Original Article

Molecular Characterization of Cytochrome P450 Genes (CYP9M10 and CYP4H34) in Insecticide-Resistant and Susceptible Strains of *Culex pipiens*

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Abstract

Background: *Culex pipiens* is widespread in Iran and is an important vector of several diseases. Although phenotypic resistance to insecticides such as DDT and pyrethroids has been reported using WHO assays, sequence-level information on metabolic resistance genes, particularly cytochrome P450 genes, remains limited. This study examined variation in two P450 genes, *CYP9M10* and *CYP4H34*, in deltamethrin- and DDT-resistant versus susceptible strains of *Cx. pipiens*, and assessed the potential impact of these differences on predicted protein structures.

Methods: Target fragments of *CYP9M10* and *CYP4H34* were amplified by PCR and sequenced using the Sanger method. Edited nucleotide sequences were aligned with CLUSTAL OMEGA, and amino acid sequences were generated using ExPASy Translate. Comparisons were conducted at both nucleotide and amino acid levels. Representative sequences were submitted to GenBank. Phylogenetic relationships among strains were inferred via maximum-likelihood (ML) analysis in MEGA6 with 1000 bootstrap replicates. Predicted amino acid substitutions were examined for structural relevance.

Results: Four nucleotide differences were detected at positions 1344, 1347, 1396 and within 1428–1442. Previously published permethrin- and pyrethroid-resistant reference sequences were identical across this region, whereas sequences from this study showed distinctions from those references and between resistant and susceptible strains. Some nucleotide substitutions led to amino acid changes, though their structural effects were only inferred computationally.

Conclusion: This study provides new sequence-level insights into variation in *Cx. pipiens* P450 genes and highlights potential genetic differences that may contribute to resistance to DDT and deltamethrin, warranting further functional investigation.

Keywords: Arboviruses; Culicidae; Disease vectors; Deltamethrin; Iran

Introduction

Mosquitoes (Family: Culicidae) are among the most important arthropod vectors of human diseases (1). *Culex pipiens*, which is widely distributed across Iran, plays a significant role in the transmission of lymphatic filariasis and several arboviruses (2). Chemical insecticides, particularly pyrethroids and DDT, have long been central to mosquito control programs; howev-

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er, the emergence of insecticide resistance has increasingly undermined their effectiveness (3–5). As one of the most important steps in managing insecticide resistance, identifying the underlying resistance mechanisms, including the involvement of insecticide-resistance–related genes, is therefore essential. Such investigations typically include sequence analysis, evaluation of structural differences and polymorphisms between susceptible and resistant strains, and examination of gene expression patterns (6, 7).

Pyrethroids and DDT exert their insecticidal effects mainly by targeting the voltage-gated sodium channel (VGSC) in the mosquito nervous system, causing prolonged neuronal excitation and paralysis. Mutations in this channel, known as knockdown resistance (kdr) mutations, are widely associated with reduced insecticide sensitivity. The voltage-gated sodium channel is the target of pyrethroids and DDT (8). In addition to target-site resistance, metabolic detoxification mechanisms involving cytochrome P450 monooxygenases (P450s), carboxylesterases (CEs) and glutathione S-transferases (GSTs) also contribute to pyrethroid resistance (9, 10). Recent studies in *Culex* mosquitoes indicate that pyrethroid resistance is a multifactorial process involving overexpression of multiple P450 and esterase genes, kdr-associated target-site insensitivity, and microRNA-mediated regulation of P450 expression (11–13). Although P450 involvment in insecticide resistance is well established, characterizing individual P450 genes remains challenging due to the size, complexity and functional diversity of this gene family (14). Thus, the specific contributions of most P450 genes are still unclear (15). Exceptions include genes such as CYP6A1, which is overexpressed in diazinon resistance in *Musca domestica* (16); CYP6G1 in *Drosophila melanogaster* in DDT-resistant strains (17); and CYP9A12 and CYP9A14 in *Saccharomyces cerevisiae* in pyrethroid-resistant strains (18.

