

Article

The G126S Mutation in the *cytb* Gene Confers Bifenazate Resistance in a *Tetranychus urticae* Koch Laboratory Strain

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Abstract

The two-spotted spider mite, *Tetranychus urticae* Koch, is a major agricultural pest with a rapid propensity for developing acaricide resistance. Bifenazate targets mitochondrial cytochrome b (CYTB). While the G126S mutation is associated with resistance, its independent role remains unclear, as it often occurs with other SNPs. This study explores the molecular basis of bifenazate resistance in a Russian laboratory strain derived from a St. Petersburg greenhouse population. Disruptive selection with increasing bifenazate concentrations generated resistant and susceptible isofemale lines. AlphaFold2 structural modeling of CYTB indicated that G126S causes a steric clash, leading to conformational destabilization, whereas other reported mutations primarily affect the ligand-binding pocket. Oxford Nanopore sequencing revealed a low initial frequency of the G126S allele (<1%; 226/35,895 reads) in the unselected population. After one year of stepwise selection (0.00005–0.031% a.i.), the mutant allele frequency surged to 90% (7272/8056 reads). No other resistance-associated mutations were found in the analyzed *cytb* fragment. We report the first identification of the G126S mutation in a Russian *T. urticae* population and demonstrate rapid fixation under bifenazate selection. Within this genetic background, G126S alone appears sufficient to confer high-level resistance, emphasizing the population-specific nature of resistance evolution and the critical need for local monitoring.

Keywords: *Tetranychus urticae*; acaricide resistance; bifenazate; allele frequency; G126S mutation; cytochrome b



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1. Introduction

The two-spotted spider mite (TSSM) *Tetranychus urticae* Koch (Acari: Tetranychidae) is one of the most destructive herbivorous pests; it damages more than 1100 plant species, about 150 of them are important crops, including vegetables, fruits, and ornamentals [1]. It has a short life cycle, high offspring production and a remarkable ability to develop pesticide resistance [2–4]. *T. urticae* females may produce over 100 eggs of different size depending on the embryo sex: males develop from unfertilized eggs while females derived from fertilized ones [5]. All of the above shows the relevance of research aimed at improving methods for controlling the populations of these pests.

Spider mites are most effectively managed when multiple control strategies (mechanical, cultivational, biological, and chemical controls) are used to reduce pest populations and maintain plant health [6]. When all other control methods have failed to keep the populations under control, chemical acaricides may be needed. Chemical control of TSSM has been only partially successful, as TSSM can rapidly develop resistance to most types of insecticides and acaricides because of its short life-cycle, resulting in rapid population growth [3]. Acaricide resistance can develop through multiple mechanisms, including enhanced metabolic breakdown of acaricides, target site insensitivity, and behavioral resistance [7]. One of the most important mechanisms for acaricide resistance development in *T. urticae* is target site insensitivity, where single-nucleotide polymorphisms (SNPs) can result in an alteration of an amino acid sequence and the translated protein which, in-turn, binds the insecticide weaklier, or not at all [8].

T. urticae has been documented to have developed resistance to over 96 acaricidal/insecticidal active ingredients [9]. Tolerance to acaricides can result after a few applications [10]. Furthermore, *T. urticae* can become fully resistant to new acaricides within two to four years, meaning that control of multi-acaricide resistant *T. urticae* has become increasingly difficult [11].

Bifenazate (D2441, N'-(4-methoxy-biphenyl-3-yl) hydrazine carboxylic acid isopropyl ester) is a hydrazine carbazate acaricide that was discovered in 1990 by Uniroyal Chemical. It is used to control all growth stages of *Tetranychus* spp. on a wide variety of crops [12]. Bifenazate was shown to be converted by carboxyl/cholinesterases into diazene and an activation pathway has been suggested [13,14].

The target of bifenazate is mitochondrial electron transport (MET) chain [3,15]. The MET chain consists of four large transmembrane enzyme complexes (Complexes I–IV) that work together to transfer electrons from NADH and succinate to molecular oxygen [15,16]. Cytochrome c oxidoreductase or the bc1 complex, catalyzes the electron transfer from reduced ubiquinone to cytochrome c [16]. Complex III consists of three core subunits: cytochrome b (*cytb*), cytochrome c1, and the Rieske iron–sulfur protein [15,17]. Notably, CYTB is the only complex III subunit that is encoded by mitochondrial DNA. CYTB contains two distinct quinone-binding sites, Qi and Qo, which are located near the inner and outer sides of the inner mitochondrial membrane, respectively [18,19].

Furthermore, a strong correlation was found between specific *cytb* point mutations and the occurrence of resistance (Table 1) [12].

Due to the characteristics of mitochondrial inheritance—namely, the absence of recombination and exclusive maternal transmission—new mutations in mitochondrial DNA are rapidly fixed in populations through a bottleneck effect [20]. This process accelerates the spread of resistance alleles, especially under strong selective pressure caused by the widespread use of bifenazate.

Metabolic resistance is generally associated with enhanced activity of enzymes that detoxify acaricides before they reach their target sites. This mechanism is driven by differential expression of detoxification genes, which can result from changes in regulatory elements (cis- or trans-regulation) or gene duplication [21].

