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Point mutations in the voltage-gated sodium channel gene associated with pyrethroid resistance in Iranian populations of the European red mite *Panonychus ulmi*.

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Abstract

The European red mite *Panonychus ulmi* (Koch) is a major pest of apple trees worldwide and causes significant damage to apple orchards in Iran. Pyrethroid

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insecticides/acaricides, such as fenpropathrin and fenvalerate, are widely used to control *P. ulmi*, but their long-term use may lead to low efficacy. Earlier studies investigating pyrethroid resistance in closely related mites such as *Tetranychus urticae* revealed that pyrethroid resistance was associated with point mutations in the voltage-gated sodium channel (VGSC) gene. The aim of this study was to investigate the biochemical and molecular mechanisms of fenpropathrin and fenvalerate resistance in Iranian populations of *P. ulmi*. Pyrethroid toxicity bioassays were carried out on different *P. ulmi* field populations. Marand (resistance ratio, RR= 149), Maraqeh (RR= 90) and Mianeh2 (RR= 71) populations exhibited high levels of resistance to fenpropathrin, compared to a susceptible field population (Shahin Dej). Resistance was also observed for fenvalerate with resistance ratio's ranging from 2- to 20-fold. Synergism experiments and enzyme activity assays predict a minor role for classical detoxification enzymes. In contrast, two amino acid mutations in the VGSC, L1024V and F1538I, that were previously shown to confer pyrethroid resistance, were detected in all three resistant *P. ulmi* populations and point towards target-site insensitivity as the most likely resistance mechanism. Furthermore, sequencing after cloning of VGSC fragments from single haploid males revealed the presence of multiple copies of VGSC in a highly resistant strain. The link between resistance mutations and VGSC copy number variation should be the subject of future study, as this might be used to develop molecular markers for monitoring pyrethroid resistance of *P. ulmi* in the field.

Keywords: pyrethroids, resistance, voltage-gated sodium channel, spider mites, gene duplication, *kdr*

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1. Introduction

The European red mite, *Panonychus ulmi* (Koch) (Chelicerata: Acari: Tetranychidae) is one of the major mite pests in apple orchards worldwide (Jeppson et al., 1975) but may also cause damage to berries, peaches, pears and plums. High population levels of *P. ulmi* can

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lead to foliar bronzing, premature leaf drop, and small, poorly colored fruits in apple trees, and subsequently cause severe economic damage (Hardman et al., 1985). The spider mite *P. ulmi*, together with its close relatives *Panonychus citri* (McGregor) and *Tetranychus urticae* (Koch), are the most economically important mite pests (Van Leeuwen et al., 2015). Their control via acaricide application becomes increasingly challenging due to rapid development of resistance (Van Leeuwen et al., 2010). This rapid selection for acaricide resistance in mite populations has been associated with several factors including arrhenotokous reproduction, the extensive use of acaricides, and a short life-cycle (Van Leeuwen et al., 2010). At present, *P. ulmi* has developed resistance to nearly all known acaricide classes (Whalon et al., 2012). Pyrethroids are a major class of neurotoxic insecticides that have been used extensively to control a wide range of agricultural pests, including mites (Davies et al., 2007; Van Leeuwen et al., 2015). Fenpropathrin and fenvalerate are Type II pyrethroids that cause membrane depolarization accompanied by suppression of cellular excitability (Narahashi, 1986). The voltage-gated sodium channel (VGSC) is the target site of pyrethroids (Soderlund and Knipple, 2003). In some insect pests, the VGSC is encoded by two genes (*vgsc1* and *vgsc2*) and both of them have a similar function (Zuo et al., 2016). Generally however, this duplication is not common in arthropods. Target site insensitivity and enhanced enzymatic detoxification are the main mechanisms of resistance to pyrethroids in insects and mites (Van Leeuwen et al., 2010; Van Leeuwen and Dermauw, 2016). In spider mites, the involvement of carboxylesterases (CarE) and cytochrome P450 monooxygenases has been reported as major enzymes involved in pyrethroid resistance (Ay and Gürkan, 2005; Van Leeuwen et al., 2005; Van Leeuwen and Tirry, 2007). However, more than 50 sodium channel mutations or combinations have been associated with pyrethroid resistance in insects, mites and ticks, and many of them have been functionally validated by functional expression in *Xenopus* oocytes (Dong et al., 2014; Feyereisen et al., 2015). Overall, a combination of mutations has been shown to result in higher levels of pyrethroid resistance, compared to individual mutations (Dong et al., 2014). High-level resistance to pyrethroids in spider mites has also been linked to point mutations in the gene encoding the VGSC (Ding et al., 2015; Kwon et al., 2010b, Nyoni et al., 2011; Tsagkarakou et al., 2009; Ya-ning et al., 2011). In the two-spotted spider mite, *T. urticae*, resistance to fenpropathrin has been linked to three amino acid substitutions: L1024V+A1215D and F1538I+A1215D (numbering according to *Musca domestica*) (Kwon et al., 2010b; Tsagkarakou et al., 2009). In addition, a fenpropathrin-resistant field strain of the citrus red mite, *P. citri*, also harbored the F1538I mutation (Ding et al., 2015). The contributions of VGSC mutations in the potentially

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complex pyrethroid resistant phenotypes has been assessed in *T. urticae* by marker assisted back crossing, and revealed an exceptional strong phenotype associated with both mutations (Riga et al., 2017).

