

# Genetic diversity and molecular variability of 'Candidatus Phytoplasma solani' based on multilocus sequence typing analysis in tomato plantations of western Turkey

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## ABSTRACT

'*Candidatus Phytoplasma solani*' (CaPso) is a very common pathogen in the Euro-Mediterranean regions and has been reported in many countries. Field surveys were conducted in tomato plantations in 2022 to determine the CaPso infections in the western part of Turkey, which has the highest vegetable production volume. A total of 67 tomato samples, 52 symptomatic and 15 asymptomatic, were collected from four different provinces. A total of thirteen CaPso strains were identified using molecular techniques from tomato samples. The *tuf/stamp/vmp1* genes molecular markers were used to perform molecular and genetic diversity characterizations of these strains. The *tuf-b1* genotype and five different *stamp* genotypes genetically closely related to Eastern European strains were determined in all strains. In addition, these strains showed more than 99% nucleotide sequence similarity with 4 different *vmp1* genotypes: V2-TA, V14, V4, and V15. Advanced bioinformatics analyses were performed with *stamp* (N = 290) and *vmp1* (N = 207) strains obtained from this study and GenBank. These analyses showed that the strains for both gene regions descended from two molecular evolutionary ancestors. In order to calculate the genetic distance relationships according to the *stamp* and *vmp1* gene regions, a hierarchical classification was done as geographical and host populations. While these analyses demonstrate the close genetic relationship between Turkish and Eastern European populations, they strongly confirm the importance of the epidemiological cycle between weed, perennial, and insect vectors. Then, molecular variance analyses (AMOVA) were performed on these classified populations. The results indicated high variation for both gene regions but showed that each strain contributed greatly to the CaPso genetic diversity. Thus, for the first time, multilocus sequence patterns of CaPso strains obtained from western Turkey were extracted. Extensive genetic analyses have strongly demonstrated the importance of horizontal transmission in the control and epidemiological cycle of CaPso.

## 1. Introduction

Tomato (*Solanum lycopersicum* L.), which is a member of the Solanaceae family, is one of the most cultivated vegetables in the world, thanks to its nutritious properties and unique aroma. The annual tomato production amount at the global level is 189 million tons, and this amount reaches 13 million tons in Turkey [1]. More than half of this production volume is carried out in the Aegean and Marmara regions, which are in the west of Turkey and where the Mediterranean climate zone is dominant.

Many phytopathogens such as viruses, fungi, bacteria, and phytoplasma, which adversely affect tomato production and yield, have been reported from different parts of the world. Among them, phytoplasmas

are bacterial plant pathogens belonging to the Firmicutes phylum (class; Mollicutes), a group of cell wall-less microorganisms genetically associated with a Gram-positive ancestor [2,3]. They are restricted to phloem tissue in plants, and they disseminate throughout the plant by migrating via the pores of the sieve plates that divide the phloem sieve tubes [3]. Insect vectors of phytoplasmas are sap-sucking hemipteran insects of the order Hemiptera, especially planthoppers (Fulgoroidea), leafhoppers (Cicadellidae), and psyllids (Psyllidae) [4]. Phytoplasmas induce disease in hundreds of plant species throughout the world, including many economically significant food, vegetable, and fruit crops, as well as ornamental plants [5]. Members of vegetable families such as Apiaceae, Asteraceae, Cucurbitaceae, Fabaceae, and Solanaceae are infected by phytoplasma groups that are geographically

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and genetically diverse and induce different and/or similar symptoms [6].

'*Candidatus* Phytoplasma solani' (CaPsoI) (subgroup 16SrXII-A), which has become quite widespread in the vineyards of the Euro-Mediterranean basin and poses a potential threat to viticulture worldwide, also causes significant infections in members of the Solanaceae and Apiaceae families [7–10]. Moreover, CaPsoI is associated with the grapevine yellow disease known as 'Bois Noir' (BN) in vineyards and 'stolbur' (STOL) disease in vegetable crop areas. Infections caused by STOL have been reported from different geographic regions of Turkey and various vegetable-growing areas such as tomato, tobacco, potato, and cucumber [11–14].

The housekeeping gene *tuf* (elongation factor Tu), which has two main types (*tuf* a/b genotypes) and one minor type (*tuf*-c genotype) throughout Europe, was one of the first STOL genes utilized in epidemiological studies [15–17]. A *tuf* type-b3 genotype was also determined in the vineyards of the Republic of Azerbaijan [18]. A *tuf* type-d genotype has recently been identified in Serbia from a variety of crops, including sugar beet, parsnip, and parsley [19]. More specifically, the *tuf*-b1 genotype was detected in chaste tree *Vitex agnus-castus*, bind-weed *Convolvulus arvensis* weeds, *Reptalus panzeri*, *Crepis foetida*, and *Hyalesthes obsoletus* insects [17,20]. The *tuf*-c genotype has been identified in hedge bindweed *Calystegia sepium* from a restricted region of Germany, while the *tuf*-a and b2 genotypes harbored by *Urtica dioica* have been reported to be transmitted only by *H. obsoletus* [16,17].