Given the wide distribution of *Cx. pipiens* in Iran and its role in disease transmission (19–24), together with documented insecticide resistance in this species (25–27), the present study aimed to characterize sequence variation in two cytochrome P450 genes, CYP9M10 and CYP4H34, in DDT- and deltamethrin-resistant and susceptible strains of *Cx. pipiens*.

Materials and Methods

Specimen collection and susceptibility test assay

All samples were collected from different parts of Urmia County, West Azerbaijan Province, Iran. Larvae were collected from diverse aquatic habitats using a standard 350-mL dipper method at four sites: Naz-Loo (37°39′24.39″N, 44°59′0.39″E; 1358 m a.s.l.), Ghahraman-Loo (37°39′10.78″N, 45°12′11.81″E; 1000 m a.s.l.), Koor-Abad (37°43′50.12″N, 44°39′33.78″E; 1545 m a.s.l.), and Issar (37°33′25.95″N, 45°0′12.52″E; 1466 m a.s.l.) between June and October 2014. Collected larvae were reared under controlled insectary conditions (27±2 °C, 70–80% relative humidity, and a 12:12 h light:dark photoperiod). Larvae were fed a finely ground fish-food diet and emerging adults were maintained in screened cages with access to 10% sucrose solution. Non-blood-fed female mosquitoes aged 3–5 days were used for susceptibility assays.

Susceptibility bioassays were performed using eight replicates per insecticide (deltamethrin and DDT), with approximately 30–32 adult female mosquitoes per replicate, resulting in 255 and 252 specimens, respectively. Control assays included four replicates with 127 mosquitoes.

WHO-supplied insecticide-impregnated papers were used according to standard WHO protocols and within their certified validity period. The sugar-fed 3–5-day-old F1-generation *Cx. pipiens* females were transferred to holding tubes, and allowed to rest for 1-hour; dead or damaged individuals were removed. Mosquitoes were then exposed for 60 minutes to WHO test tubes containing insecticide-impregnated papers at the discriminating concentrations of 0.05% deltamethrin and 4% DDT (26).

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DNA extraction and amplification of cytochrome P450 genes (CYP9M10 and CYP4 H34) Genomic DNA was extracted from susceptible and resistant individuals using the DNA Extraction Mini Kit (YT9030; Yekta Tajhiz Azma, Iran). The extracted DNA served as the template for PCR amplification of the cytochrome P450 genes (CYP9M10 and CYP4H34) using previously described primers (29). PCR reactions were performed in a final volume of 25 µL volums containing 1 µL of genomic DNA, 12.5 µL of 2× PCR Master Mix, 1 µL of each primer (10 pmol), and nuclease-free water to final volume. Thermal cycling conditions included an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 7 min.

PCR products were visualized on a 1% agarose gel, and high-quality amplicons were purified and subjected to Sanger sequencing.

Sequences and bioinformatics analysis

Raw sequences were edited using BioEdit and compared against reference sequences available in the NCBI database (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were generated using CLUSTAL OMEGA (www.ebi.ac.uk/Tools/msa/clustalo). Representative sequences were deposited in GenBank (Accession Numbers for CYP9M10: MF740771–MF740782; for CYP4H34: MF740783–MF 740788).

The sequences and translated amino acids were analyzed and compared using MEGA 6 (30) to evaluate sequence similarity and variation. Phylogenetic relationships for CYP9M10 and CYP4H34 were inferred using the Maximum Likelihood (ML) method implemented in MEGA 6, with 1000 bootstrap replicates to assess node support. Amino acid substitutions were identified through sequence-based comparisons; no structural modeling analyses were performed.

Results

Post-exposure mortality rates at 24 hours were 81.21% for deltamethrin and 15.62% for DDT. Based on WHO criteria, which classify resistance when mortality is below 90%, mosquitoes were determined to be resistant to these insecticides.

