns	0.11388 ns
South-East Asia	nd	nd	nd
Other Asia	− 0.62605 ns	− 0.22631 ns	− 0.38720 ns
Oceania	nd	nd	nd
Europe	− 0.92571***	− 0.36733 ns	− 0.50544 ns
Africa	− 1.04247***	− 0.34495 ns	− 0.50872 ns
America	0.35555 ns	0.27426 ns	0.32779 ns
CP			
All	− 0.10932 ns	− 0.13498 ns	− 0.14855 ns
S-type	− 0.10059 ns	0.32402 ns	0.19817 ns
L-type	− 0.83795 ns	− 1.11911 ns	− 1.21285 ns
N-type	− 1.17661 ns	− 1.01313 ns	− 1.23401 ns
Regions¹			
East Asia	0.06523 ns	0.48996 ns	0.40947 ns
South-East Asia	0.45640 ns	0.66386 ns	0.67838 ns
Other Asia	− 0.63866 ns	− 0.14124 ns	− 0.36622 ns
Oceania	− 0.00033 ns	0.52888 ns	0.45009 ns
Europe	− 0.09854 ns	0.06839 ns	0.03267 ns
Africa	nd	nd	nd
America	0.03798 ns	0.41558 ns	0.36855 ns
ORF			
All	− 0.05263 ns	0.12458 ns	0.08537 ns
S-type	− 0.27410 ns	0.04410 ns	− 0.03743 ns
N-type	− 0.73228 ns	− 0.66126 ns	− 0.74488 ns

¹East Asia: China, Japan, South Korea, Taiwan; South-East Asia: Indonesia, Myanmar, Vietnam; Other Asia: India, Iran, Israel, Turkey, UEA; Europe: Spain, Germany, Netherlands, Italy; America: Argentina, Brazil, Mexico, Uruguay; Oceania: Australia, New Zealand; Africa: Tunisia, Ethiopia

ns not significant

nd not determined, at least four data sets are needed for calculation

Statistical significance: Not significant, $P > 0.10$; Statistical significance: Not significant, $0.10 > P > 0.05$; *, $P < 0.05$; **, $P < 0.01$

levels and genetic difference between the three phylogroups, which was supported by the statistically significant maximum Snn values (1.000) for each region (Table 5). These findings confirmed the existence of phylogenetic lineages and that S, L, and N types differ from each other and have unique genetic characteristics. Among the LYSV lineage populations based on P1, CP, and ORF, significant Z^* values were calculated between all main lineages (S, L, and

N types). Furthermore, the Z^* value of the S-type for both genes was statistically significant and higher than that of other lineages. F_{ST} values between main lineages for all gene regions were greater than 0.33 (Table 5). These results indicated that the virus may be transmitted and spread by plant materials between geographic regions.

Discussion

Although early molecular studies on LYSV revealed that leek isolates form a distinct L group (from leek) [15, 31], the genomic data of leek isolates surprisingly remained scarce because most of the newly sequenced isolates were from garlic, which belongs to either the S or N group [1, 2, 4, 5, 32]. Therefore, this population study involving recent additions of complete CP gene sequences of L isolates from onion [33] and leek [7] and P1 and CP gene sequences of L isolates could help elucidate LYSV evolution. The new Turkish isolates shared high nt and aa identities and were dispersed in the same phylogroups of previously characterized Turkish isolates in the P1 and CP trees [7, 33] (Fig. 1). LYSV was identified for the first time in Aydın, İzmir, Osmaniye, Bursa, Kastamonu, and Düzce provinces of Turkey.

The previous P1 gene-based phylogenetic tree divides isolates into two major lineages (S and N types). The S-type is further split into S and O groups, and the N type into N, U, Sp and L groups [15]. However, this phylogrouping is not used widely because P1 is rarely targeted in the molecular detection of LYSV. Furthermore, phylogenetic analysis based on P1 is generally not reliable because the high variations in the gene could potentially generate new phylogroups every time new isolates are characterized. Given that the P1- and CP-based phylogenetic trees generated in the current study clustered isolates in the same manner, a new phylogrouping was proposed: isolates are simply clustered into S, L, and N types, and future phylogrouping of LYSV isolates should be based on CP, which is more conserved and frequently sequenced in LYSV characterization than P1.