Based on the sequencing and amplification of non-ribosomal genes, multilocus sequencing analyses (MLSA) are widely performed to better understand the evolutionary processes, population structure, epidemiological cycles, and taxonomies of phytoplasmas [16,21,22]. Furthermore, the sequences of the *vmp1* gene, which codes for a variable membrane protein [23], and the *stamp* gene, which codes for the antigenic membrane protein AMP [24], have been used as molecular markers for insight into the genetic diversity and epidemiological cycle of BN, which has a devastating effect on vineyards [18,25–27]. Similarly, these molecular markers have been used to investigate the epidemiological pathway of 'Ca. P. solani' -associated rubbery taproot disease (RTD) of sugar beet, occurs at an epidemic level in northern Serbia and southern Slovakia [19]. Recently, 56 *stamp* and 23 *vmp1* genotypes have been characterized with nucleotide sequences obtained from these epidemiologically informative markers in the Euro-Mediterranean basin [28]. In another study, it was reported that 80 *vmp1* and 46 *stamp* genetic variants could be identified based on the nt sequence similarities of CaPsoI strains [27].

Molecular typing and genetic diversity studies on the non-ribosomal gene sequences of CaPsoI in Turkey are limited. STOL strains with a different V-genotype and closely related to the V1 genotype have been reported in infected potatoes and tomatoes from the eastern Mediterranean region [14]. Maize plants from the same region have also been reported to be infected with the V15 genotype [29]. A strain closely related to the Serbia *Reptalus quinquecostatus* strain (V7-A genotype) has been documented in an infected tomato plant from the Eastern Anatolia region [12]. In the present study, a multilocus sequence typing approach, based on the molecular typing of *vmp1* and *stamp* genes, was used to determine the genetic diversity between 'Ca. P. solani' strains in tomato cultivation areas of the Aegean and Marmara regions, which have the highest volume of vegetable production in Turkey. In addition, in this study, using the non-ribosomal gene regions of strains obtained from Turkey and other global strains, how the epidemiological cycle of the pathogen is reflected in the population genetics and how it shapes it has been comprehensively discussed in this study.

## 2. Materials and methods

### 2.1. Phytoplasma sources

Between May and September 2022, surveys were carried out in three

provinces of the Marmara region and one province of the Aegean region, which is the main vegetable-growing area of Western Anatolia (Fig. 1). Tomato fields were randomly selected and a total of 67 samples were taken from 52 symptomatic and 15 asymptomatic plants (Fig. 2).

### 2.2. Phytoplasma detection

Total nucleic acid (TNA) was extracted from about 250 mg of tomato leaves using the cetyltrimethylammonium bromide (CTAB) procedure described by Li et al. (2008) [30] with minor modifications. For the molecular detection of phytoplasmas, TNAs extracted from a total of 67 tomato samples were diluted 1:50 in nuclease-free water (NFW) to be used in PCR assays. Samples were tested by nested PCR to amplify partial 16S ribosomal DNA gene region using universal primers R16mF2/R16mR1 [31] followed by R16F2n/R16R2 primer pairs [32]. The PCR products were checked through a 1% agarose gel in a 1X TAE buffer stained with ethidium bromide and visualized under a UV imaging device (Syngene, UK). In addition, TNAs from tobacco and carrot infected by 'Tobacco phytoplasma' (*Ca. P. solani*; subgroup 16SrXII-A) strain Tut-2 (GenBank access no OK336716) and 'Carrot yellows phytoplasma' (*Ca. P. asteris*; subgroup 16SrI-B) strain KM3-1 (GenBank access no MZ457916) were used as positive controls, respectively.

### 2.3. Molecular identification of non-ribosomal genes

The *tuf*, *stamp*, and *vmp1* gene amplifications were conducted using a nested PCR strategy from all tomato samples in an automated thermal cycler (Blue-Ray, Taiwan). All molecular assays were performed in a final volume of 25 µL containing 1 µL template (TNA diluted 1:50), 1 µL of each primer at 10 µM (final concentration 0.4 µM), and 12.5 µL of Emerald PCR Master Mix (2X) (Takara, Japan).

The *tuf* gene was amplified using the primers fTuf1/rfTuf1, followed by fTufAY/rTufAY, as described by Schneider et al. (1997) [33]. Amplification of the *stamp* gene was performed using the StampF-/StampR0 and StampF1/StampR1 gene region primers, following the defined reaction conditions as previously described by Fabre et al. (2011) [24]. Amplification of the *vmp1* gene was performed using primers H10F1/R1 [23], followed by nested PCR with TYPH10/R pairs [34].