CYP4H34 gene analysis

Partial sequences of the CYP4H34 gene from DDT-susceptible and DDT-resistant *Cx. pipiens* strains were analyzed and compared to previously reported permethrin-resistant (GenBank accession no. JQ001927) and pyrethroid-resistant (GenBank accession no. AB334746) reference sequences. Four nucleotide differences were detected at positions 1344, 1347 and 1396, along with a variable region spanning nucleotides 1428–1442. The two reference sequences were identical in the analyzed region. In contrast, sequences generated in this study exhibited variations compared to the reference sequences and also showed differences between the resistant and susceptible strains (Fig. 1A). Nucleotide substitutions at positions 1344, 1347 and 1396 were synonymous, resulting in no amino acid changes. However, variations in the 1428–1442 region led to predicted amino acid substitutions (Fig. 1B). Overall, three amino acid differences were observed between the DDT-resistant and DDT-susceptible strains (Fig. 1C).

CYP9M10 gene analysis

Sequence analysis of the *CYP9M10* gene in DDT- and deltamethrin-resistant strains from this study, compared to a permethrin-resistant reference sequence from Japan (GenBank accession no. AB724265), revealed multiple nucleotide differences across several regions (Fig. 2A). Nucleotide substitutions occurred at positions 3136–3151, 3153–3154, 3174–3176, 3178, 3184, 3207–3209, 3205–3214, 3217 and 3244. Translated amino acid sequences showed six amino acid differences between the DDT-

resistant and DDT-susceptible strains (Fig. 2B). Five amino acid substitutions were identified when comparing DDT-resistant strains to the permethrin-resistant reference (Fig. 2C), and at least eight amino acid differences were observed between the DDT-susceptible strain and the permethrin-resistant reference (Fig. 2D).

Phylogenetic analysis

Phylogenetic analysis employing the Maximum Likelihood method indicated that *CYP4 H34* sequences from resistant and susceptible strains clustered together, suggesting limited divergence at this locus (Fig. 3). Conversely, greater sequence divergence was apparent in *CYP9M10*, particularly among deltamethrin-resistant strains.