Cytochrome P450 enzymes (CYPs) are the most extensively studied detoxification enzymes in various species, including insects and mites. Most functionally validated CYPs belong to the CYP392 family, which exhibits increased diversity in *T. urticae* [21]. For instance, the P450 enzyme CYP392A11 is capable of metabolizing bifenazate through hydroxylation of its ring structure [22]. This enzymatic modification significantly reduces the toxicity of bifenazate, thereby contributing to the development of resistance.

Despite frequent increases in the expression of detoxification genes in response to pesticides, the molecular mechanisms underlying these changes remain largely unclear.

Researchers rarely succeed in identifying mutations that directly trigger the upregulation of these enzymes [20].

The high levels of resistance observed in some populations of *T. urticae* are likely the result of a combination of mechanisms, including target-site mutations and enhanced detoxification mediated by P450 enzymes [22–25]. These mechanisms may act synergistically, significantly increasing resistance levels compared to the effects of each mechanism individually [22].

Table 1. Mutations in the two-spotted spider mite *cytb* gene, associated with bifenazate resistance.

Gene	Mutation	Phenotypically Shows Up as Being Associated with	Location	Reference
<i>cytb</i>	G126S	A133T, I136T, S141F, D252N, L258F	Cyprus; The Netherlands; Finland; Japan; USA; China; Australia	[26–29]
<i>cytb</i>	G132A		Japan; The Netherlands; Belgium	[20,28]
<i>cytb</i>	A133T	G126S	UK	[30]
<i>cytb</i>	I136T	G126S	The Netherlands	[26,31]
<i>cytb</i>	S141F	G126S	laboratory strain	[31]
<i>cytb</i>	D161G		laboratory strain	[28,31]
<i>cytb</i>	D252N	G126S	Australia	[29]
<i>cytb</i>	L258F	G126S	The Netherlands	[22]
<i>cytb</i>	I260V	N326S	Republic of Korea	[32]
<i>cytb</i>	P262T		UK; Belgium; The Netherlands; Asia	[22,26,30,31]
<i>cytb</i>	A269V		China	[33]
<i>cytb</i>	N326S	I260V	Republic of Korea	[32]
CYP392A11 (P450)	differential expression		Japan	[22,24]

SNPs frequently interact with other genetic and physiological factors. This complicates the study of their role in isolation and the interpretation of research results. Of particular interest is the G126S mutation in mitochondrial cytochrome b, which has long been considered an important factor responsible for resistance in *T. urticae* to bifenazate and related compounds. However, later studies based on sequencing and functional tests indicate that this mutation is unlikely to be the sole cause of resistance, and its effect appears only in combination with other mutations.

For example, one study showed that lines of the mite *Panonychus citri* with the G126S mutation exhibit only moderate levels of resistance to bifenazate, whereas the combination of G126S with an additional A133T mutation significantly increases resistance [34]. In another study, cross-resistance in a *T. urticae* line carrying the G126S + S141F combination conferred high levels of resistance to both acequinocyl and bifenazate. In contrast, a line with the single G126S mutation remained susceptible to acequinocyl, casting doubt on its role in resistance mechanisms [35].

Additional data showed that G126S was also detected in *T. urticae* populations susceptible to bifenazate. This led to the hypothesis that this mutation is not a reliable marker for bifenazate resistance. Nevertheless, resistance was observed in populations where G126S occurred in combination with I136T and S141F mutations. It was also noted that five mutations conferring bifenazate resistance (A133T, I136T, S141F, L258F, D252N) were always found alongside G126S, yet none of them appeared independently or in other combinations [29].

The complexity of interpreting the role of G126S is further compounded by its interaction with metabolic detoxification mechanisms. Experiments selecting acequinocyl-resistant populations revealed the G126S mutation; however, it was also detected in bifenthrin-susceptible populations, calling into question its key role in resistance to this compound. While resistance to acequinocyl showed maternal inheritance, resistance to bifenthrin depended on additional factors. Studies using synergists, such as piperonyl butoxide (PBO), identified the role of cytochrome P450 enzymes in enhancing resistance, supporting its polygenic nature and the interaction of mutations with metabolic mechanisms [24]. Finally, it was concluded that the G126S mutation may serve as a marker of resistance, but its effect largely depends on the presence of other mutations or metabolic detoxification mechanisms. These findings emphasize the need for a comprehensive approach to studying resistance, incorporating analyses of both genetic and physiological factors and their interactions.

To our knowledge, no studies have been conducted on the molecular genetic control of resistance of Russian spider mite wild populations and/or laboratory cultures to bifenthrin.

The purpose of our study was to investigate the molecular mechanisms of bifenthrin resistance in two-spotted spider mite (TSSM) *Tetranychus urticae* Koch laboratory cultures of the All-Russian Institute of Plant Protection (VIZR).

2. Materials and Methods

2.1. TSSM Cultures

Experiments were conducted on females of the common spider mite *Tetranychus urticae* Koch. Mite cultures were selected using a diagnostic concentration of the acaricide bifenthrin, a carbamate. The line was developed from a laboratory culture selected with dimethoate (BI-58 40% e.c.), originally brought from the greenhouse of JSC “Flowers” in St. Petersburg from a Solo rose.

The general scheme of the experiment is shown in Figure 1A.