Serological methods have low sensitivity for LYSV detection have been reported. Immunocapture-RT-PCR and standard RT-PCR are 10^2 – 10^4 times more sensitive than DAS-ELISA in LYSV detection [34]. One study described that DAS-ELISA results were erratic and unreliable for the detection of isolates no. GQ475411–13 (N type) but consistent and reliable for the detection of onion yellow dwarf virus (OYDV, genus *Potyvirus*) and garlic common latent virus (GarCLV, genus *Carlavirus*) [1]. DAS-ELISA was also found to be less sensitive than RT-PCR in the detection of isolates no. MN070130–31 and MN864794–95 (L-type) [33]. These phenomena could be caused by the antibodies used in DAS-ELISA that are produced from the CP of isolates of one of the types and failed to cross-react with

Table 5 Genetic differentiation estimates for LYSV lineages, based on the comparison partial P1 and complete CP gene sequences

Comparison	^a K_S^*	^a K_{ST}^*	^a Z^*	Snn	^b F_{ST}
P1 gene					
S-type (<i>n</i> = 31)/L-type (<i>n</i> = 11)	4.56981 (0.0000***)	0.06711 (0.0000***)	5.11292 (0.0000***)	1.00000 (0.0000***)	0.46419
S-type (<i>n</i> = 31)/N-type (<i>n</i> = 18)	4.29568 (0.0000***)	0.10724 (0.0000***)	5.31652 (0.0000***)	1.00000 (0.0000***)	0.53040
L-type (<i>n</i> = 18)/N-type (<i>n</i> = 25)	4.07356 (0.0000***)	0.12906 (0.0000***)	4.39955 (0.0000***)	1.00000 (0.0000***)	0.60744
Regions¹					
EastAsia (<i>n</i> = 26)/Other Asia (<i>n</i> = 14)	4.76785 (0.0000***)	5.48983 (0.0000***)	5.48983 (0.0020**)	0.82500 (0.0010**)	0.12394
EastAsia (<i>n</i> = 26)/Oceania (<i>n</i> = 3)	4.75023 (0.4960 ns)	− 0.00063 (0.4960 ns)	5.01308 (0.3990 ns)	0.91379 (0.0300*)	− 0.01830
EastAsia (<i>n</i> = 26)/Europe (<i>n</i> = 4)	4.75745 (0.2090 ns)	0.00486 (0.2090 ns)	5.06919 (0.2650 ns)	0.90000 (0.0220*)	0.00430
EastAsia (<i>n</i> = 26)/Africa (<i>n</i> = 4)	4.71040 (0.1130 ns)	0.00844 (0.1130 ns)	5.04565 (0.1250 ns)	0.93333 (0.0080**)	0.01854
EastAsia (<i>n</i> = 26)/America (<i>n</i> = 8)	4.69783 (0.0230*)	0.02095 (0.0230*)	5.25599 (0.0500 ns)	0.79902 (0.0240*)	0.09881
CP gene					
S-type (<i>n</i> = 43)/L-type (<i>n</i> = 28)	4.47571 (0.0000***)	0.07421 (0.0000***)	6.24524 (0.0000***)	1.00000 (0.0000***)	0.40952
S-type (<i>n</i> = 43)/N-type (<i>n</i> = 17)	4.48495 (0.0000***)	0.08135 (0.0000***)	5.88205 (0.0000***)	1.00000 (0.0000***)	0.47446
L-type (<i>n</i> = 28)/N-type (<i>n</i> = 17)	3.69765 (0.0000***)	0.14209 (0.0000***)	5.26988 (0.0000***)	1.00000 (0.0000***)	0.65472
Regions¹					
EastAsia (<i>n</i> = 31)/South-East Asia (<i>n</i> = 5)	4.81443 (0.0160*)	0.01288 (0.0160*)	5.38414 (0.0440*)	1.00000 (0.0000***)	0.11413
EastAsia (<i>n</i> = 31)/Other Asia (<i>n</i> = 28)	4.58418 (0.0000***)	0.04165 (0.0000***)	6.28347 (0.0000***)	0.86885 (0.0000***)	0.16604
EastAsia (<i>n</i> = 31)/Oceania (<i>n</i> = 8)	4.82480 (0.3630 ns)	0.00022 (0.3630 ns)	5.66711 (0.4120 ns)	0.6500 (0.4830 ns)	− 0.00944
EastAsia (<i>n</i> = 31)/Europe (<i>n</i> = 8)	4.74016 (0.0750 ns)	0.00964 (0.0750 ns)	5.52633 (0.0170*)	0.84615 (0.0080**)	0.04819
EastAsia (<i>n</i> = 31)/Africa (<i>n</i> = 2)	4.80248 (0.0180*)	0.00986 (0.0180*)	5.26597 (0.0160*)	0.92647 (0.0310*)	0.28008
EastAsia (<i>n</i> = 31)/America (<i>n</i> = 6)	4.79641 (0.0260*)	0.01148 (0.0260*)	5.45808 (0.0860 ns)	0.91892 (0.0040**)	0.04734
ORF					
S-type (<i>n</i> = 9)/N-type (<i>n</i> = 6)	6.95861 (0.0010**)	0.05648 (0.0010**)	2.90015 (0.0010**)	1.00000 (0.0010**)	0.46169