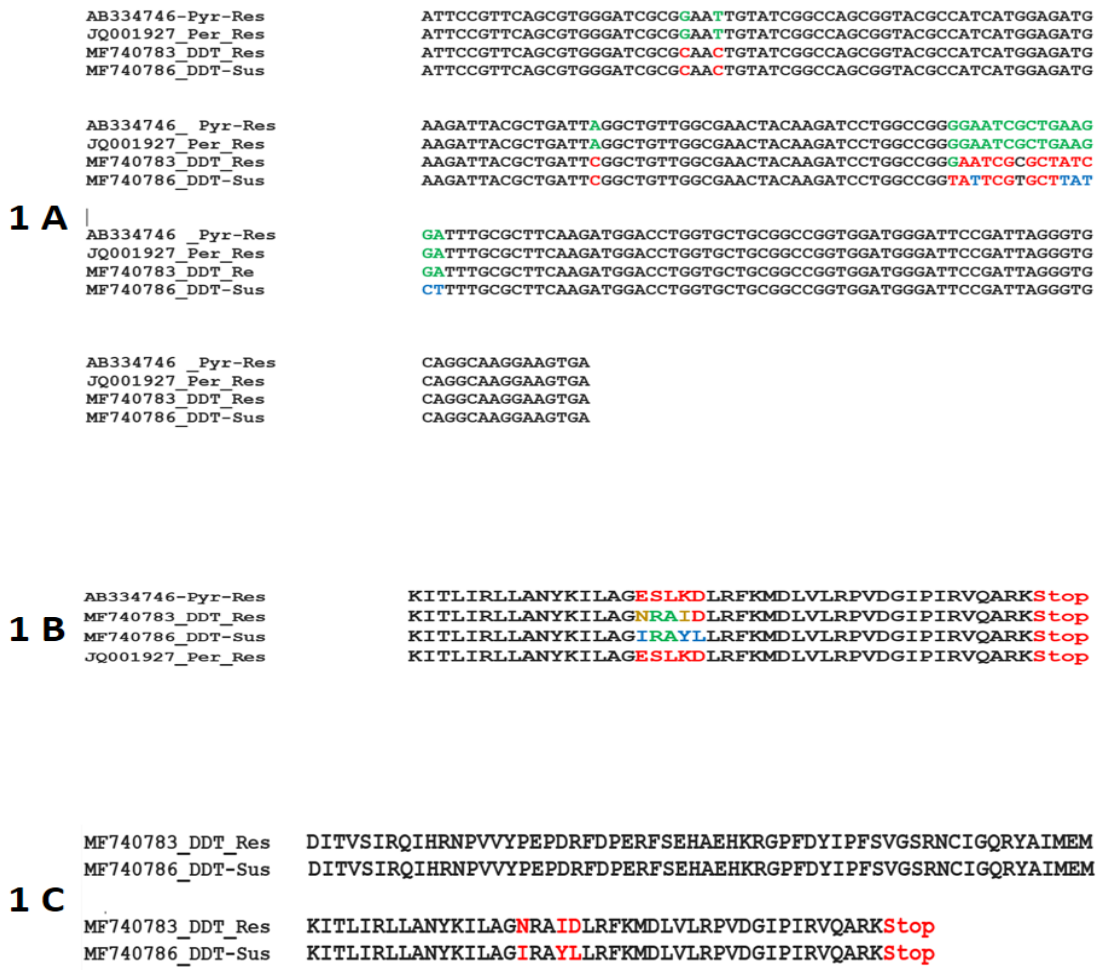

Fig. 1. Sequence analysis of the CYP4H34 gene. (A) Nucleotide sequence alignment of CYP4H34 fragments from DDT-resistant (MF740783) and DDT-susceptible (MF740786) *Cx. pipiens* strains, with permethrin-resistant (JQ001927) and pyrethroid-resistant (AB334746) reference sequences. Variable nucleotide positions are indicated. (B) Corresponding predicted amino acid sequence alignment derived from the nucleotide sequences shown in panel A, highlighting non-synonymous substitutions. (C) Pairwise amino acid sequence comparison between DDT-resistant and DDT-susceptible strains, illustrating the specific amino acid differences identified in this study

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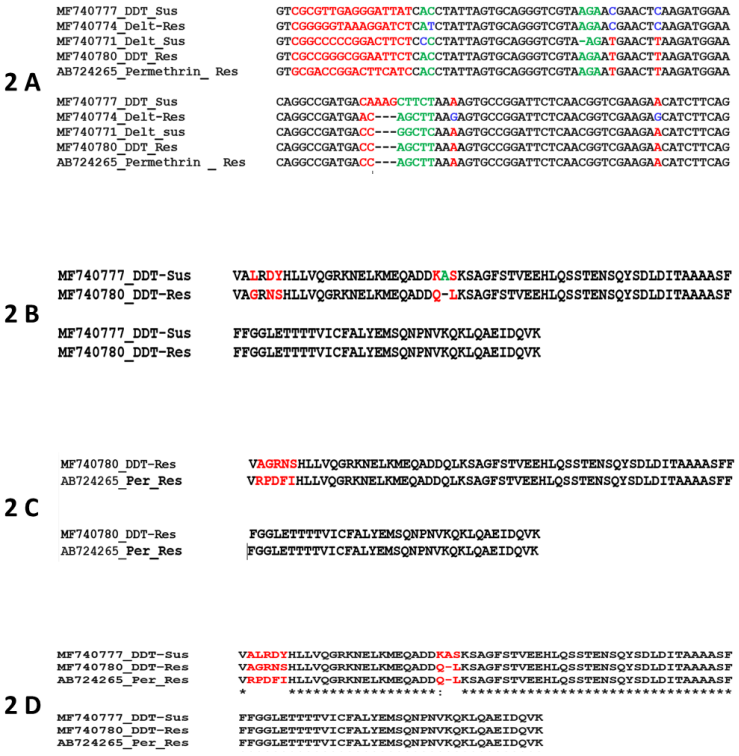