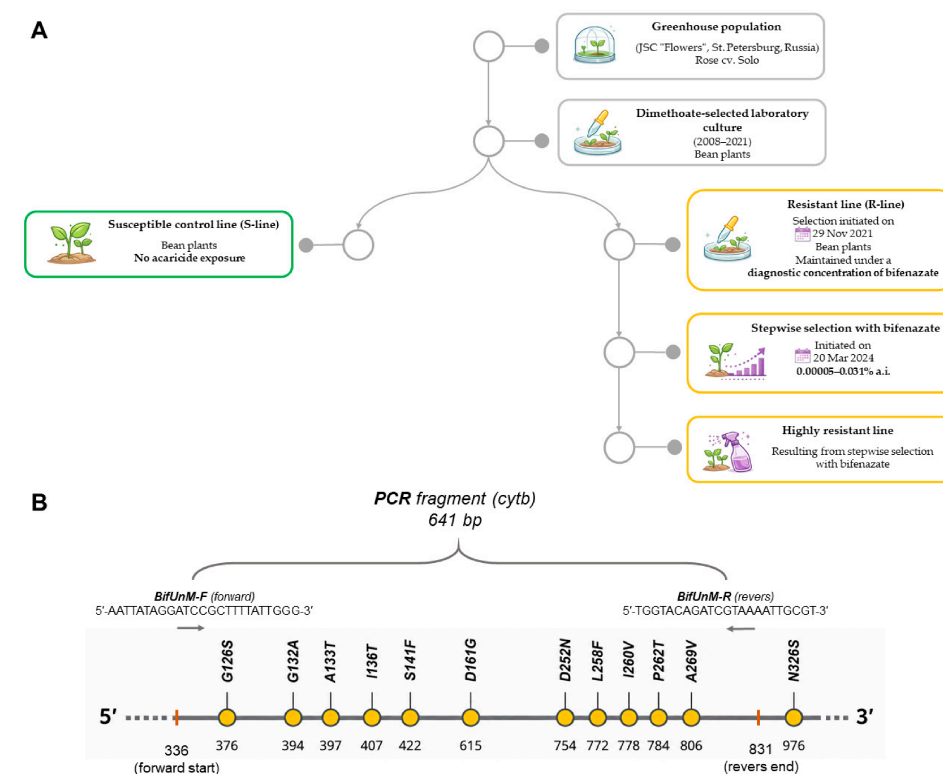


Figure 1. Experimental design. (A) Schematic representation of the sampling and selection procedure. (B) Schematic diagram of the diagnostic fragment within the *CYTB* gene.

2.2. Maintaining of TSSM

Homozygous laboratory strains of *T. urticae* were obtained by disruptive selection during inbred propagation. Resistant (R) and susceptible (S) strains of mites were cultured as separate colonies on isolated leaves of the common bean (*Phaseolus vulgaris*) placed in a cuvette on a layer of moistened cotton wool. The mites were maintained at 22 °C and 18 h of daylight to optimally support their development.

Mites were treated by immersing infested leaves in an aqueous solution of the toxicant bifenazate, specifically using the commercial pesticide Fenobi at a concentration of 240 g/L SC. The treatment concentration was maintained at 0.00005%, resulting in a 75% mortality rate among female mites. Mite colonies exhibiting 90–100% survival after acaricide treatment were used to create R-lines, while S-lines were developed through sister selection from colonies that experienced 100% mortality following treatment. The offspring of a single female, demonstrating either resistance or sensitivity, were individually placed on leaf rafts after bifenazate treatment to obtain age-matched offspring. The PCR experiment involved females treated with bifenazate from 1–3 rafts, showing the lowest mortality rate for R-strains and 90–100% for S-strains. Adult females that had undergone two molting stages (proto- and deutonymph) were used in the study, which was conducted every 15–20 days, depending on the season.

2.3. Selective Treatments

Treatments were conducted on whole bean plants grown in special containers filled with a manganese solution. When the plants began to wilt or became heavily infested with mites, a new container with freshly sprouted plants was placed nearby to encourage the pests to migrate to the new shoots. The treatments involved spraying the infested plants, with the frequency varying from 8 to 15 days, depending on how quickly the mite population recovered to the level necessary for the next treatment. Every eighth treatment, the concentration of the product was increased fivefold. In total, 28 treatments were administered, with the bifenazate concentration rising from 0.00005% to 0.031% of the active ingredient over the entire period.

2.4. DNA Isolation and PCR

An adult female mite was transferred to a separate 0.2 mL tube containing 20 µL of ddH₂O and heated to 90 °C for 10 min. The solution was then carefully pipetted and either used immediately as a PCR template or stored at –20 °C.

Each PCR reaction was carried out in 20 µL volume containing 8 µL of ddH₂O, 10 µL of 2x Dream Taq Green PCR Master Mix (Thermo Scientific, Vilnius, Lithuania), 5 picomoles of each forward and reverse primer (BifUnM-F: 5'-AATTATAGGATCCGCTTTTATTGGG-3'; BifUnM-R: 5'-TGGTACAGATCGTAAAATTGCGT-3'), and 1 µL of template. PCR was performed in a GeneExplorer Thermal Cycler 96 × 0.2 mL (Bioer, Hangzhou, China). The amplification program included a 3 min preincubation at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C.

The PCR products were separated electrophoretically in an agarose gel, and the results were visualized using the GelDoc Go gel documentation system (Bio-Rad, Hercules, CA, USA).

The location and structure of the diagnostic fragment within the target gene are illustrated in Figure 1B.