### 2.4. Sequencing and phylogenetic inference

All fragments of 16S rDNA and specific gene regions obtained from molecular assays were sequenced bi-directionally by Bmlabs Company (Ankara, Turkey). The raw sequence data were edited and assembled

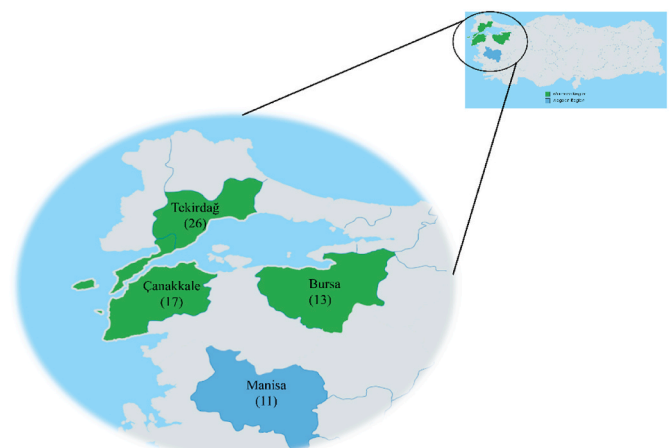


Fig. 1. Provinces of intensive tomato production and surveys in the western part of Turkey.



**Fig. 2.** Symptoms exhibited by ‘Ca. *P. solani*’ infected tomato plants (a) Growth retardation, (b) yellowing and flower malformations, (c) sterile flowers and the proliferation of axillary branches, (d) shortening of nodes and malformation, (e) anthocyanin coloration and curling on leaves.

using the software BioEdit version 7. 2. 5 [35]. Nucleotide similarities of data were also determined by sequence comparison using BlastN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Then, all data were loaded into the GenBank and assigned accession numbers to ‘Ca. *P. solani*’ tomato strains (Table 1).

The molecular evolutionary relationships were inferred based on the *stamp* and *vmp1* gene sequences of CaPsol strains determined in the present study and previously defined in other studies [19,27,36–38]. Nucleotide sequences of the *vmp1* and *stamp* genes were aligned utilizing ClustalW in the MEGA 11 software [39]. The unrooted phylogenetic trees were generated using the Neighbour-Joining (NJ) statistical method according to the Tamura-3 parameter model [40] with gamma distribution and the Tamura-Nei model [41], for the *vmp1* and *stamp* genes, respectively. Analyses were supported by the bootstrap method with 1000 replications.

## 2.5. Analysis of molecular variance (AMOVA) and genetic distance

Molecular analysis of variance (AMOVA) [42] was performed to predict the effects of genotype distributions on the total variation of the tomato CaPsol strains based on the *stamp* and *vmp1* genes. Nucleotide sequences of the *vmp1* (N = 207) and *stamp* (N = 290) genes of CaPsol subgroup strains obtained from this study and other studies were prepared to generate datasets. Analyses were implemented in Arlequin version 3.5.2.2 software [43]. The total variance for each gene region was split into its components as among- and within-population variance. Populations were formed according to hosts (perennial and annual plants, insect vectors, and weeds) and geographical regions (Asia, Central Europe, Turkey, Italy, and Eastern Europe) of strains. Additionally, countries according to the geographies from which the ‘Ca. *P. solani*’ strains were obtained; It is classified as Asia (Azerbaijan, Iran, Georgia), Eastern Europe (Hungary, Montenegro, Slovenia, Macedonia, Greece,

**Table 1**

Molecular genotyping of CaPsol strains based on *tuf/stamp/vmp1* genes and their GenBank accession numbers.

Strain	Province	Genotype			GeneBank accession number			
		<i>Tuf</i>	<i>Stamp</i>	<i>Vmp1</i>	16SrDNA	<i>Tuf</i>	<i>Stamp</i>	<i>Vmp1</i>
TT-27	Tekirdağ	b1	BG4560	V2-TA	OR095673	OR119799	OR119777	OR119806
TB-11	Bursa	b1	Rqg50	V14	OR095674	OR119797	OR119786	OR119808
TC-12	Çanakkale	b1	Rqg31	V14	OR095675	OR119790	OR119778	OR119813
TC-13	Çanakkale	b1	Rqg31	V14	OR095676	OR119796	OR119779	OR119810
TT-19	Tekirdağ	b1	Rqg50	V4	OR095677	OR119791	OR119780	OR119811
TT-20	Tekirdağ	b1	STOL	V2-TA	OR095678	OR119792	OR119789	OR119812
TT-28	Tekirdağ	b1	Rqg31	V4	OR095679	OR119798	OR119781	OR119805
TB-29	Bursa	b1	Rqg50	n.d.	OR095680	OR119800	OR119787	n.d.
TT-30	Tekirdağ	b1	Rqg31	V14	OR095681	OR119794	OR119782	OR119807
TT-31	Tekirdağ	b1	Rqg31	V4	OR095682	OR119793	OR119783	OR119814
TC-33	Çanakkale	b1	Rqg31	V4	OR095683	OR119801	OR119784	OR119804
TB-35	Bursa	b1	Rqg50	V14	OR095684	OR119802	OR119785	OR119809
TM-48	Manisa	b1	GGY	V15	OR095685	OR119795	OR119788	OR119803