Fig. 2. Sequence analysis of the CYP9M10 gene. (A) Nucleotide sequence alignment of *CYP9M10* fragments from deltamethrin-susceptible (MF740771), deltamethrin-resistant (MF740774), DDT-susceptible (MF740777), and DDT-resistant (MF740780) *Cx. pipiens* strains analyzed in this study, compared with the permethrin-resistant reference strain (AB724265). Variable nucleotide positions are indicated. (B) Predicted amino acid sequence comparison between DDT-susceptible (MF740777) and DDT-resistant (MF740780) strains analyzed in this study, highlighting amino acid differences. (C) Predicted amino acid sequence comparison between the DDT-resistant strain (MF740780) analyzed in this study and the permethrin-resistant reference strain (AB724265). (D) Multiple alignment of predicted amino acid sequences from DDT-susceptible (MF740777), DDT-resistant (MF740780), and permethrin-resistant (AB724265) strains, illustrating amino acid variation among groups

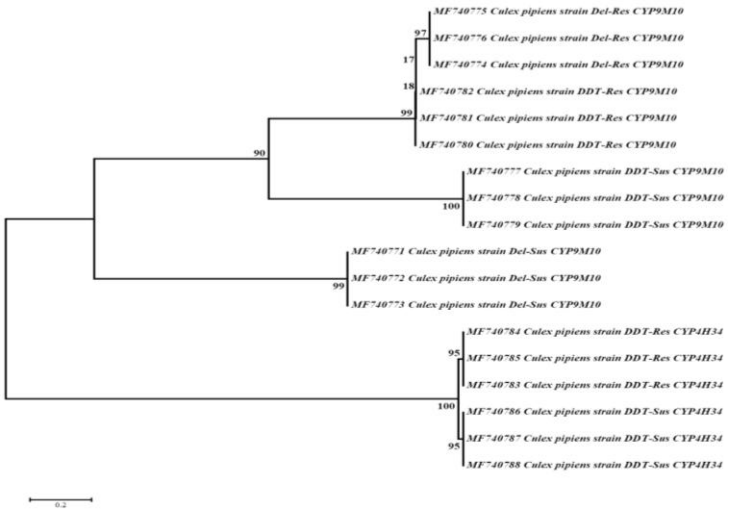

Fig. 3. Maximum likelihood phylogenetic trees of CYP4H34 and CYP9M10 genes.
Separate trees are shown for *CYP4H34* and *CYP9M10* sequences obtained from DDT- and deltamethrin-susceptible and resistant *Culex pipiens* samples analyzed in this study, together with reference sequences retrieved from GenBank. Trees were inferred using the Maximum Likelihood method with 1000 bootstrap replicates. Bootstrap support values are indicated at the corresponding nodes. The trees are rooted using [outgroup name, if applicable], and branch lengths represent evolutionary distances

Discussion

This study characterized the cytochrome P450 genes *CYP9M10* and *CYP4H34* at the sequence level in DDT- and deltamethrin-resistant and susceptible *Culex pipiens* populations. Nucleotide substitutions were identified in both genes, and several of these variations resulted in predicted amino acid differences between resistant and susceptible samples. However, these observations are based solely on sequence comparison and do not establish functional consequences.

