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RESEARCH ARTICLE

Molecular evolution and adaptation of the effector protein from *Candidatus* Phytoplasma trifolii associated with brinjal little leaf disease

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Abstract

Brinjal little leaf (BLL) disease, caused by phytoplasmas, leads to significant yield losses, but its pathogenic mechanisms remain poorly understood. This study characterizes SAP54LP/S54LP (SAP54-like protein of BLL), an effector protein associated with the 16SrVI-D phytoplasma strain linked to BLL in Tamil Nadu, India. Molecular analyses, including nested PCR and virtual RFLP, confirmed the phytoplasma's classification within the 16SrVI group. The S54LP effector gene was amplified from infected brinjal, confirming its presence. Sequence comparisons showed that S54LP was 100 % identical to homologs in the 16SrVI-A group and 98 % identical to those in the 16SrI-B group (*phyl*1 and S54LP), indicating group-specific divergence. Comparative analysis identified 13 single nucleotide polymorphisms (SNPs), 69 % of which led to nonsynonymous substitutions in the mature protein, potentially affecting host interactions. Haplotype network analysis revealed a globally distributed Hap_6 lineage and region-specific haplotypes, suggesting localized evolutionary dynamics. Evolutionary analysis indicated strong purifying selection on the coding sequence (Ka/Ks ≈ 0.3), reflecting the conservation of functional domains essential for pathogenicity. Conversely, positive selection was detected in the signal peptide region, implying adaptive evolution related to secretion and host targeting. Additionally, AT-rich codon usage at specific sites highlights multiple layers of evolutionary constraints shaping S54LP. Overall, these findings establish S54LP as a model for studying host-phytoplasma coevolution, providing insights into effector biology and informing strategies to manage phytoplasma diseases in crops.

Keywords: adaptation; brinjal little leaf; evolution; phytoplasmas; SAP54 effector

Introduction

Brinjal (*Solanum melongena* L.), often referred to as the "king of vegetables," is a vital solanaceous crop native to the Indian subcontinent, with a cultivation history spanning over 4000 years (1-3). Valued for its culinary versatility, brinjal is a good source of dietary fiber, vitamins (B1, B6, K) and minerals like potassium, magnesium and copper. Additionally, it contains bioactive compounds such as nasunin, chlorogenic acid and flavonoids, which confer antioxidant, anti-inflammatory, anticancer and cardioprotective properties (4-8). Traditionally, brinjal fruit extracts have been used in traditional remedies to soothe skin conditions, address women's health concerns, especially related to the uterus and act as a gentle natural laxative (9).

Globally, China leads brinjal production with approximately 36 million tonnes (about 60 % of global output), followed by India with 12.5 million tonnes (around 25 %) (10). Within India, West Bengal is the top producer (23.72 %), while Tamil Nadu contributes 2.76 % (2021-22), although yields remain below the national average owing to biotic stresses (11-14). A major constraint is BLL disease, caused by phytoplasmas, which are cell-wall-less, phloem-inhabiting bacteria of the class Mollicutes. Phytoplasmas are transmitted by leafhoppers, grafting or dodder and induce severe symptoms including stunting, phyllody, virescence and excessive branching, often resulting in yield losses of up to 100 % (12, 15-17).

BLL is associated with multiple phytoplasma groups (16Sr I, II, III, VI, IX and XII) and subgroups (e.g., 16Sr VI-D), with several weeds serving as alternate hosts (17-23). Phytoplasmas have highly reduced genomes (530-1530 kb) and depend entirely on host-derived nutrients for survival, as they lack many essential metabolic pathways. They secrete effector proteins such as SAP54 and its homolog *Phyl1* (Phyllogen gene), which hijack the host's RAD23-proteasome system to degrade MADS-box transcription factors, triggering phyllody and increasing attractiveness to insect vectors (24-30). Despite their critical role, SAP54-like effectors in

Indian brinjal phytoplasmas have remained poorly characterized. This study aimed to detect and characterize SAP54LP in 16SrVI-D phytoplasma from Tamil Nadu, providing molecular and evolutionary insights into host-pathogen interactions for improved disease management.

Materials and Methods

Source of phytoplasma

Symptomatic brinjal plants exhibiting phytoplasma infection were collected from fields in the Madurai district of Tamil Nadu, India. Sampling was conducted during the active growing season (October 2024 to January 2025), coinciding with the stage when disease symptoms were most prominent. Tissue samples including leaves, flowers and shoots exhibiting characteristic little leaf symptoms (Fig. 1), were collected from infected plants and immediately stored at -20 °C to preserve nucleic acids. These samples were subsequently used for DNA extraction and molecular characterization of the phytoplasma isolates.

Genomic DNA extraction and phytoplasma detection using PCR

Genomic DNA was extracted from three symptomatic samples using the CTAB (cetyltrimethylammonium bromide) method (31). To ensure an enriched phytoplasma DNA yield, only petioles, midribs and prominent leaf veins were selected. The quality and quantity of the extracted DNA were assessed using 1.8 % agarose gel electrophoresis and quantified using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). Samples with an OD260/280 ratio between 1.8 and 2.0 were used for further analysis. For phytoplasma detection, PCR amplification was conducted using universal 16S rDNA primers P1/P7 (32), followed by a nested PCR using the R16F2/R2n primer set (33). The PCR conditions for amplification with P1/P7 included initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 10 min. For amplification with R16F2/R2n, all conditions remained the same except for an increased annealing temperature of 60 °C. The final PCR products were electrophoresed on a 1.2 % agarose gel for visualization.

Gel extraction, sequencing and *in silico* RFLP

The PCR product was purified using a standard ethanol precipitation protocol (34). The purified PCR products were bidirectionally sequenced using the Sanger sequencing platform at Medauxin Pvt. Ltd. (Bengaluru, India). The consensus sequence was examined for homology using NCBI BLASTn. The aligned 16S rRNA gene sequence of the phytoplasma isolate, approximately 1.25 kb in length was obtained in FASTA format. This sequence was then uploaded into the iPhyClassifier tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) for analysis. The tool performs a virtual restriction enzyme digestion using 17 reference enzymes (BamHI, KpnI, EcoRI, AluI, BfaI, BstUI, DraI, HaeIII, HhaI, HinfI, HpaI, HpaII, MboI, MseI, RsaI, SspI and TaqI) commonly used for phytoplasma classification. iPhyClassifier will then generates a virtual RFLP profile for the sequence by comparing the restriction patterns with the reference profiles for known phytoplasma groups and subgroups.