Fenpropathrin and fenvalerate are the main pyrethroids that are extensively used to control *P. ulmi* and the pear-leaf blister moth, *Leucoptera scitella* (Zell), respectively, in apple orchards in Iran. The objective of the present study was to study whether resistance to fenpropathrin has evolved in Iranian populations of *P. ulmi* collected in apple orchards. In addition, we aimed at elucidating the resistance mechanisms and report the presence of two mutations (L1024V and F1538I) that co-occur in populations and are putatively associated with fenpropathrin resistance in *P. ulmi*.

2. Materials and methods

2.1. Populations of European red mites

During 2016-2017, twelve field populations (Fig. 1) were collected from apple orchards of three provinces of East Azerbaijan (Mianeh, Marand, Ahar, and Maraqeh), West Azerbaijan (Urmia, Salmas, Shahin Dej, and Mahabad), and Isfahan (Semirom and Khafr). Selected apple orchards except Khafr, had a history of frequent spraying with different acaricides and insecticides. All populations of *P. ulmi* were grown on *Malus domestica* var. Fuji leaf discs, at 25 ±1°C, a photoperiod of 16:8h (L:D) and 60% relative humidity.

2.2. Chemical materials, bioassay and synergism assay

Commercial formulations of fenpropathrin (EC 10%, Golsam Gorgan, Iran), and fenvalerate (EC 20%, Golsam Gorgan, Iran) were provided from local distributors and used in all bioassays. Synergists piperonyl butoxide (PBO) and diethyl maleate (DEM) were purchased from Sigma-Aldrich (Bornem, Belgium), and triphenyl phosphate (TPP) was purchased from Merck (Darmstadt, Germany).

Bioassays were conducted using a method described by Van Leeuwen et al. (2004). Briefly, 10–20 young adult female mites were transferred to the upper side of square-cut apple leaf discs (12.25 cm^2) on wet cotton wool, which had been sprayed with 1.5 ml of spray fluid (1 bar pressure, 1.46±0.05 mg acaricide deposit cm^{-2}) by using a Potter spray tower (Burkard Scientific Ltd, Uxbridge, UK). The plates were then placed in a climatically controlled room at 25 ±1°C, 60% RH and 16/ 8 h (light/dark) photoperiod. Four replicates of

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at least five concentrations of each acaricide and a control (distilled water) were tested. Mortality was assessed after 24 h. Control mortality was always lower than 10%.

For synergism experiments, mites were exposed for 4 h to leaf discs sprayed with a non-toxic concentration of synergists (PBO= 1000 mg L^{-1}, DEM= 500 mg L^{-1}, TPP= 1500 mg L^{-1}), after which female mites were used in toxicity experiments according to a method as described above (Tsagkarakou et al., 2009). LC_{50}-values, slopes, resistance ratios (RR), synergistic ratios (SR) and 95% confidence limits were calculated using POLO-Plus software (Robertson et al., 2017).

2.3. Enzyme activity assays

For the preparation of enzyme source for carboxylesterases (CarEs), glutathione-S-transferases (GSTs) and cytochrome P450 monooxygenases (P450s) assays, about fifty female mites were homogenized in 300 µl of sodium phosphate buffer 0.1 M (300 µl, pH 7.4) with 0.1% Triton X-100, sodium phosphate buffer 0.1 M (300 µl, pH 7.4), and phosphate potassium buffer 0.1 M (400 µl, pH 7.1), respectively. The homogenates were subsequently centrifuged at 12,000 g (CarE and GSTs) and 10,000 g (P450s) for 15 min at 4°C.

CarE assays were conducted according to Van Leeuwen et al. (2006). with little modifications, using α-naphthyl acetate (64 mM) as substrate (diluted in phosphate sodium buffer 0.1 M) was used for evaluation of CarE activity. Enzyme samples (30 µl) were added to α-NA (200 µl), Fast Blue RR 0.2% (120 µl) and phosphate sodium buffer 0.02 M (200 µl, pH 7.3). Finally, absorbance was read at 450 nm every 30 s for 5 min by using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

GST activity was quantified based on the method of Habig et al. (1974). Briefly, enzyme samples were placed in each well (25 µl) plus 200 µl of 1-chloro-2,4-dinitro benzene mixture (CDNB; 63 mM) and 200 µl of reduced glutathione (GSH; 10 mM). Then, absorbance was read at 340 nm every 30 sec for 5 min using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA).