2.5. RNA Extraction and Reverse Transcription

Total RNA was extracted from 40 pooled adult female *T. urticae* collected from approximately three to four infested bean leaves. Mites were transferred into a 0.2 mL

microcentrifuge tube containing 10 µL of nuclease-free water and incubated at 90 °C for 10 min. Subsequently, 100 µL of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added, and the sample was thoroughly homogenized by pipetting. The lysate was transferred to a 0.5 mL tube and incubated for 10 min at room temperature.

Samples were centrifuged at 13,000 rpm for 7 min at room temperature. The supernatant was transferred to a new tube, mixed with 20 µL of chloroform, incubated for 7 min at room temperature, and centrifuged at 13,000 rpm for 15 min at 4 °C. The aqueous phase was carefully transferred to a fresh tube, mixed with 60 µL of isopropanol, and centrifuged at 13,000 rpm for 15 min at 4 °C. The RNA pellet was washed with 60 µL of 70% ethanol and centrifuged at 13,000 rpm for 15 min at 4 °C. After removing the supernatant, the pellet was air-dried for 5–7 min and resuspended in 20 µL of nuclease-free water. RNA samples were used immediately for cDNA synthesis or stored at –80 °C until further analysis. First-strand cDNA was synthesized using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. All reaction mixtures were prepared on ice. qPCR was performed using SYBR Green chemistry.

2.6. qPCR

For qPCR, a reaction mixture was prepared using the SYBR®Green intercalating dye. The 20 µL reaction mixture consisted of 8 µL of ddH₂O, 10 µL of SsoAdvanced Universal SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA), 5 picomoles of each forward and reverse primer, and 1 µL of template. qPCR was conducted using a Gentier 96R Real-Time PCR System (Tianlong, Wuhan, China).

1. To detect the G126S mutation, we employed the diagnostic system described by Maeoka et al. [36], which includes two pairs of primers: allele-specific and universal. The amplification program comprised a 3-min preincubation step at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 61 °C, and 40 s at 72 °C. DNA of no less, then 12 individual mites were analyzed in each line at each stage. For allele frequency assessment, we used two complementary analytical approaches: the ΔC_t method, which reflects the raw C_t difference between the reference and alternative alleles, and the $2^{-\Delta\Delta C_t}$ method, which normalizes these differences to a calibrator sample R-before selection [37]. This dual approach allowed us to estimate intra- and inter-culture variability in allele representation.

2. For gene expression analysis RT-qPCR was conducted on cDNA generated as described in Section 2.5 using primers P450_F AGGAGACCGTTTCGTGTATGC, P450_R TAACGTGGCTGAAGTCGAGG, GAPDH-2Fb GCACCAAGTGCTAAAGCATGGAG, GAPDH-2Rb GAACTGGAACACGGAAAGCCATAC

The amplification program comprised a 3 min preincubation step at 95 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 63 °C, and 40 s at 72 °C.

Relative expression of the target gene was calculated according to the $2^{-\Delta\Delta C_t}$ method [37]. All reactions were run in triplicate, and *GAPDH* was used as the endogenous control.

2.7. Oxford Nanopore Sequencing and Data Analysis

A MinION Mk1B nanopore sequencer (Oxford Nanopore Technologies, Oxford, UK) was employed for sequencing the amplicons, utilizing a R10.4.1 flow cell. Libraries were prepared using the Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK), and sequencing was conducted over a period of 120 h.

Primary data processing was performed using Guppy version 6.5.7 (Oxford Nanopore Technologies). The superprecision model (SUP) was applied to convert electrical signals into nucleotide sequences (basecalling), and samples were automatically separated by index.

Subsequently, the resulting reads were mapped to the reference sequence (locus JAPRAS010000022.1:11775–12839) using the minimap2 algorithm [38]. The quality and structure of the resulting alignment were verified through visualization in IGV [39]. Finally, the consensus sequences were generated using the samtools toolkit [40].

2.8. Statistical Analysis

Statistical analyses were performed using the two-tailed exact Mann–Whitney U test. Data are presented as mean $\pm$ SE for bar graphs and as box-and-whisker plots showing the median, interquartile range (IQR), and whiskers extending to $1.5 \times$ IQR, where appropriate. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed in Microsoft Excel using the Real Statistics Resource Pack software (Release 9.7.6) add-in [41].

2.9. Databases and Software

The original amino acid sequence of cytochrome b (CYTb) from *Tetranychus urticae* was obtained from GenBank (ID: WQH82754.1). A search for homologous structures was performed using BLASTp 2.16.0 with default parameters (word size = 5, BLOSUM62 matrix, gap opening/extension penalty of 11/1) against the Protein Data Bank (PDB). Chicken complex III (PDB ID: 1BCC) was used as a reference. The new structure of CYTb from TSSM was predicted using AlphaFold2 with default parameters, using 1BCC as the template. Molecular visualization and structural analysis were conducted in PyMOL 3.0.0 [42]. For modeling subunit complexes, MODELLER 10.6 (Conda version) was used [43].

2.10. Methods of Bioinformatics Analysis

Homologous Modeling. Using BLASTp, homologues of the cytochrome c1 subunit (XM_015925946) and heme protein (XP_025016154.1) were identified in *T. urticae*. The sequences were aligned to the corresponding structures from 1BCC, with unnecessary N-terminal fragments removed. For mutation analysis, structural variants were generated in three ways: (1) all mutations combined, (2) each mutation separately, and (3) pairs of linked mutations.

Search for Coevolving Residues. Using BLASTn (default parameters), sequences were searched against a database of mites from the same genus. The sequences listed in the table and the sequence WQH82754.1 served as the initial queries for the search. Deduplicated results and sequences with known mutation effects were then included in the analysis.