Effector gene amplification and sequencing

Primers (SAP5LP of BLL_F 5'-TGCTCATCATAATCTTGCTC-3' and SAP54LP_R 5'-CGGAATATCATTAACGTAGT-3') were manually designed from *Phyl1*, a homolog of SAP54 identified in various phytoplasma groups. These primers were specifically crafted to include a 24 bp homology to the *Phyl1* sequence. For amplification of the SAP54H, symptomatic brinjal plants confirmed to harbour phytoplasma were selected. Three symptomatic samples were used in total. The resulting PCR product underwent gel extraction and purification, quantified and sequenced.

Sequence analysis

BLAST analysis was conducted to identify sequences with the highest homology to the SAP54 ortholog from little leaf infected brinjal. The sequences were aligned using CLUSTAL W (Table 1), phylogenetic relationships were inferred under the maximum likelihood framework using the Jukes-Cantor (JC) model, which was selected based on the observed low sequence divergence among taxa. Node support was evaluated using 1000 bootstrap replicates in MEGA11 (35). Sequence analysis and comparisons of the effector gene from brinjal and *Phyl1* were carried out at both nucleotide and amino acid levels to determine synonymous and nonsynonymous substitutions between the two effector proteins encoded within the same phytoplasma subgroup. SignalP 5.0 (36) was utilized to validate the cleavage site of the SAP54 homolog.

Fig. 1. Phytoplasma-infected brinjal plants displayed characteristic disease symptoms: flower stalk elongation, phyllody (conversion of floral organs to leaf-like structures), virescence (abnormal greening of flowers), chlorosis (yellowing), witches' broom (abnormal shoot proliferation) and excessive vegetative growth.

Table 1. SAP54 homologs and orthologs from phytoplasma strains across nine different (sub) groups infecting twelve plant families in ten countries

S. No.	*Candidatus* Phytoplasma species	16Sr subgroup	Phytoplasma strain	Place of isolation	GenBank accession
1.	*Candidatus* Phytoplasma trifolii	16SrVI-D	BLL (S54LP of BLL)	Madurai, India	PV610804
2.	*Ca.* P. trifolii	16SrVI-A	BLL (BLL)	Sudan	AB897827.1
3.	*Ca.* P. trifolii	16SrVI-A	BLL (BLL)	Japan	LC740446.1
4.	*Ca.* P. asteris	16SrI-L	Aster yellows	Germany	AB862480.1
5.	*Ca.* P. pruni	16SrIII	X-disease	Japan	LC740445.1
6.	*Ca.* P. asteris	16SrI-M	Apricot atypic aster yellows phytoplasma (AVUT)	Germany	AB862478.1
7.	*Ca.* P. asteris	16SrI	Onion yellows phytoplasma	Delhi, India	MK858224.1
8.	*Ca.* P. asteris	16SrI-B	Gladiolus witches'-broom phytoplasma	Japan	LC740447.1
9.	*Ca.* P. asteris	16SrI-B	Carrot yellows phytoplasma	Italy	AB862481.1
10.	*Ca.* P. asteris	16SrI	Onion yellows phytoplasma	Japan	AB812838.1
11.	*Ca.* P. pruni	16SrIII-A	Clover proliferation (CP)	Italy	AB862489.1
12.	*Ca.* P. asteris	16SrI	Leontodon yellows phytoplasma	Italy	AB862484.1
13.	*Ca.* P. asteris	16SrI-B	Peach yellows phytoplasma	Japan	AB862486.1
14.	*Ca.* P. asteris	16SrI-F	Apricot aster yellows phytoplasma	Spain	AB862477.1
15.	*Ca.* P. phoenicium	16SrIX-A	Pigeon pea witches'-broom	Italy	AB862490.1
16.	*Ca.* P. asteris	16SrI-B	Tomato yellows (TY)	Japan	EF200537.1
17.	*Ca.* P. asteris	16SrI-B	Gladiolus witches'-broom (GLAWB)	The Netherlands	AB897828.1
18.	*Ca.* P. asteris	16SrI-B	Oilseed rape virescence (RV)	France	AB862487.1
19.	*Ca.* P. asteris	16SrI-B	Severe western aster yellows (SAY)	USA	AB862488.1
20.	*Ca.* P. asteris	16SrI-B	Maryland aster yellows (AY1)	Maryland, USA	DQ837760.1

Minimum spanning haplotype network of *Ca. Phytoplasma* SAP54H based on the *Phyl* gene

The evolutionary relationships among *Candidatus* Phytoplasma SAP54LP haplotypes were analyzed by constructing a minimum spanning haplotype network using PopArt (37). The analysis included 14 *Phyl* gene (SAP54H) sequences representing diverse geographic isolates from several countries.

Test for selection

To assess the evolutionary constraints and selection pressures acting on SAP54 orthologs, the rates of nonsynonymous (Ka) and synonymous (Ks) substitutions were estimated across all amino acid sites. A total of 20 SAP54 ortholog sequences, representing distinct phytoplasma strains from nine subgroups and affecting host plants in ten countries were analyzed. Nucleotide sequence polymorphisms were evaluated using DnaSP v6.10.04 (38). Pairwise comparisons were conducted to calculate the Ka, Ks and Ka/Ks ratios for the full-length protein and separately for the mature peptide and signal peptide regions. A total of 125 pairwise sequence comparisons were analyzed to derive the average Ka, Ks and Ka/Ks values across the dataset.

To identify site-specific selection pressures, including positive or negative selection at individual codon sites, the Fixed Effects Likelihood (FEL) model was applied using DataMonkey, a web-based phylogenetic analysis platform designed for evolutionary inference (39).

Results

Detection of group and subgroup classification of brinjal phytoplasma strain

The symptomatic brinjal sample tested positive for phytoplasma infection based on nested PCR analysis, confirming the diagnosis. Using the phytoplasma-specific primers P1/P7 in the first round and R16F2n/R2 in the second round, an amplicon of approximately 1200 bp was obtained (Fig. 2a). A similar product was amplified from a known phytoplasma-infected sample used as a positive control, whereas no amplification was observed in the negative control (non-template) and in healthy brinjal samples. The 16S rDNA sequence of the phytoplasma isolate from brinjal (GenBank Accession Number PV040784) showed 99 % sequence identity to members of the 16SrVI-D subgroup, including the brinjal phyllody phytoplasma strain (GenBank Accession Number EF186820.1).