Croatia, Bosnia, Slovakia), Southern Europe (Italy), Central Europe (France, Austria, and Germany), and Turkey. Genetic differentiation of CaPsol strains between the host and geographic populations was estimated using the *F*-statistic, and its significance was evaluated using a non-parametric permutation analysis based on 10,000 repetitions. Also, population comparisons were evaluated based on geographic and host levels by computing pair-wise population differentiation estimates (Fixation indices-  $F_{ST}$ ) among populations. The distance method was used, and the significance level was determined at  $P < 0.05$  [44]. Thus, genetic distance (D) [45] analyses were performed in the same software according to the geographical and host populations of each gene region.

### 3. Results

#### 3.1. Field surveys and molecular determination of 'Ca. P. Solani' strains

During the field surveys, 52 symptomatic and 15 asymptomatic tomatoes were collected from plants exhibiting foliar and flower symptoms typically caused by phytoplasmas, such as yellowing, leaf curling, purpling, flower, and leaf deformation (Fig. 2). 1.2 kb long amplicons were obtained using universal phytoplasma primers in one sample of the Aegean region and 12 samples from the Marmara region in PCR assays. No positive results were obtained for the asymptomatic plants. All thirteen CaPsol tomato samples were identical to 'Ca. P. solani' 16SrXII-A related strain based on the BLAST analyses. All tomato strains from Turkey were assigned to GenBank under the accession number OR095673- 85 (Table 1).

#### 3.2. Multilocus gene amplification of 'Ca. P. Solani' strains

Amplifications of the *stamp*, *vmp1* (except for one sample), and *tuf* genes of thirteen CaPsol-infected samples were successfully performed in molecular assays. However, no gene amplicons were detected using any primer pair during the first step of the nested- PCR assay. The GenBank accession numbers (OR119777- OR119814) for the specific gene regions of each strain obtained in this study were detailed in Table 1.

#### 3.3. Molecular genetic variability and phylogenetic inferences

Thirteen new tomato CaPsol strains in total, from all positive fragments were chosen for *tuf*, *stamp*, and *vmp1* gene sequencing. The sequencing results revealed that the length of the *vmp1* gene region was about 1.293 nt, the *stamp* gene region was 470 nt, and the *tuf* gene region was 827 nt for all Turkish tomato strains. The *tuf* gene region of the thirteen tomato CaPsol strains had 100% nt homology with the *tuf*-b1 genotype (access no OP231787; Sb567/21 sugar beet strain from Serbia).

The *stamp* gene sequence similarity analyses indicated that the four Turkish strains (TB-11, TT-19, TB-29, and TB-35) shared over 99% nt identity with the Rqg50 genotype (access no KC703019; *Reptalus quinquecostatus* from Serbia), and six strains (TC-12, TC-13, TT-28, TT-30, TT-31, and TC-33) shared over 99% nt identity with the Rqg31 genotype (access no KC703017; *R. quinquecostatus* from Serbia). In addition, one strain (TT-27) had over 99% nt similarity with the BG4560 genotype (access no FN813252; *Vitis vinifera* from Bulgaria), one strain (TT-20) had over 99% nt similarity with the STOL genotype (access no FN813261; *Capsicum annum* from Serbia), and one strain (TM-48) had over 99% nt similarity with the GGY genotype (access no FN813256; *V. vinifera* from Germany).

The *vmp1* gene sequence similarity analyses revealed that the five Turkish strains (TT-30, TB-11, TB-35, TC-13, and TC-12) shared over 99% nt homology with the V14 genotypes (access no OK041406, sugar beet strain 1595/20 from Austria and access no OK041402 sugar beet strain 1221/20 from Serbia), and other four strains shared over 99% nt homology with the V4 genotypes (access no OK041398 sugar beet strain

1216/20 from Serbia and access no OK041397 sugar beet strain 1204/20 from Serbia). Additionally, two Turkish strains (TT-27 and TT-20) shared more than 99% nt similarity with the V2-TA genotype (access no KF957604 grapevine strain MK29 from Macedonia). However, only the tomato stolbur strain (TM-48) obtained from the Aegean region showed more than 99% nt similarity with the V15 genotype.

To insight into the evolutionary relationships between the non-ribosomal gene regions of CaPsol strains, aligned nucleotide datasets from Turkey and other global strains were analysed separately at the *stamp* and *vmp1* gene levels. Phylogenetic trees for both gene regions were constructed unrooted without using an outgroup. Also, in the phylogenetic analyses for the *stamp* and *vmp1* genes, the distribution of the CaPsol strains to the major phylogroup and subgroups was given in detail in the Supp. Table 1 and Supp. Table 2.