Correlating positions and binding pockets were identified using visual CMAT with default parameters, using the AlphaFold2 prediction as the input structure [44].

Determination of Membrane Topology. The positions of transmembrane domains were predicted using DeepTMHMM 1.0.42 based on the sequence [45]. These predictions were then refined using OPM 3.0 (AWS version), analyzing both with and without membrane curvature.

Validation of Results. The quality of the models was assessed by comparing them with the experimental structure (1BCC) and conducting steric clash analysis in PyMOL Version 3.0.0.

For mutant variants, we checked for preservation of the overall fold and the absence of steric overlaps, except in the case of G126S, where the mutation was found to be structurally incompatible.

Computational Resources. Structure prediction was performed using the Google Colab AlphaFold2 interface (ColabFold 1.6.1, AlphaFold 2.0) [46].

3. Results

3.1. Structural Modeling of Cytochrome b from *Tetranychus urticae*

Since the alleles associated with resistance to bifenthrin in Russian two-spotted spider mite populations have not been studied, we first compiled a list of mutations documented in the literature that confer resistance to this substance. We then modeled the protein structures to identify which mutations might significantly impact their interaction with the active ingredient.

The structure of *T. urticae* cytochrome b (CYTb) was predicted using AlphaFold2 and exhibits high similarity to chicken cytochrome b (PDB ID: 1BCC; RMSD = 1.2 Å).

Two subunits were identified in complex with cytb:

- Cytochrome c1 (homologue: XM_015925946), which was successfully modeled based on the chicken template.
- Heme protein (homologue: XP_025016154.1), whose structure was predicted with lower confidence (pLDDT < 70 for the N-terminal domain).

3.2. Analysis of Mutations of Cytb at the Protein Level

The desired mutations were introduced into the structure using PyMod 3.0.2 (Figure 2A). The G126S substitution created a steric conflict that prevented its stable integration into the structure. The mutations do not interact with neighboring subunits, as the minimum distance to cytochrome c1/heme protein is greater than 8 Å.

Additionally, the structures of the mutant proteins were predicted using AlphaFold. The mutant variants—single, paired, and full set—did not exhibit significant structural rearrangements, with an average RMSD of less than 0.8 Å relative to the native model (Supplementary Files S1 and S2).

Twenty AlphaFold structures were obtained for the mutant protein (4 seeds, max_msa 64:128). Residues within 15 Å of residue 126 were tested, excluding residues 126 and 247. Residues 239–242 and 245 changed their conformation in all mutated structures, exhibiting at least two different orientation types. These amino acids do not appear to make contact with the ligand, heme, or other subunits and seem to comprise a loop, predicted with a low level of certainty (Figure 2B).

Approximately 20 additional residues differ in states between the various structures; however, none of these were found to be significant in any other context.

Additionally, the spatial interaction between residues S141 and K279 is illustrated in Figure 2C. This analysis demonstrates that while the S141F mutation disrupts the original contact, a cation– π interaction between K279 and F141 may still occur, potentially contributing to structural stabilization.

3.3. Coevolution and Membrane Topology

Coevolutionary Analysis. VisualCMAT revealed significant evolutionary correlations among 345 residues (Supplementary File S3). Notably, only the 161st residue, which correlates with the 157th, has been previously identified as playing a role in resistance (Figure 2D). VisualCMAT also did not predict the locations of resistance-associated mutations within the binding pockets (Supplementary File S4).

Membrane Topology. Deep TMHMM and OPM predicted eight transmembrane helices, which is in agreement with previous findings (Figure 2E).

The curvature of the membrane did not influence the positioning of mutations, as all are situated on the positive side of the membrane. The substitution of hydrophilic serine with hydrophobic phenylalanine near the predicted membrane surface may lead to slight changes in complex conformation or positioning (Supplementary File S5).

A summary of the protein structure modeling results is provided in Table 2.