Virtual RFLP analysis of the amplified 16S rDNA sequence using iPhyClassifier confirmed the classification, displaying a restriction profile matching that of the 16SrVI-D subgroup strains reported previously (Fig. 2b). This subgroup is also known as the Clover proliferation (*Ca.* P. trifolii*)* group, which is associated with diverse plant hosts worldwide.

Effector amplification and sequence analysis of brinjal phytoplasma effector

A PCR product of approximately 550 bp, corresponding to the *Phyl1* gene (a homolog of SAP54), was amplified from all confirmed phytoplasma-infected brinjal plants (Fig. 3). Sequence analysis revealed significant homology to known SAP54-like effector genes. A BLASTn search identified 15 closely related sequences with nucleotide identities ranging from 96 % to 100 % (Table 2). The amplicon derived from BLL-affected plants in Tamil Nadu, India, showed 100 % sequence identity to known SAP54-family effector homologs, including *Phyl1* from *Ca.* P. trifolii (16SrVI -A subgroup; GenBank Accession No. AB897827.1) and a SAP54-like sequence annotated as "*hflB-like*" in GenBank (Accession No. LC740446.1), which we infer based on sequence homology and conserved domain analysis. Based on these findings, this effector was designated as S54LP of BLL (SAP54-like protein of BLL). The nucleotide sequence has been deposited in GenBank under accession number PV610804.

Interestingly, S54LP of BLL exhibited 100 % nucleotide identity with SAP54 homologs from phytoplasma strains belonging to the 16SrVI-A group, but only 98 % identity with *Phyl1* (GenBank: AB862489.1), a SAP54 homolog from the X-disease phytoplasma group (16SrIII-A subgroup), indicating group-specific divergence among SAP54-like effectors.

Phylogenetic analysis

Phylogenetic analysis of S54LP of BLL, 15 homologous and orthologous SAP54-like sequences retrieved via BLASTn was

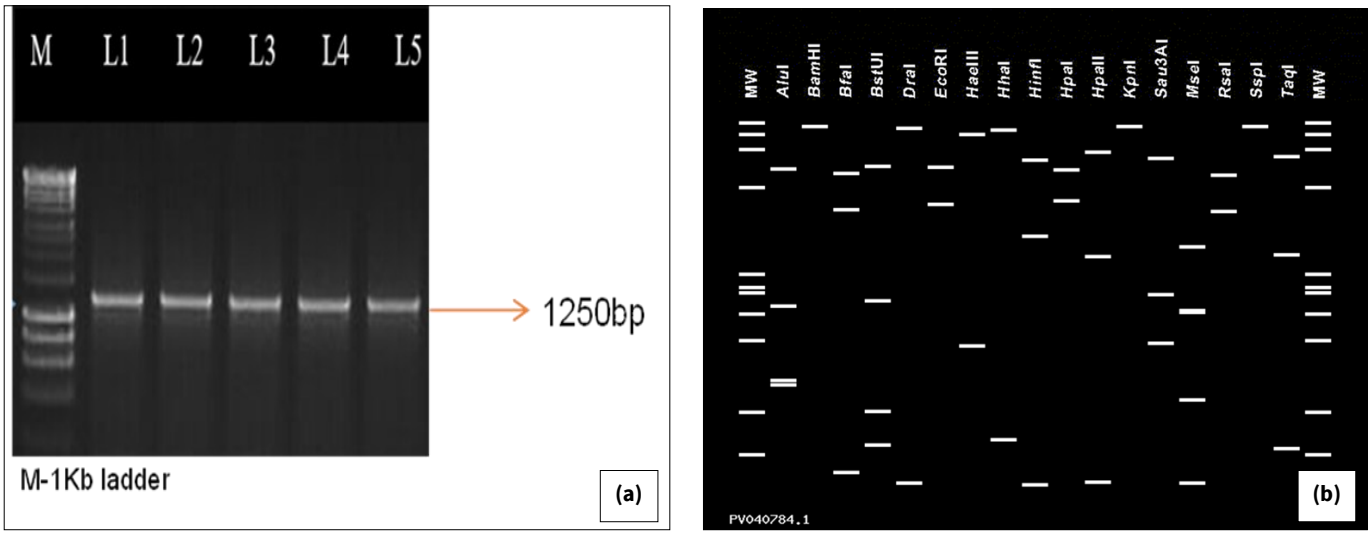

Fig. 2. Molecular detection and characterization of phytoplasma associated with BLL disease. (a) Agarose gel (1 %) showing a 1.25 kb nested PCR amplification product using phytoplasma-specific 16S rDNA primers (P1/P7 and R16F2n/R2), with lanes 1-5 (BLL) and M (1 kb DNA ladder). (b) Virtual RFLP profile of the R16F2/R16R2 gene fragment from a phytoplasma strain causing BLL in Madurai, Tamil Nadu generated by *in silico* digestion using 17 restriction enzymes with MW-100 bp Plus DNA ladder (Fermentas) using iPhyClassifer.

Table 2. BLASTn result of effector gene (S54LP of BLL) from phyllody affected brinjal displaying similarity ranging from 96 % to 100 % with other SAP54 homologs and orthologs

S. No.	Phytoplasma strain	Accession no.	Query cover (%)	E-value	Identity (%)
1.	*Ca.* P. trifolii *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB897827.1	74	0	100
2.	*Ca.* P. trifolii *hflB-like*, *phyllogen* genes for hypothetical protein, phytoplasmal effector causing phyllody, partial and complete cds	LC740446.1	100	0	100
3.	*Ca.* P. pruni *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862489.1	74	0	98.94
4.	*Ca.* P. pruni *hflB-like*, *phyllogen* genes for hypothetical protein, phytoplasmal effector causing phyllody, partial and complete cds	LC740445.1	74	0	98.94
5.	Apricot atypic aster yellows phytoplasma AVUT *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862478.1	74	0	98.68
6.	Onion yellows phytoplasma (OY) isolate *SLP*_Si SAP54-like protein (*slp*) gene, complete cds	MK858224.1	74	0	97.88
7.	Gladiolus witches'-broom phytoplasma *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862483.1	74	0	97.62
8.	Onion yellows phytoplasma OY-W *Phyl1* gene for phytoplasmal effector causing phyllody 1, complete cds	AB812838.1	74	0	97.62
9.	Gladiolus witches'-broom phytoplasma GLAW *hflB-like*, *phyllogen* genes for hypothetical protein, phytoplasmal effector causing phyllody, partial and complete cds	LC740447.1	74	0	97.62
10.	Carrot yellows phytoplasma CA-76 *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862481.1	74	7e-180	97.35
11.	Aster yellows phytoplasma AY2192 *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862480.1	74	7e-180	97.35
12.	Leontodon yellows phytoplasma LEO *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862484.1	74	7e-180	97.35
13.	Peach yellows phytoplasma PYR *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862486.1	74	2e-179	97.35
14.	Apricot aster yellows phytoplasma A-AY *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862477.1	74	3e-178	97.09
15.	*Ca.* P. phoenicium *Phyl1* gene for phytoplasmal effector causing phyllody symptoms 1, complete cds	AB862490.1	74	1e-176	96.83