Previous investigations have largely focused on the transcriptional regulation of P450 genes in resistant mosquito populations. Elevated expression of CYP9M10, CYP4H34 and CYP6Z10 has been reported in resistant strains (29). Specifically, CYP9M10 expression was found to be significantly increased in permethrin-resistant *Culex quinquefasciatus*, with promoter polymorphisms potentially driving this up-regulation (31, 32). In contrast, evidence for consistent differential expression of CYP4H34 between resistant and susceptible strains has been limited or inconsistent (33). These findings collectively suggest that regulatory mechanisms may be a primary driver of resistance phenotypes. Our study complements these expression-based findings by examining coding sequence variation and identifying amino acid substitutions in CYP9M10, indicating that structural variation may coexist with previously described transcriptional changes.

Resistance in *Culex* mosquitoes is widely recognized as a multifactorial phenomenon. Beyond metabolic detoxification mediated by P450 monooxygenases, resistance phenotypes can involve target-site mutations in the voltage-gated sodium channels (e.g., kdr L1014F) and post-transcriptional regulation, including microRNA-mediated modulation of detoxification genes (11–13). Therefore, sequence variation in individual P450 genes should be interpreted within this broader genetic and regulatory framework, rather than as an isolated determinant of resistance.

Gene-specific patterns of divergence were observed in the present study. Phylogenetic analysis revealed that CYP4H34 sequences from resistant and susceptible samples clustered within the same clade, indicating limited sequence divergence at this locus. This pattern suggests that structural variation in CYP4H34 is less strongly associated with resistance phenotypes in the studied populations. Moreover, as CYP4H34 has not been conclusively demonstrated to metabolize insecticides, the identified amino acid substitutions may reflect background genetic variation rather than resistance-specific adaptation.

In contrast, greater sequence divergence was observed in CYP9M10, particularly among deltamethrin-resistant samples. Comparative analysis with a permethrin-resistant reference strain from Japan also revealed additional amino acid differences, indicating sequence divergence across geographically distinct resistant populations. While these differences might associated with resistance phenotypes, structural variation alone does not confirm functional impact; the precise relationship between these amino acid substitutions and detoxification efficiency requires experimental validation.

Overall, the findings suggest a comparatively stronger association of sequence divergence in CYP9M10 resistance phenotypes than observed for CYP4H34 in the studied populations. However, definitive conclusions regarding functional relevance of these sequence variations necessitate integrative investigations. Future research should combine coding sequence analysis with promoter characterization, gene expression profiling, screening for *vgsc* mutations, and biochemical characterization of recombinant proteins.

Limitations

This study is subject to several limitations that warrant consideration when interpreting the findings. First, only partial coding regions of

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CYP9M10 and CYP4H34 were analyzed, and promoter regions were not examined. Second, the absence of gene expression level measurements and functional assays precludes a direct assessment of how observed structural variations translate into altered enzymatic activity or biological significance. Consequently, the biological significance of the observed amino acid substitutions remains uncertain. Third, functional assays were not performed to evaluate the enzymatic activity of the identified variants, and therefore, the biological significance of the observed amino acid substitutions remains uncertain. Furthermore, the analysis was restricted to sequences available in GenBank, limiting the sample size and potentially introducing ascertainment bias. Consequently, the observed findings should be interpreted as preliminary, sequence-based associations rather than definitive evidence of functional resistance mechanisms.

Conclusions

This study provides structural evidence that sequence divergence in CYP9M10, but not CYP4H34, may be associated with insecticide resistance in *Cx. pipiens*. These findings complement previous reports on transcriptional regulation, microRNA involvement and *kdr*-mediated target-site insensitivity, supporting a multifactorial basis for pyrethroid resistance. Further functional studies are required to clarify the precise role of CYP9M10 in resistance mechanisms.

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Ethical considerations

This study was conducted on *Cx. pipiens* mosquitoes and did not involve human participants or vertebrate animals. All procedures followed standard laboratory practices for insect handling and experimentation and were approved by the National Institute for Medical Research Development (NIMAD) Ethics Committee (IR.NIMAD.REC.1398.250).

Conflict of interest statement

The authors have declared that no competing interests exist.

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