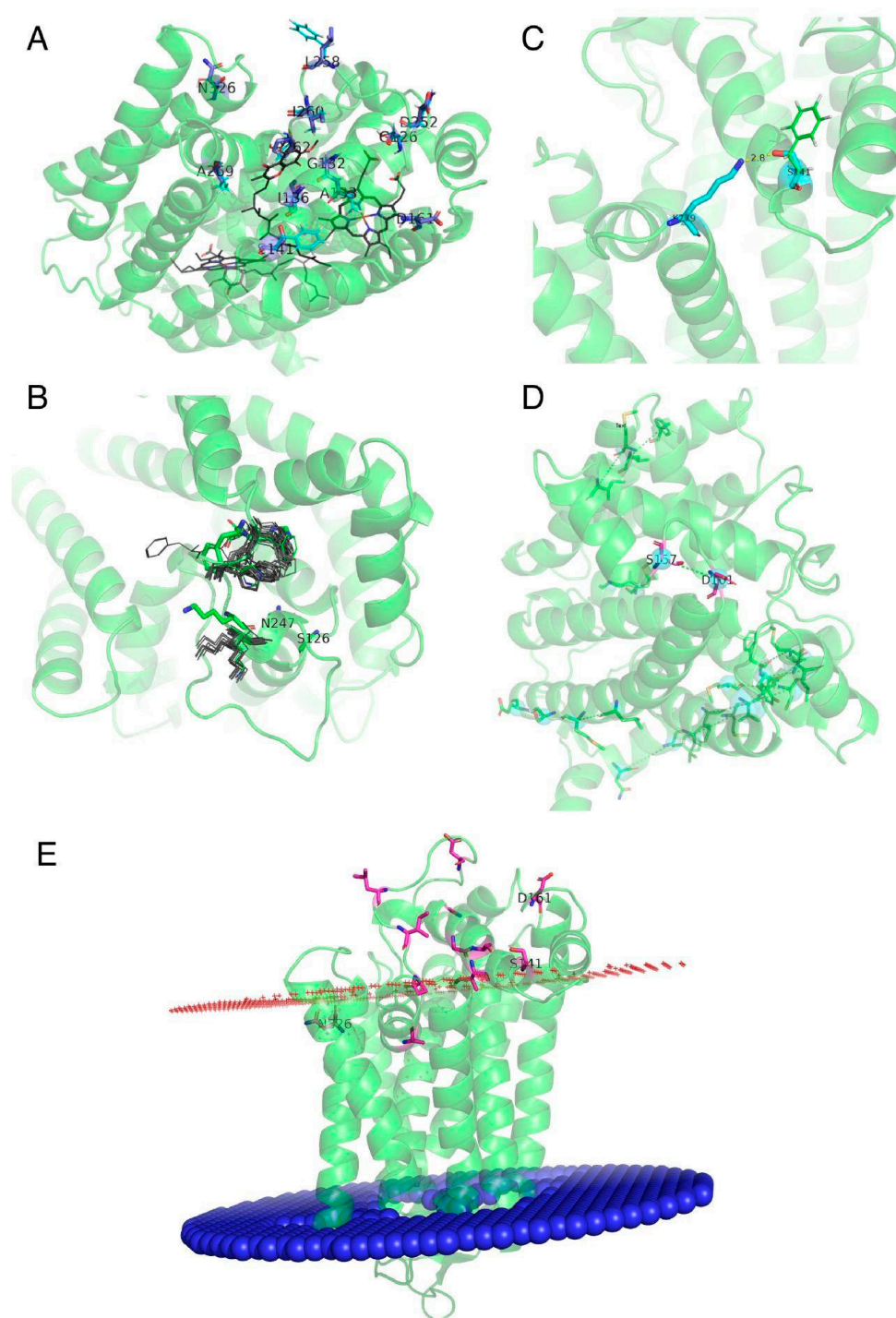


Figure 2. Spatial location of resistance-associated mutations in cytochrome b. **(A)** Location of candidate residues relative to cytochrome b. Hemes are shown as well. Residues of interest are shown in blue, with mutated residues shown in cyan. **(B)** Predicted conformations of selected residues in 20 G126S mutants. Wild-type conformations are shown as sticks. Straightforward mutation G126S in original structure leads to a clash with N247. **(C)** Interaction between S141 and K279 (shown in cyan). This interaction is disrupted by the S141F mutation; however, cation– π interaction between K279 and F141 may still occur. **(D)** Coevolving residue pair D161–S157. Irrelevant pairs could be seen in the background. Cyan transparent spheres represent the significance of coevolution. **(E)** Position of mutations relative to membrane topology. Unsurprisingly, mutations are found on the positive side of the membrane marked by red dots. N326 and A269 are located inside the membrane. S141 is predicted to lie close to the phosphodiester layer; S141F mutation is a hydrophilicity–hydrophobicity switching.

Table 2. Structural changes in *cytb* gene mutants in two-spotted spider mite (*Tetranychus urticae* Koch).

Mutation	Phenotypic Manifestation	Predicted Structural/ Functional Effect	Notes
G126S	Bifenazate/acequinocyl resistance	Steric clash in structure; incompatible with wild-type fold due to N247	Often co-occurs with A133T, I136T, S141F, L258F, D252N
A133T	Bifenazate/acequinocyl resistance	OH-group may interact with S129 backbone; no direct ligand contact	Found alongside G126S; radical buried in globule
G132A	Bifenazate resistance	Binds bifenazate; hydrophobic pocket alteration; no interaction with ubiquinone radical	Located in binding site
I136T	Bifenazate resistance in laboratory strains	Proximity to ligand (no direct contact); hydrophobic face may interact with bifenazate	Near binding site
S141F		Interacts with K279; bridges distant regions; membrane boundary location	Structural stabilization; potential allosteric effect
D161G		Faces hydrophilic region; no interactions; membrane boundary	Co-evolves with residue 157
D252N		Hydrophilic region; no ligand contact; membrane boundary	Found alongside G126S; no functional impact detected
L258F	Acequinocyl/bifenazate resistance	May interact with heme protein	Found alongside G126S
I260V		Conservative replacement in binding site	Found alongside N326S
P262T		Binding site disruption; proline substitution alters backbone conformation; alkyl group points toward active site	High structural impact
A269V	Minor bifenazate resistance	Slightly narrows binding pocket surface	Indirect effect
N326S		Faces solvent; potential interaction with unknown protein	Outside binding site; found alongside I260V

Our data align with the molecular docking results obtained by Kewedar and co-authors [47] which indicated that the G132A mutation alters the structure of the binding pocket.

Thus:

Mutation-induced structural changes in cytochrome b do not account for stability through direct effects on protein–protein interactions. Instead, they suggest conformational changes within the protein itself (G126S) or in the ligand-binding pocket (G132A, I260V, P262T).