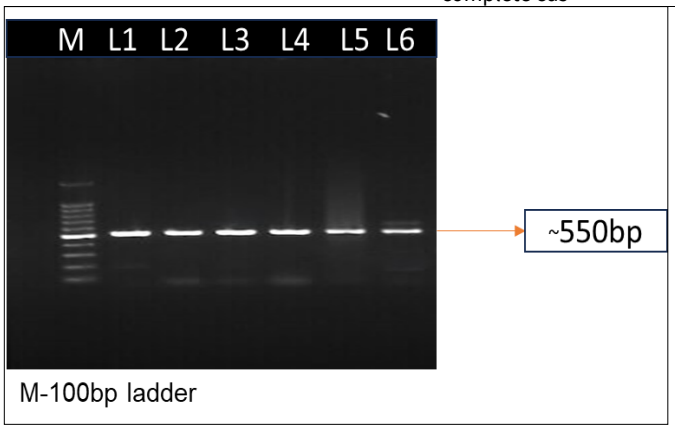

Fig. 3. Agarose gel (1 %) showing a 550 bp SAP54LP of BLL band from BLL infected plants amplified via PCR with lanes 1-6 (BLL infected sample) and M (100 bp DNA ladder).

conducted using the maximum likelihood method. The resulting tree revealed distinct clustering patterns among the analyzed sequences (Fig. 4). S54LP from the BLL-associated 16SrVI-D phytoplasma clustered with SAP54 homologs from the 16SrVI-A subgroup, albeit with low bootstrap support, possibly reflecting functional or structural conservation despite unresolved evolutionary relationships. Another homolog from the 16SrVI-A group was also grouped with S54LP of BLL, showing a relatively higher bootstrap support of 69, reinforcing their close evolutionary relationship.

Notably, orthologs from *Ca.* P. pruni (16SrIII group) and *Candidatus* Phytoplasma asteris (GLAW strain, 16SrI-B group) formed separate clades with strong bootstrap support, indicating potential genetic divergence among the SAP54-like effectors across different phytoplasma groups.

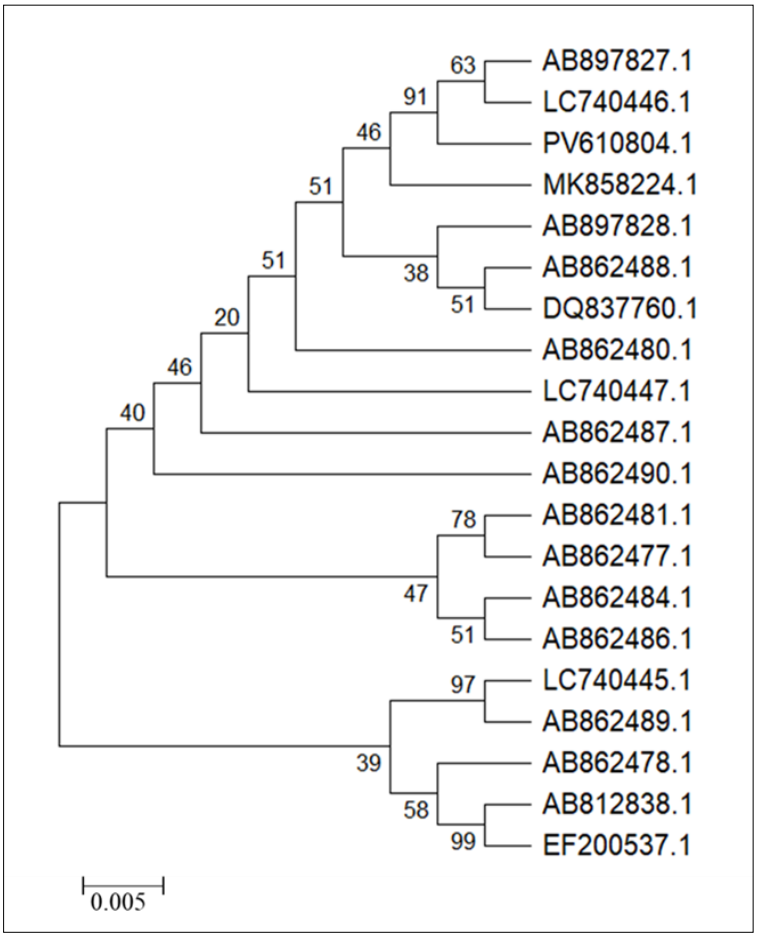

Fig. 4. Phylogenetic relationships of SAP54LP from BLL and orthologs identified through BLASTn were analyzed using the Maximum Likelihood method with 1000 bootstrap replicates in MEGA 11 software.

Comparative sequence analysis of S54LP of BLL and OY

The S54LP proteins associated with BLL phytoplasmas differ in length between groups: the variant from the 16SrI group consists of 125 amino acids, including a 91-amino acid N-terminal signal peptide that is cleaved to yield a 34-amino acid mature protein. In contrast, the variant from the 16SrVI group is shorter, comprising 70 amino acids with a 26-amino acid signal peptide cleaved to produce a 44-amino acid mature effector. The cleavage site of S54LP of OY (onion yellow phytoplasma) and BLL was identified using SignalP 5.0. A comparison of the full-length ORFs of S54LP of OY and BLL revealed 8 SNPs (Fig. 5a), of which 6 (75 %) were nonsynonymous and 2 (25 %) were synonymous. Further analysis showed that 31 % of nonsynonymous SNPs were located in the signal peptide region, potentially affecting secretion efficiency. While, 69 % of nonsynonymous SNPs were found in the mature (secreted) protein, suggesting functional implications (Fig. 5b). The analysis of SNPs between revealed several significant amino acid property changes between S54LP of OY and BLL. Analysis of the protein sequence revealed several significant mutations. Four changes from hydrophilic to hydrophobic residues were found, which could alter the protein's folding and stability. Conversely, three hydrophobic-to-hydrophilic changes might disrupt its interactions with other cellular components. Additionally, we observed an acidic-to-neutral change that could affect charge-based binding and a critical stop codon mutation that is predicted to cause a truncated, non-functional protein. Additionally, three synonymous mutations preserved the original protein structure despite sequence variations, highlighting the potential effects of SNPs on protein secretion efficiency and host interactions (Table 3).