^a K_S^* , K_{ST}^* , Z^* and Snn are test statistics of genetic differentiation

^b F_{ST} is coefficient of gene differentiation, which measures inter-population diversity

¹East Asia: China, Japan, South Korea, Taiwan; South-East Asia: Indonesia, Myanmar, Vietnam; Other Asia: India, Iran, Israel, Turkey, UEA; Europe: Spain, Germany, Netherlands, Italy; America: Argentina, Brazil, Mexico, Uruguay; Oceania: Australia, New Zealand; Africa: Tunisia, Ethiopia

ns not significant

*0.01 < *P*-value > 0.05; **0.001 < *P*-value > 0.01; ****P*-value < 0.001

the other types due to large genomic sequence differences (Table 2). Therefore, specific antibodies for the detection of each LYSV type must be developed.

Among all the isolates, the mean evolutionary distance of P1 was significantly higher than that of CP. This finding indicated that the P1 gene region plays active roles in the formation of new types/variants of LYSV through host selection and environmental adaptation. The evolutionary processes of the P1 gene are highly affected by recombination and gene duplication and help potyviruses adapt to different host species [13]. In addition, the evolutionary distance for both gene regions was particularly high within the S-type lineage. The distance of S-type to L and N-types was also high, suggesting the larger genetic variation of S-type than that of L and N-types. The high evolutionary distance of S-type to L and N-types may be related to host species selection instead of geographic adaptation. Current attempts to identify resistant *Allium* spp. varieties only involved field observations [35,

36]. The findings of the present study showed that leek is highly resistant to S and N types and garlic is highly resistant to L type. This observation should be considered in future breeding programs using advanced DNA marker techniques.

The obtained ω values were all < 1, showing that the P1 and CP genes were both under negative (purifying) selection pressure, with the CP gene being under stronger pressure compared with the P1 gene. The evolutionary rates of P1 and CP genes were estimated to be 0.40 and 0.16, respectively, similar to those obtained in [8] at 0.49 and 0.19, respectively. Evolutionary analysis on soybean mosaic virus, another *Potyvirus*, also revealed that its CP gene (ω = 0.02) was more constrained for diversification than its P1 gene (ω = 0.2) [14].

In this study, neutrality tests performed on both genes assigned a positive number to Eastern Asian populations (China, Japan, South Korea, and Taiwan) and a negative number to other Asian, Oceanian, European, and African

populations (Table 4). Analysis of genetic differentiation and gene flow between East Asia versus other regions consistently produced F_{ST} values < 0.33 (Table 5). These results suggested that the ancestors of current LYSV isolates may have evolved with garlic while they were endemic in East Asia and then spread to different regions of the world through garlic propagation materials. This finding was in line with the conclusion in [15] and the assumption that the dissemination of garlic started from its genetic center in Central Asia and then spread to two major destinations: China/India and Europe/Russia [37]. In particular, S-type might represent the strained isolates showing strict host selection; so far, onion is not a natural host for these viruses because they were mostly detected in garlic and sometimes in leek (no. DQ925452-53) (Supplementary material 1-Table 1). The extensive adaptation of S-type isolates to garlic as a natural host was also confirmed in the phylogenetic analysis of the current study.

The large F_{ST} values between main nodes (S, L, and N) strongly supported the existence of three LYSV types, the high genetic diversity among them, and the high reliability rates of dendrograms created for both genes. These values also indicated a frequent gene flow from S-type to L and N-types, and these phylogroups differentiate from each other over time. Therefore, neutrality analysis may also corroborate the conclusion in [15] stating that the sequences missing in the P1 of S-type are present in the common ancestor of S and N types.

Conclusion

Host differences, inconsistent serological test results, substantial nt and aa variation, and phylogenetic and diversity analyses supported earlier studies on the separation of LYSV into three distinct evolutionary lineages: S, L, and N types [15, 31]. Recognition of these three types and other new variants that might emerge among LYSV isolates could lead to the development of accurate and specific diagnostic tools and the identification of resistant genes, which in turn will help epidemiologically control the virus.

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Author contributions All authors contributed to the study conception and design. P1 and CP gene sequence alignments and phylogenetic and evolutionary distance analyses were performed by AIS. Genetic diversity and evolutionary selection pressure, neutral selection, and genetic differentiation analyses were performed by FR-Z. DAS-ELISA, RT-PCR, and nucleotide sequencing were performed by AK. Sample collection and maintenance were performed by SK. Phylogenetic analysis was supervised by SH. Funding acquisition and experiments were supervised by FE. The first draft of the manuscript was written by AIS and FR-Z. All authors commented on previous versions of the manuscript and have read and approved the final manuscript.

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Data availability The data supporting the findings of this study are included in this published article (and/or) its supplementary materials. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest relevant to the content of this article.

Ethical approval This article does not contain any studies with the human participant or animal performed by any of the authors.

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