The identified coevolutionary patterns and membrane localization provide new targets for functional studies, with amino acid residues 161 and 157 being particularly noteworthy.

3.4. Search for the Presence of SNP G126S in the Mitochondrial Genomes of Mites from the Resistant Culture of VIZR

To detect the G126S mutation in the CYTB gene, we employed a diagnostic system previously described in the literature [36], which included both allele-specific primers and primers that amplify a gene region regardless of the mutation's presence. Analysis of 32 *T. urticae* females (19 from the resistant line (R line) and 13 from the susceptible line (S line)) revealed 17 individuals carrying the G126S mutation. The threshold cycle (Ct) values for both primer types allowed us to estimate the relative copy number of the mutant alleles. Given that the mitochondrial genome exists in multiple copies (ranging from hundreds

to thousands of copies per cell) [48], variability in the frequency of mutant alleles was detected. The difference in threshold cycles (ΔC_t) between reactions with allele-specific and universal primers varied from 9.4 to 3.15, the average ΔC_t was 4.46 cycles, which, assuming equal reaction efficiency, corresponds to a proportion of the mutant allele of approximately 0.15% to 11.26%, average 4.54%. The low frequency of mutant alleles was likely associated with an insufficient concentration of the selective agent used.

3.5. Estimation of Mutant Allele Frequency Using Oxford Nanopore Sequencing

To estimate the allele frequency of the single-nucleotide substitution corresponding to the G126S mutation (G>A substitution at position 376), sequencing was conducted on a diagnostic fragment of the *CYTB* gene, amplified using the primers BifUnM-F and BifUnM-R on DNA template of a typical individual of culture R. The analysis showed that the frequency of the allele with the G126S mutation was less than 1 percent (226 out of 35,895 reads). No other resistance-inducing substitutions described in the literature were identified, nor were any associated substitutions found during the coevolutionary analysis. In the analyzed region of the gene, three previously undescribed synonymous substitutions were identified that do not alter the amino acid sequence of the protein (441A>G, c.475G>A, and c.624T>C—numbering from the start of the coding sequence). The raw sequencing data are available in the NCBI Sequence Read Archive (SRA) under accession number SRR37490508. These substitutions can be used for molecular labeling of the laboratory culture of spider mites from the VIZR collection.

3.6. Selection at Elevated Concentrations of Bifenazate

To assess the role of the identified allele with the G126S substitution in the development of bifenazate resistance, we conducted selection treatments on beans infected with individuals from the R line over the course of a year. The concentration of the drug was gradually increased throughout the series of treatments. Upon completion of the experiment, we performed toxicological testing on the crop subjected to the selection treatments using both the diagnostic and maximum concentrations of bifenazate. At the diagnostic concentration, the average mortality rate was 18.4%, indicating the development of resistance to the drug. At a concentration of 0.031%, survival was limited to just a few individuals.

3.7. Estimation of Mutant Allele Frequency After Selection at Elevated Concentrations of Bifenazate

After a series of selection treatments with stepwise increasing drug concentrations, real-time PCR was repeated using the diagnostic system described above [36]. The difference in threshold cycles between reactions with allele-specific and universal primers (ΔC_t) decreased, indicating an increase in the proportion of the resistant allele (Figure 3A,B).

Similar to the previous analysis, Oxford Nanopore sequencing was performed on laboratory mite cultures after completing the bifenazate treatments. Before selection (initial culture), the wild-type allele prevailed, accounting for 99% of the total 35,895 reads. After a series of selection treatments with bifenazate, the allele ratio reversed. Out of 8056 reads, the mutant allele was detected in 90% (7272), while the wild-type allele was present in 10% (770). The raw sequencing data are available in the NCBI Sequence Read Archive (SRA) under accession number SRR37490507. Thus, the selection treatments resulted in a significant increase in the proportion of the mutant allele, establishing its prevalence (Figure 3A–C). No other mutations conferring resistance were identified. The data indicate rapid fixation of the resistant allele under chemical selective pressure.