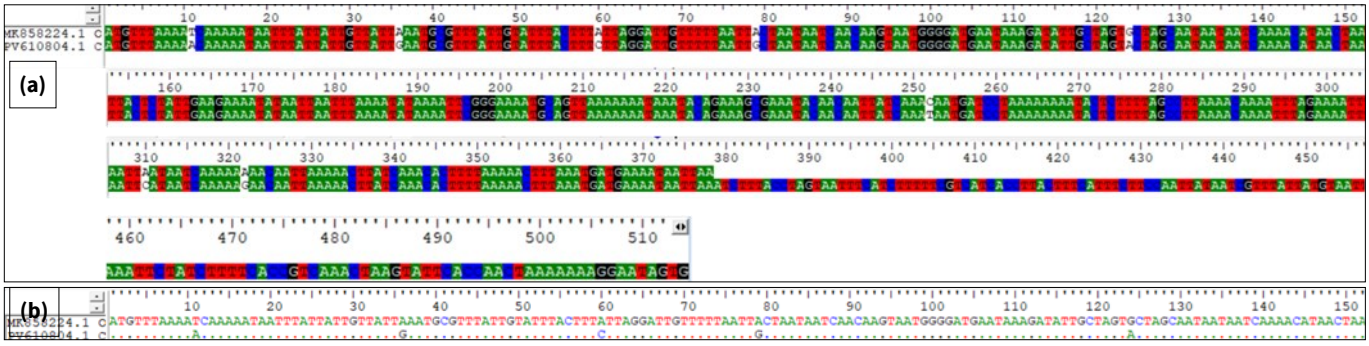

Fig. 5. Sequence alignment and SNP analysis of SAP54LP gene from OY and BLL phytoplasma strains. (a) Multiple sequence alignment of SAP54LP nucleotide sequences from OY and BLL phytoplasma strains using Clustal W showing eight single nucleotide polymorphisms (SNPs). (b) Nucleotide sequence alignment of SAP54LP showing the first five nonsynonymous substitutions identified between OY and BLL phytoplasma isolates.

Table 3. Characterization of SNPs in signal peptide and mature protein regions showing synonymous and nonsynonymous mutations in S54LP of BLL

S. No.	SNP position	Change	Codon change	Amino acid change	Position of amino acid change	Synonymous/ nonsynonymous	Change in property
1.	5	T → A	ACC → GCC	Threonine → alanine	Signal peptide	Nonsynonymous	Hydrophilic → hydrophobic
2.	12	C → A	GCT → GAT	Alanine → aspartic acid	Signal peptide	Nonsynonymous	Neutral → acidic
3.	19	A → T	ATT → ATC	Isoleucine → isoleucine	Signal peptide	Synonymous	No change
4.	24	A → T	GCA → GTA	Alanine → valine	Mature protein	Nonsynonymous	Hydrophobic → hydrophilic
5.	26	T → G	ACT → GCT	Threonine → alanine	Mature protein	Nonsynonymous	Neutral → hydrophobic
6.	33	A → T	GCA → GGA	Alanine → glycine	Mature protein	Synonymous	No change
7.	40	G → C	GGG → CGG	Glycine → arginine	Mature protein	Nonsynonymous	Hydrophobic → hydrophilic
8.	47	T → A	ACC → GCC	Threonine → alanine	Mature protein	Nonsynonymous	Hydrophilic → hydrophobic
9.	55	A → C	GCA → GGA	Alanine → glycine	Mature protein	Synonymous	No change
10.	62	T → A	ACA → GCA	Threonine → alanine	Mature protein	Nonsynonymous	Neutral → hydrophobic
11.	65	G → A	GGA → GAA	Glycine → glutamic Acid	Mature protein	Nonsynonymous	Hydrophobic → neutral
12.	68	T → G	ACT → GCT	Threonine → alanine	Mature protein	Nonsynonymous	Hydrophilic → hydrophobic
13.	70	G → T	GGA → TGA	Glycine → STOP codon	Mature protein	Nonsynonymous	Functional disruption

Minimum spanning haplotype network

The global distribution of haplotypes highlights the diversity in the phytoplasma population structure. Haplotype 6, comprising 5 sequences from Japan, the Netherlands, France and 2 sequences from the USA, emerged as the most widespread, suggesting extensive geographical dissemination (Table 4). Haplotype 1, which includes three sequences from India, Sudan and Japan, demonstrated significant distribution. Meanwhile, haplotype 3, with two sequences from Japan and Italy, highlights regional diversity. Additionally, haplotype 8, consisting of two sequences from Japan, further underscores genetic variation within the region. The remaining haplotypes were represented by single sequences. The small perpendicular marks on these connections represent mutational steps, suggesting the minimum number of mutations required to transform one haplotype into another (Fig. 6).

Selection analysis

Polymorphism exists in SAP54 orthologs from diverse strains

Alanine remains the predominant amino acid across all sequences (Supplementary material 1), with an average frequency exceeding 46 %, closely followed by threonine at approximately 32 % (Supplementary table 1). Cysteine levels fluctuate between ~9 % and ~16 %, suggesting variations in structural composition, while glycine exhibits similar variability (Fig. 7). Amino acid substitutions encompass various chemical classifications, including hydrophobic residues such as phenylalanine (F), leucine (L), alanine (A), proline (P) and isoleucine (I), as well as polar residues such as asparagine (N), serine (S), tyrosine (Y), threonine (T), histidine (H), lysine (K) and arginine (R) (Fig. 8a). The weblogo representation illustrates site-specific amino acid conservation and the relative frequency of each residue (http://weblogo.threeplusone.com/create.cgi) (Fig. 8b).