Molecular evolutionary analyses indicated that *vmp1* gene strains were distributed into two major branches, *vmp1*-I and *vmp1*-II. More specifically, the *vmp1*-I clade was split into five subclades, *vmp1*-I (a/b/c/d/e). The main clade *vmp1*-II and subclade *vmp1*-Ie included only some of the Italy strains. Besides, *vmp1*-I (a/b/c/d) subclades contained strains from Turkey and other global strains. For the *stamp* gene, phylogenetic analyses revealed that all strains were clustered in two main branches, *stamp*-I and *stamp*-II. In addition, the *stamp*-I main branch was divided into three separate sub-branches as *stamp*-I (a/b/c), and the strains from Turkey were grouped only in *stamp*-I (a/c) sub-branches.

#### 3.4. Molecular variance and genetic distance

AMOVA was performed to evaluate the genetic variation distribution among and within the host and geographic groups of populations for the *stamp* and *vmp1* genes of CaPsol. All molecular variance results were shown in detail in Table 2. A statistically significant genetic difference was calculated between the host-level populations of both non-ribosomal genes ( $F_{ST}$ ; *stamp* gene 0.03147; *vmp1* gene 0.04184;  $P < 0.05$ ). In particular, this genetic differentiation was highly due to each individual strain forming the interior of the host clusters rather than among the host populations (*stamp* gene % 3.14721, *vmp1* gene % 4.18353). However, genetic variation values at the geographic level for both genes showed significant differentiation among populations from Italy, Central Europe, Asia, Eastern Europe, and Turkey, with 8.93% (*vmp1* gene) and 14.39% (*stamp* gene). This differentiation may be due to the genetic diversity of each strain forming geographic clusters of 91.07% (*vmp1* gene) and 85.61% (*stamp* gene) of the total variation within populations.

The genetic distance method was used to determine the genetic relationships between populations at both the geographic and host levels for the *stamp* and *vmp1* gene regions. All results were shown in detail in Table 3 and Table 4. Thus, strikingly, results for the *stamp* gene were obtained among geographic populations that were statistically significant and consistent. While the Turkish population demonstrated a closer genetic distance from the Eastern European population, the *stamp* gene populations of Italy, Central Europe, and Eastern Europe showed very closely related genetic distances among themselves (Table 3). On the other hand, genetic distance results were obtained for the *vmp1* gene with inconsistent variation in physical distances between populations. Moreover, at the host level, insect vector populations for both gene regions showed statistically insignificant genetic distances with perennial and weed populations (Table 4).

### 4. Discussion

'Ca. P. solani' has high genetic variability in the ecosystem as it infects a wide range of plant hosts, is transmitted by insects, and replicates both in their vector bodies and in the hosts. Therefore, to better understand this genetic variability in the ecological niches of CaPsol, the non-ribosomal genes *vmp1* and *stamp*, which encode membrane proteins

**Table 2**

Analysis of molecular variance (AMOVA) representing the population genetic structure of the CaPsoI non-ribosomal genes.

Population definition	Source of variation	Sum of squares	Variance components	Percentage variation	Fst and p- value
<i>vmp1</i> gene Geographic populations	Among Populations	811.288	4.24530	8.92793	0.08928
	Within Populations	8747.707	43.30548	91.07207	0.00000*
	Total	9558.995	47.55078	100.00	
<i>vmp1</i> gene Host populations	Among Populations	420.192	1.96561	4.18353	0.04184
	Within Populations	9138.803	45.01873	95.81647	0.00000*
	Total	9558.995	46.98434	100.00	
<i>Stamp</i> gene Host populations	Among Populations	97.776	0.32217	3.14721	0.03147
	Within Populations	2835.524	9.91442	96.85279	0.00000*
	Total	2933.300	10.23659	100.00	
<i>Stamp</i> gene Geographic populations	Among Populations	352.163	1.52220	14.38916	0.14389
	Within Populations	2581.137	9.05662	85.61084	0.00000*
	Total	2933.300	10.57883	100.00	

d.f.: degrees of freedom. Statistical significance: P < 0.05\*- Significance tests (1023 permutations).

**Table 3**

Genetic distances of geographic populations of *stamp* and *vmp1* gene regions of CaPsoI strains.

Gene region	Population groups					
		Italy	Central Europe	Asia	Turkey	Eastern Europe
<i>vmp1</i> gene	<b>Italy</b>	*	+(0.00901 ± 0.0091)	+(0.00901 ± 0.0091)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Central Europe</b>	0.05857	*	+(0.00000 ± 0.0000)	+(0.00901 ± 0.0091)	+(0.01802 ± 0.0121)
	<b>Asia</b>	0.07924	0.07748	*	+(0.04505 ± 0.0203)	+(0.00000 ± 0.0000)
	<b>Turkey</b>	0.10793	0.07270	0.07727	*	-(0.09910 ± 0.0286)
	<b>Eastern Europe</b>	0.11628	0.05435	0.11251	0.02879	*
<i>stamp</i> gene	<b>Italy</b>	*	+(0.00901 ± 0.0091)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Central Europe</b>	0.07435	*	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Asia</b>	0.17447	0.24785	*	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Turkey</b>	0.17136	0.33171	0.33427	*	+(0.00000 ± 0.0000)
	<b>Eastern Europe</b>	0.09473	0.17933	0.14139	0.10365	*

Significance Level = 0.0500 (+/significant -/non-significant).