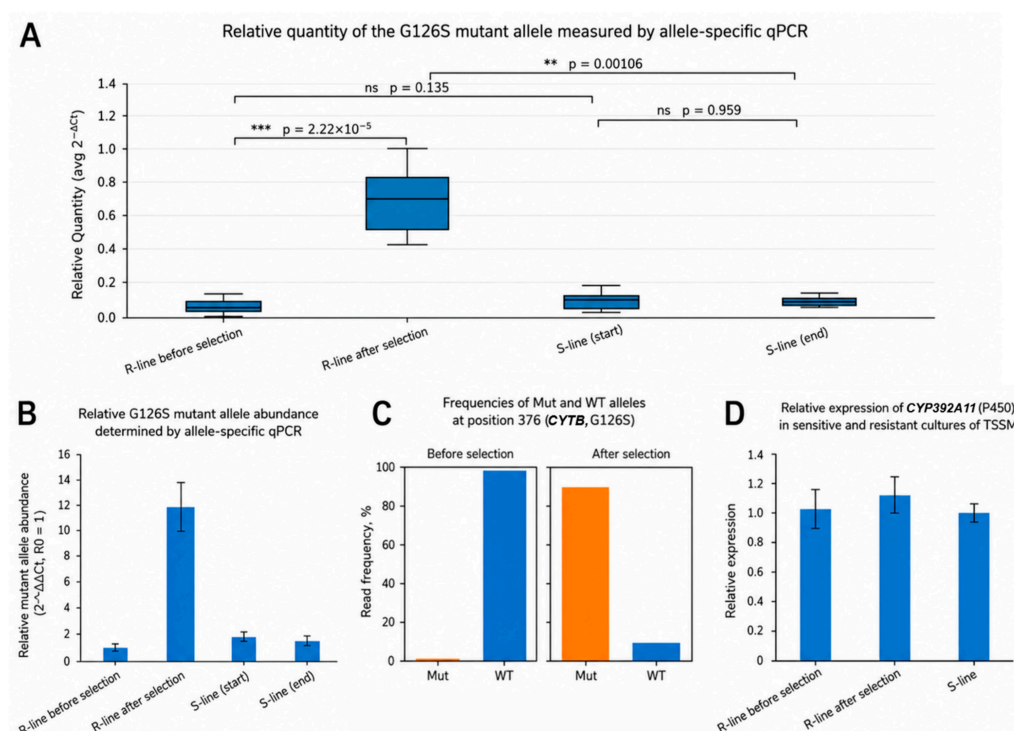


Figure 3. *CYTB* allele frequencies and relative P450 expression before and after bifentazate selection. (A) Variability of relative allele frequencies within and between cultures, assessed by the ΔCt method. (B) Mean allele frequencies across cultures, calculated as $2^{\Delta Ct}$ values. (C) Allele frequencies derived from Oxford Nanopore sequencing; error bars denote standard error of the proportion (SE). (D) Relative expression of CYP392A11 (P450) in different TSSM cultures.

3.8. Estimation of Expression of CYP392A11 (P450) in Sensitive and Resistant Cultures of TSSM

Given that acquired resistance may arise not exclusively from target-site mutations but also from enhanced metabolic detoxification, we examined the transcript levels of P450, a key enzyme responsible for bifentazate metabolism. Relative expression was measured using RNA extracted from pooled samples of 40 individuals per line. As presented in Figure 3D, no significant differences in CYP392A11 (P450) expression were detected between the susceptible and resistant lines, neither prior to nor following bifentazate selection. These findings indicate that metabolic detoxification is unlikely to contribute to the resistance phenotype selected under increasing bifentazate concentrations.

4. Discussion

To our knowledge, this study is the first to document the G126S mutation in a laboratory culture originating from a Russian TSSM population. The functional significance of this mutation in bifentazate resistance is substantiated by comparative in silico modeling of the wild-type and variant protein structures. Moreover, the absence of significant differences in the expression of detoxification-associated gene CYP392A11 (P450) between resistant and susceptible lines further excludes metabolic resistance as an alternative mechanism, thereby reinforcing the primary role of the target-site mutation.

Our findings align with the data presented by Shi et al. [49] and contradict the views of some other authors [34,35]. This discrepancy may stem from the different genetic backgrounds in which mutations manifest in TSSM from various populations. The *cytb* gene product is a protein that functions as part of a complex multisubunit assembly. Consequently, we can hypothesize the existence of compensatory mutations outside the gene under investigation that could affect its functionality. To elucidate the mechanisms of

the mutation's action, more detailed studies are needed that extend beyond the structure of the gene and cytochrome b protein.

Furthermore, it is not only important to detect the G126S substitution in the mite population but also to assess the frequency of the mutant allele relative to the wild-type variant. Our experiment showed a two-order-of-magnitude increase in the frequency of the mutant allele in the culture maintained at high concentrations of the active ingredient.

Thus, our findings reconcile conflicting literature by emphasizing the population-specific nature of resistance evolution. The discrepancy regarding the indispensability of G126S likely stems from differing genetic backgrounds, where its effect may be modulated by unlinked compensatory mutations or metabolic mechanisms not examined here. This research underscores the necessity of monitoring local pest populations for resistance alleles and confirms the G126S mutation as a potent and selectable marker for bifenthrin resistance. Future work should focus on comprehensive genomic and metabolomic analyses to identify potential contributing factors outside the *cytb* gene and to develop robust diagnostic tools for resistance management in agricultural settings.

5. Conclusions

This study provides the first molecular genetic characterization of bifenthrin resistance in a Russian population of the two-spotted spider mite, *Tetranychus urticae*. We successfully established resistant and susceptible isogenic lines and applied structural bioinformatics, targeted genotyping, and next-generation sequencing to elucidate the resistance mechanism. Our key finding is the identification and validation of the G126S mutation in the mitochondrial cytochrome b gene as a central factor conferring resistance in this specific genetic background. Structural modeling indicated that this substitution causes significant steric conflict, likely destabilizing the protein fold or its interaction with bifenthrin.

Critically, longitudinal selection experiments with escalating bifenthrin concentrations demonstrated a direct correlation between selection pressure and the frequency of the G126S allele, which increased from less than 1% to 90% dominance in the population. This rapid fixation under chemical stress strongly supports a causal role for G126S in the observed resistance phenotype. Furthermore, the absence of other known resistance-associated mutations in the analyzed *cytb* fragment suggests that, contrary to some reports from other global populations, G126S alone can be a primary determinant of resistance in certain genetic contexts.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae12070825/s1>, File S1: PyMol session with all mutations and components marked; File S2: PyMol session with *cytb* and Rieske iron-sulfur protein; File S3: VisualCMAT output; File S4: PyMol session with VisualCMAT results; File S5: PyMol session with *cytb* location in the membrane.

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