SAP54 orthologs are under purifying selection

Ka and Ks values were calculated across the entire ORF sequences of SAP54 orthologs. Analysis of 190 pairwise comparisons across 20 sequences revealed all pairs had Ks values greater than Ka, indicating strong purifying/stabilizing selection across diverse phytoplasma strains. The silent mutation rate (Ks) ranged from 0 to 0.1034 substitutions per site, whereas the non-silent mutation rate (Ka) was lower, between 0 to 0.0413 substitutions per site (Table 5). The Ka/Ks ratio (ω) was significantly less than 1 across all pairwise comparisons, ranging from 0 to 0.39 with an average of ≈0.3. Most values fell below 0.5 (85.8 %) and a significant portion (58.9 %) was under 0.1, indicating strong purifying selection and strong structural and evolutionary stability of the protein. The highest Ka/Ks ratio(ω = 0.39) occurred between PV610804.1 and AB862490.1, indicating persistent purifying selection. Similarly, the sequence pairs AB862477.1 vs AB862490.1 and PV610804.1 vs AB862477.1 both showed ω = 0.33 (Ka = 0.027, Ks = 0.081), indicating moderate but constrained divergence under purifying selection.

SAP54 amino acid sites are under pervasive purifying selection

SAP54 amino acid sites are predominantly subject to site specific selection, as assessed using the FEL approach with a *p*-value threshold of 0.1 (Table 5). Analysis revealed one site under positive/diversifying selection (in the signal peptide) and five sites under negative/purifying selection. Among six sites, nonsynonymous substitution rates (β) exceeded neutral expectations, with the 42nd codon showing the highest β value, 12.014, indicating pervasive positive selection. Codon 64 exhibits strong purifying selection by synonymous substitution rates (α = 15.188), highlighting its evolutionary constraint and potential significance in SAP54 function. The codon 42 showed positive

Table 4. Identification of various haplotypes of *Ca.* P. based on the SAP54H gene in the current study

Haplotype	number of sequences	Accession numbers and countries
Hap_1	3	PV610804.1 (India), AB897827.1 (Sudan), LC740446.1 (Japan)
Hap_2	1	AB862480.1 (Germany)
Hap_3	2	LC740445.1 (Japan), AB862489.1 (Italy)
Hap_4	1	AB862478.1 (Germany)
Hap_5	1	MK858224.1 (India)
Hap_6	5	LC740447.1 (Japan), AB897828.1 (The Netherlands), AB862487.1 (France), AB862488.1 (USA), DQ837760.1 (USA)
Hap_7	1	AB862481.1 (Italy)
Hap_8	2	AB812838.1 (Japan), EF200537.1 (Japan)
Hap_9	1	AB862484.1 (Italy)
Hap_10	1	AB862486.1 (Japan)
Hap_11	1	AB862477.1 (Spain)
Hap_12	1	AB862490.1 (Italy)

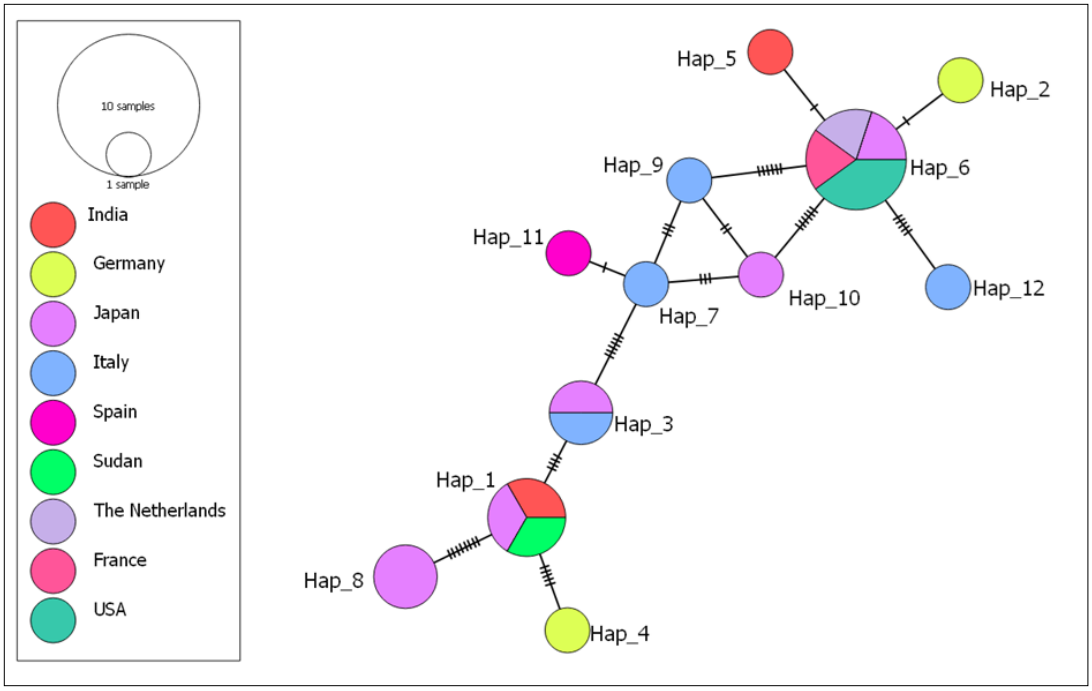

Fig. 6. The haplotype network of *Ca.* P. based on SAP54 orthologsequences (n = 20), from nine countries. Each circle represents a unique haplotype, with size proportional to sequence count. Nucleotide differences are marked by hatch lines, each indicating a single nucleotide variation.