**Table 4**

Genetic distances of host populations of *stamp* and *vmp1* gene regions of CaPsoI strains.

Gene region	Population groups				
	*	Perennial	Insect vector	Weed	Annual
<i>vmp1</i> gene	<b>Perennial</b>	*	-(0.30631 ± 0.0364)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Insect vector</b>	0.00440	*	-(0.11712 ± 0.0430)	+(0.01802 ± 0.0121)
	<b>Weed</b>	0.03456	0.01971	*	+(0.00000 ± 0.0000)
	<b>Annual</b>	0.06334	0.02965	0.09716	*
<i>stamp</i> gene	<b>Perennial</b>	*	-(0.09910 ± 0.0252)	+(0.00000 ± 0.0000)	+(0.00000 ± 0.0000)
	<b>Insect vector</b>	0.01029	*	-(0.18018 ± 0.00407)	+(0.00901 ± 0.0091)
	<b>Weed</b>	0.02508	0.00814	*	-(0.13514 ± 0.0389)
	<b>Annual</b>	0.07651	0.04135	0.01246	*

Significance Level = 0.0500 (+/significant -/non-significant).

that are essential for host and vector interactions, were thoroughly investigated in vineyards [18,27,37]. In this study, stolbur strains were obtained from the tomato-growing areas of the Western Region, which

have the highest vegetable production volume in Turkey. The molecular genetic diversity and population dynamics of these tomato strains and other global CaPsoI strains based on the *vmp1* and *stamp* gene sequences were examined at the host and geographic levels using bioinformatic tools.

The genetic distance (D) is a commonly used formulation to quantify accumulated allele differences per locus and, in some migration models, to generate estimates using data that are linearly related to geographic distance or area [45]. In this study, genetic distance values were calculated in detail according to geographical and host populations by using partial *vmp1* and *stamp* gene regions. For the *stamp* gene, pairwise population distance comparison analysis at the geographic level revealed statistically significant values (P < 0.05). More specifically, the Turkish population was most closely associated with the Eastern European populations (0.10365); it showed the most distant relationship with the Asian (0.33427) and Central European (0.33171) populations. While the Italian population showed values closely related to the Central (0.07435) and Eastern European (0.09473) populations, it showed the most distant values from the Asian (0.17447) and Turkish (0.17136) populations. On the other hand, compared to the data for the *stamp* gene, the genetic distance values for the *vmp1* gene were more inconsistent (Table 3). For the *vmp1* gene, the Italian population showed the furthest value from the Eastern European (0.11628) and Turkish (0.10793) populations, whereas it showed the closest values for the Asian (0.07924) and Central European (0.05857) populations. The lowest value was also determined between Turkey and Eastern Europe, and it was statistically insignificant (0.02879) (Table 3). In addition, several

studies have reported that environmental and ecological characteristics in different geographical areas may be directly or indirectly effective in determining the strain diversity of phytoplasma populations [46,47]. Thus, in the present study, genetic distance values calculated for both gene regions provide evidence that environmental and ecological features may be very important in shaping geographic populations of phytoplasmas.

As for the genetic distances at the host level, statistically insignificant values were calculated for perennial-insect (*vmp1*-0.0044 and *stamp*-0.01029) and insect-weed (*vmp1*-0.01971 and *stamp*-0.00814) binary population comparisons for both gene regions (Table 4). In studies on the epidemiology of CaPsoI in European vineyards, it has been reported that *H. obsoletus* is a vector that plays a key role in the transmission of Bois Noir disease [15,20,48]. Another major vector effective in the transmission of 'Ca. P. solani', especially, was identified as the *R. panzeri*, and in addition, at least eight alternative vector species (*Euscelidius variegatus*, *Laodelphax striatella*, *Philaenus spumarius*, *Aphrodes makarovi*, *Dicranotropis hamata*, *Dictyophara europaea*, *Euscelis incisus*, and *Psammotettix alienus/confinis*) were reported in vineyards by Ref. [10]. Besides, in the epidemiological cycle of the pathogen, its vector has been particularly associated with two main host weeds: *C. arvensis* (field bindweed) and *U. dioica* (stinging nettle) [15,20,48]. Herein, population clusters were generated based on the *stamp* and *vmp1* gene regions of the strains obtained from vector insects, weeds, and perennial plants, including different species along with the aforementioned main epidemiological factors. Since alternative species other than major vectors and weeds may play an important role in this cycle, the genetic distance values of these host populations for both gene regions strongly support this hypothesis. Additionally, results for the annual-perennial (*vmp1*-0.06334 and *stamp*-0.07651) and annual-insect (*vmp1*-0.02965 and *stamp*-0.04135) binary clusters for both gene regions were statistically significant (Table 4). These findings may be explained by the pathogen contamination of the plant seeds that form the annual population, which has the potential to significantly shape the population structure by spreading over long distances. Therefore, although there has been no direct connection between phloem and seed embryo for a long time, and seed transmission of phytoplasmas has been controversial, recent studies have revealed that the pathogen may be seed-borne [49–53].