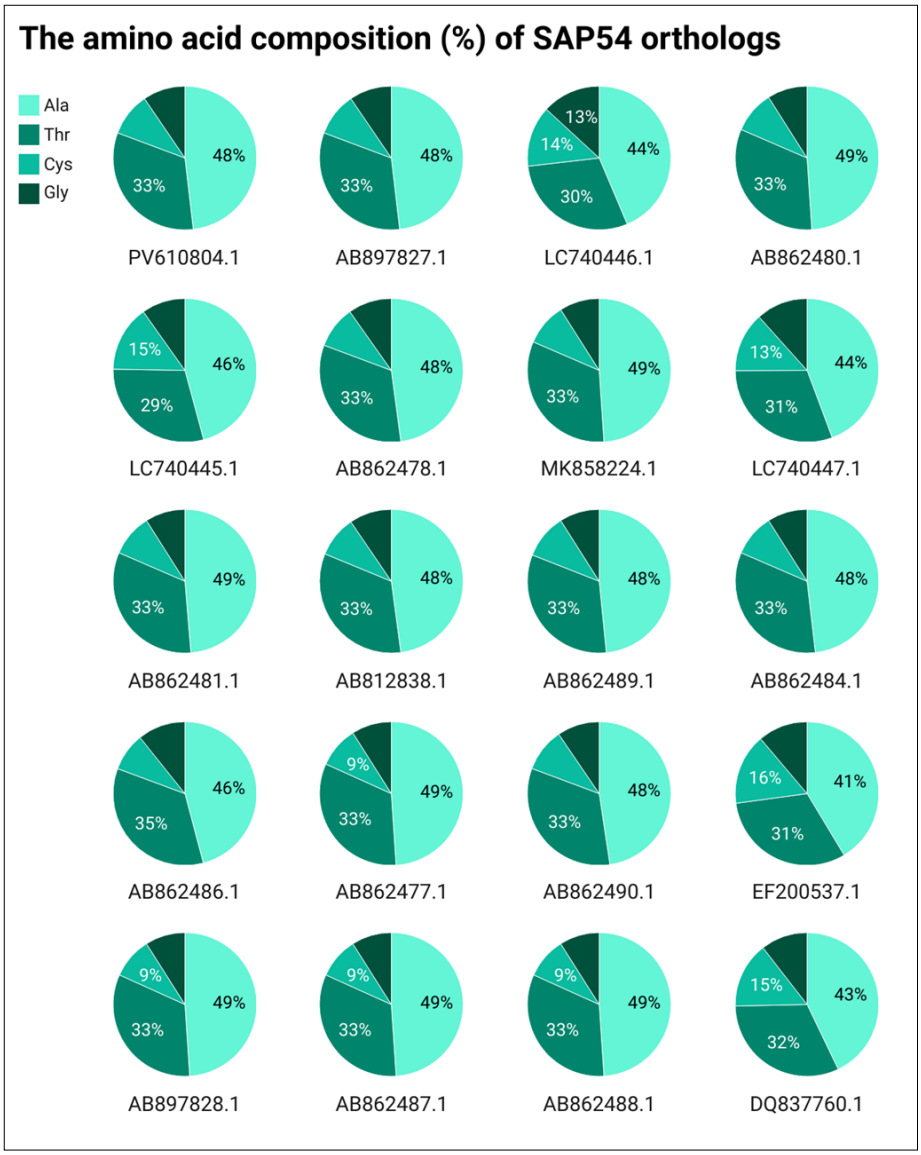

Fig. 7. Major amino acid composition of SAP54 orthologs, the percentage composition of four amino acids alanine (Ala), threonine (Thr), cysteine (Cys) and glycine (Gly) across different groups and subgroups of phytoplasma strains.

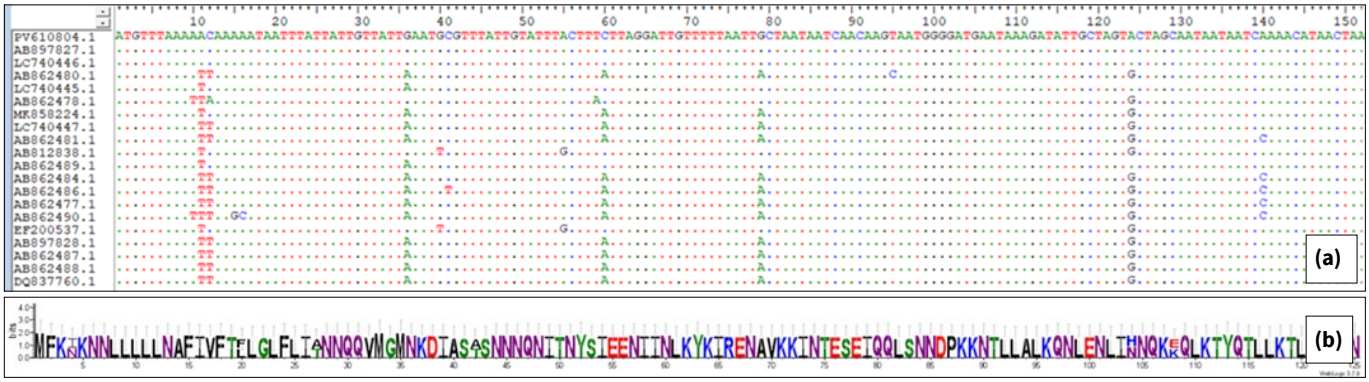

Fig. 8. Comparative analysis of amino acid polymorphisms and conservation patterns in SAP54 orthologs from diverse phytoplasma groups. (a) A multiple sequence alignment of Polymorphism in 20 SAP54 orthologs from phytoplasmas spanning nine different groups and subgroups, affecting plant species from across nine countries. Conserved amino acid residues are marked by dots. (b) WebLogo representing amino acid conservation and relative frequency at specific sites.

Table 5. Evolutionary selection of SAP54 sites identified by FEL analysis

Partition	Codon	α (dN)	β (dS)	α=β	LRT	*p*-value	Total branch length	Selection class
1	12	1.876	0.003	1.697	5.293	0.0214	0.249	Purifying
1	17	0.441	0.001	0.075	6.180	0.0129	0.039	Purifying
1	42	0.000	12.014	5.356	4.999	0.0254	0.695	Diversifying
1	53	0.352	0.001	0.050	5.900	0.0151	0.044	Purifying
1	64	15.188	0.000	7.280	5.672	0.0172	0.823	Purifying
1	108	0.337	0.000	0.248	5.867	0.0154	0.305	Purifying

selection (LRT = 4.999), while the codon 64 was negatively selected (LRT = 5.672).

Discussion

In this study, we characterized a SAP54-like protein (SAP54LP) encoded by a phytoplasma strain associated with BLL disease in Madurai, Tamil Nadu, India. Molecular analyses confirmed that the phytoplasma belonged to the 16SrVI-D subgroup based on 16S rDNA sequence similarity and virtual RFLP profiling (40). However, the SAP54LP effector gene exhibited 100 % nucleotide sequence identity with homologs from 16SrVI-A strains, but only 98 % identity with the *Phyl1* effector from 16SrI-B phytoplasmas. This incongruence between effector gene similarity and 16S rRNA-based phylogeny suggests that *SAP54*-like genes do not strictly follow vertical inheritance. Instead, the pattern is consistent with horizontal gene transfer (HGT) or intergroup recombination, which may enable the spread of this virulence factor across phylogenetically distant phytoplasma lineages. Such events are biologically plausible given the mobile nature of phytoplasma effector genes, often located on potential mobile units (PMUs) or plasmid-like elements and are supported by prior studies reporting HGT of SAP54/PHYL1-family genes among divergent phytoplasma groups (41).

The functional role of SAP54 in phytoplasma pathogenicity and vector interactions has been well documented (20, 23). Our findings support the hypothesis that SAP54LP enhances phytoplasma transmission by promoting host attractiveness to insect vectors, possibly through the degradation of *MADS*-box transcription factors (MTFs), which prolong the vegetative phase of the host. Leaf-like floral phenotypes, often observed in phytoplasma-infected plants may be an unintended consequence of SAP54 adaptation to target MTFs (41). This dual role, enhancing vector colonization and altering host development-underscores SAP54LP's importance in the epidemiology of phytoplasma diseases.

Sequence analysis revealed that the SAP54LP gene from BLL-affected brinjal was sequenced with 3x coverage and although the start and stop codons were not definitively identified, the high sequence similarity and low E-values in BLASTn searches suggest that SAP54LP functions as a functional ortholog of SAP54, rather than a pseudogene. The absence of insertions, deletions or disruptive mutations further supports its potential effector functionality. This aligns with previous reports indicating that SAP54 orthologs are functionally conserved across phytoplasma species, despite some sequence variations (24).

Comparative sequence analysis between SAP54LP of BLL and its homolog from onion yellows (OY) phytoplasma (16SrI-B) revealed 16 SNPs, with nonsynonymous substitutions occurring threefold more frequently than synonymous ones. These mutations were distributed across both the signal peptide and mature protein regions, suggesting their functional relevance. Notably, amino acid substitutions in the signal peptide, such as Thr5→Ala (hydrophilic-to-hydrophobic) and Ala12→Asp (neutral-to-acidic), may affect membrane interactions, secretion efficiency or protease recognition, potentially influencing host-pathogen interactions. Moreover, the absence of the canonical AxA motif and its replacement with a VxG motif suggest a non-canonical cleavage mechanism, supporting the idea of evolutionary flexibility in signal peptide motifs (42).

In mature proteins, mutations such as Gly70→STOP (premature termination) and Thr→Ala substitutions (introducing hydrophobic residues) may alter protein folding, stability or host-binding affinity. Charge-altering mutations such as Gly65→Glu could further modify protein-protein interactions. These findings are consistent with reports showing that frameshifts and premature stop codons in PMU-associated genes, such as *hflB,* can lead to functionally modified proteins that contribute to host -specific virulence (17). Thus, the Gly70→STOP mutation in our identified SAP54LP of BLL may represent an adaptive truncation,

potentially optimizing SAP54 functionality for host manipulation or immune evasion.

Haplotype network analysis revealed clear geographic structuring of SAP54LP sequences. Hap_6, the most widespread and diverse haplotype, included isolates from India, Japan, Italy, the Netherlands, France and Sudan, suggesting that it may represent a globally circulating lineage. The presence of region-specific haplotypes, such as Hap_2 (Germany), Hap_5 (India), Hap_8 (Japan) and Hap_11 (Spain), indicates localized evolutionary events or recent mutations. Mutational distances between haplotypes provide insight into pathogen spread: short mutational steps among multi-country haplotypes suggest rapid transmission with minimal genetic change, whereas longer branches imply more isolated or slower evolution. These patterns are consistent with those of earlier studies on other phytoplasma species, in which geographic proximity and vector-mediated dispersal have been shown to shape population structures (43, 44).

Evolutionary pressure analysis using Ka/Ks ratios and site -specific selection models (FEL) revealed that SAP54LP is under strong purifying selection, with an average Ka/Ks value of 0.36. This indicates that the gene is functionally constrained, likely due to its critical role in host manipulation and pathogenicity. Only 4 % of codons were under pervasive purifying selection, while 0.8 %, primarily in the signal peptide region, were under positive selection, suggesting adaptive diversification in regions involved in host interaction or secretion. It is important to note that these conclusions are derived from computational predictions. Therefore, future experimental validation, such as through transient expression or gene knockout studies, is essential to directly confirm the impact of these substitutions on host targeting and response. This pattern is consistent with findings for other phytoplasma effectors, where functional domains are conserved, but surface-exposed or regulatory regions evolve rapidly to optimize host-pathogen interactions (45, 46).

Interestingly, all positively and negatively selected codons were encoded by AT-rich codons, indicating multi-level evolutionary constraints. The high α values at specific sites such as Site 64 suggest additional selective pressures beyond protein structure including mRNA stability and codon usage bias. For instance, Site 6 showed evidence of overlapping selection at both the protein and RNA levels, reinforcing the idea that SAP54 is an ideal model for studying multi-level evolutionary adaptations in host-associated pathogens (47).

Conclusion

In conclusion, this study provides valuable insights into the molecular evolution and functional adaptation of the SAP54LP effector in the 16SrVI-D phytoplasma strain associated with BLL disease. While SAP54LP shares a high sequence identity with SAP54 homologs from the 16SrVI-A subgroup, it exhibits distinct nonsynonymous substitutions that may reflect adaptive evolution driven by host and vector interactions. The haplotype network highlights both global dissemination and localized evolution, while the dominance of purifying selection underscores the functional importance of SAP54LP in effector-mediated pathogenicity. However, the presence of positively selected sites, particularly in the signal peptide, indicates ongoing diversification that may enhance transmission efficiency and host manipulation.

These findings position SAP54LP as a promising model system for studying effector evolution in phytoplasmas, with implications for understanding host-pathogen-vector interactions and the epidemiology of phytoplasma diseases. Future studies should focus on the transient expression or gene knockout studies, functional validation of these adaptive mutations including their effects on host gene expression, vector behavior and pathogen transmission, to further elucidate the molecular mechanisms underlying SAP54LP-mediated pathogenicity.

Authors' contributions

MN, EP, MS and GS conceived and wrote the manuscript. TA, GK, NM, VBRP contributed to improving the manuscript. All the authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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