Phylogenetic studies were performed to reveal the molecular evolutionary relationships of the *stamp* gene of the CaPsoI strains determined from different geographical regions and hosts, and it was demonstrated that the strains clustered in different main clades [18,27,38,54]. Herein, it was determined that Turkey and the global *stamp* gene of 'Ca. P. solani' strains (N = 290) evolved from two main branches

(*stamp* I/II) and distributed in three subgroups (*stamp* I-a/b/c) in main branch *stamp* I (Fig. 3). All *stamp* gene strains determined from Turkey were also located in *stamp* I-a and I-c sub-branches. Moreover, the phylogenetic relationships of the *vmp1* gene region of the strains have been detailed in previous studies, and it has been shown that the strains were clustered in different subgroups [27,38]. In this study, two main groups (*vmp1*-I and II) and five subgroups (*vmp1*-Ia/b/c/d/e) in which the strains (N = 207) were distributed were determined, and it was shown that all strains determined from Turkey were clustered in *vmp1* (a/b/c/d) subgroups. Consequently, the phylogenetic trees generated for both partial gene regions showed that all strains evolved into two main branches (*stamp*-I/II and *vmp1*-I/II) [27,36,38,54] however, these main branches did not completely reflect any geographical or specific host clustering. On the other hand, the *vmp1*-II major and *vmp1*-Ie subgroups consisted entirely of some Italian region populations, including weed, insect, and perennial hosts. Moreover, there was an absolute host vector insect in both the main branches and all sub-branches for the *vmp1* and *stamp* genes. This strongly supports the hypothesis that the molecular evolutionary relationship dynamics of CaPsoI strains could be shaped by vector insects.

In previous studies, multilocus sequence typing (MLST) analyses were performed based on the *tuf*, *stamp*, and *vmp1* gene regions to provide information about the distribution pathways and the molecular ecology of 'Ca. P. solani' strains in vineyards [18,26,27,55]. However, in a recent study conducted by Ćurčić et al. (2021) [19], the dSTOLg (*tuf*-d/STOL/V2-TA) genotype was determined to be dominant in the sugar beet cultivation areas of Serbia, Slovakia, Croatia, and Hungary in the MLS analyses of rubbery taproot disease of sugar beet (RTD) associated with 'Ca. P. solani'. Furthermore, one of the two important epidemiological cycles of BN disease has been associated with the transmission and spread of the pathogen by the vector *H. obsoletus* from infected *tuf*-b-genotype bindweed (*C. arvensis*) plants [15]. In this study, the *tuf*-type of thirteen stolbur strains obtained from tomato cultivation areas in western Turkey was determined molecularly as the *tuf*-b1 genotype. *C. arvensis* is widely distributed in every region of our country [56], and the *tuf*-b genotype has also been reported in CaPsoI infected maize from the eastern Mediterranean region of Turkey [29]. On the other hand, it is known that the *vmp1* gene region has great genetic diversity, and different genotypes have been reported from previous studies, especially from vineyards. While four different genotypes, V2-TA, V14, V4, and V15, were determined from the strains obtained from this study, V7-A, V15, and V1 genotypes were determined from infected corn, tomato, and potato plants from the Eastern and Mediterranean regions of Turkey. The strains identified in this study were obtained from the region of Turkey closest to the Eastern European

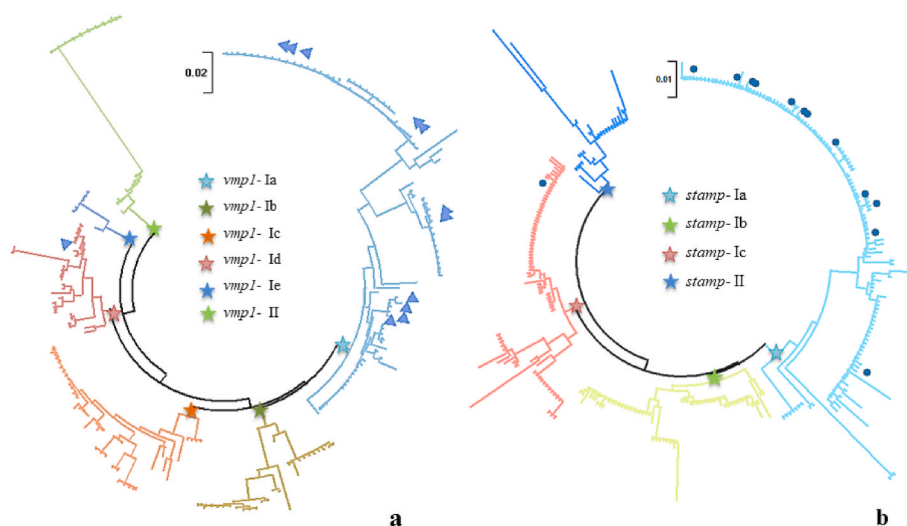


Fig. 3. The unrooted phylogenetic trees were constructed using the Neighbour-Joining (NJ) statistical method according to the Tamura-3 parameter model with gamma distribution and Tamura-Nei model, for the *vmp1* (a) and *stamp* (b) genes. A blue triangle (a) and a navy-blue circle (b) were used to identify Turkish tomato strains. The main branches of the phylogenetic groups were colored and marked with the same color star shapes. The strains and their groups in both phylogenetic trees were given in detail in the Supplementary Material Tables 1 and 2

region, and the nucleotide diversity of the Turkish strains showed more than 99% similarity with the Eastern European strains. In addition, the statistical values indicating that there was no appreciable difference between the populations of Turkey and Eastern Europe were further supported by genetic distance analyses. These findings strongly support the hypothesis that global warming may contribute to the regional migration of agricultural pests or vector insects, thereby increasing the likelihood of phytoplasma infections spreading to different hosts [57–59]. The *stamp* gene has been frequently used as an epidemiological molecular marker in previous studies to understand the phytoplasma transmission pathways in vineyard agroecosystems and to reveal the population structure of BN [17,18,26,27]. This marker was also used to determine the genetic diversity of RTD associated with ‘*Ca. P. solani*’ in sugar beet cultivation areas and 14 new *stamp* genotypes were determined. Herein, 5 different *stamp* genotypes were determined that are molecularly closely related to BG4560 (*Vitis vinifera*- Bulgaria), Rqg50 (*R. quinquecostatus*- Serbia), Rqg31 (*R. quinquecostatus*- Serbia), STOL (*Capsicum annum*- Serbia) and GGY (*V. vinifera*- Germany) strains. Findings about the *stamp* genotypes for ‘*Ca. P. solani*’ infections seen in Turkey were obtained for the first time in this study. Therefore, for future studies, genotypes of strains to be obtained from different geographical regions of Turkey, various plant patterns, and vector insects will provide more information in terms of genetic diversity.

In this study, molecular variance analyses (AMOVA) were applied at the geographic and host hierarchical levels based on the *stamp* and *vmp1* gene regions to better understand which parameters are effective in the population structure and genetic diversity of CaPsol. The AMOVA results clearly demonstrated that each of the strains forming the populations for both gene regions contributed greatly to the genetic diversity. In addition, the variation values obtained among the populations forming the hierarchical levels were not high.

During the field studies in this study, symptoms such as growth retardation, yellowing of leaves, curling, and anthocyanin coloration were observed in 52 symptomatic plants (Fig. 2). However, only 13 of these plants were found to be infected with CaPsol molecularly. In previous studies, it has been reported that agents such as potato virus Y (PVY), tomato yellow leaf curl virus (TYLCV), and southern tomato virus (STV) on tomato plants cause co-infections with together CaPsol or single [60,61]. As a result, it can be thought that the symptoms seen in other plants are caused by physiological or any viral factor rather than phytoplasma.

## 5. Conclusion

Studies on population genetics of ‘*Ca. P. solani*’ have been carried out using non-ribosomal gene regions to better understand its epidemiology and increase control methods, especially due to the damage it causes in vineyards. Thus, research has often focused on vineyard agroecosystems. In this study, *vmp1*, *stamp*, and *tuf* genotyping and population dynamics of CaPsol strains collected from tomato-growing areas in the Marmara and Aegean regions were revealed for the first time in Western Turkey. In future studies, the analysis of strains to be obtained from different geographical regions of Turkey will provide more information about the population structure of Turkish CaPsol. To understand the inter-geographical flow and adaptation of genotypes, more reliable estimations will be made with more non-ribosomal gene information and it will be used as a piece of important genetic information in the prevention of the spread of the disease. Also, these findings might indicate that CaPsol has a very dynamic epidemiological cycle and that horizontal transmission is more efficient, particularly in this dynamic structure. Therefore, this high genetic diversity may require our further investigation of vector insects and application of alternative methods for pest control, as well as more intensive enforcement of legislative measures in propagation material movements.

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## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

## Author contributions

FRZ designed the study; FRZ collected samples; FRZ conducted the molecular experiments and designed the phylogenetic trees. FRZ prepared the manuscript and performed population genetic analysis.

## Declaration of competing interest

The author declare that they have no conflict of interest.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pmpp.2023.102120>.

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