Cytochrome P450 enzyme levels were measured using the heme peroxidation method (Wang et al., 2018; William and Janet, 1997). Briefly, the total reaction volume was 650 µl, consisting of enzyme sample (40 µl), potassium phosphate buffer (160 µl, 0.625 M, pH 7.0), 50 µl H_2O_2 (3%) and 400 µl of 3,3',5,5'-tetra-methylbenzidine (TMBZ) solution (Sigma

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Aldrich, USA). The reaction was incubated at room temperature for 2 h. The optical density was measured at 450 nm using a Unico 1200 Spectrophotometer (UNICO, Dayton, USA). Cytochrome C (Merck, Germany) from equine heart was used as a standard.

The Bradford method (1976) was used with bovine serum albumin (Merck, Germany) as standard to measure the protein concentration of each enzyme sample. About 5.25 µg of protein was used in each enzymatic reaction. Three technical replicates were performed for each enzyme activity assay. Data represent the mean values of three replicates with the standard error.

2.4. Genomic DNA extraction and detection of pyrethroid resistance mutations

Genomic DNA was extracted from 300-400 adult female mites of each *P. ulmi* population (resistant and susceptible to fenpropathrin) by using the Muray and Tompson method (1980). In order to perform DNA extraction of single *P. ulmi* males, the method of Bryon et al. (2017) was used. Briefly, individual male adults were homogenized in 20 µl STE buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 1 mg ml^{-1} proteinase K) and were subsequently incubated at 37°C for 30 min, followed by an incubation at 95 °C for 5 min. To determine the presence of previously reported pyrethroid-associated point mutations in spider mites, L1024V, F1538I and A1215D (numbering according to *M. domestica*), three partial segments of the *P. ulmi* VGSC gene were sequenced. The primers were designed based on the *P. ulmi vgsc* sequence provided by Bajda et al. (2015) (Table 1). For pooled samples, PCRs were conducted in 25 µl final volume with 12.5 µl 2x Master mix (Ampliqon, Denmark), 0.6 µM each primer, 3 µl template DNA, with cycling conditions: 5 min at 94°C followed by 38 cycles of 45 s at 94°C, 45 s at 56°C (L1024F, A1215D) and 60°C (F1538I), 1 min at 72°C and 10 min of final extension. For single males, PCRs were conducted in a final volume of 50 µl with 10 µl 5x GoTaq® G2 reaction buffer, 0.3 µM each primer, 1 µl template DNA, with cycling conditions: 2 min at 92ºC, followed by 40 cycles of 30 s at 92ºC, 1 min at 55ºC, 2 min at 72ºC, and 5 min of final extension. Reactions were performed in Bio-Rad thermocycler (Bio-Rad Laboratories Inc., Hercules, USA). PCR products were purified and sequenced either 1) at Microsynth (Switzerland) for amplicons from PCRs using DNA of pooled females as template or 2) at LGC Genomics (Germany) for amplicons from PCRs using DNA of single males as template. A selection of amplicons from single male PCRs was cloned into the pJET1.2 plasmid (Thermo Fisher Scientific) and transformed into *E. coli.*

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Plasmids of liquid cultures were purified after which the sequence was determined by sequencing (LGC Genomics, Germany). Sequencing data were analyzed using BioEdit 7.0.1 software (Hall, 1999).

2.5. Discriminating concentration bioassay

Adult females of *P. ulmi* populations were sprayed (1.5 ml, 1 bar pressure, 1.46±0.05 mg deposit cm^{-2}) with a discriminating concentration of fenpropathrin (80 mg a.i. L^{-1}). This concentration was derived from the fenpropathrin concentration that resulted in 95% mortality of the most susceptible population (Shahin Dej, based on the bioassay data). Each test was replicated four times.

3. Results

3.1. Fenpropathrin and fenvalerate toxicity in field populations

The toxicity of fenpropathrin on various *P. ulmi* populations collected from apple orchards of three provinces in Iran was determined by a leaf disc bio-assay (Table 2). The Marand, Maraqeh, and Mianeh2 populations from East Azerbaijan Province showed about 149-, 90-, and 71-fold resistance to fenpropathrin respectively, while the other populations were not highly resistant to fenpropathrin (RR less than 6) compared to the Shahin Dej population (field susceptible population). The Marand, Maraqeh, and Mianeh2 populations showed resistance to fenvalerate with LC_{50} values around 2000 mg/L and RRs of 21, higher than 20 and 19 respectively. The Shahin Dej population was also found to be susceptible to fenvalerate (Table 3), albeit with an LC_{50} of 99 mg/L, which is 5-fold higher compared to fenpropathrin (20 mg/L) in the same population. In addition, the $\log_{10}LC_{50}$ values of fenpropathrin and fenvalerate in the eight field populations of *P. ulmi* were significantly correlated (Pearson coefficient, $r = 0.94$, $P < 0.0006$) (Fig. 2).

3.2. Synergism assay

The effect of synergists (PBO, TPP and DEM) on fenpropathrin toxicity against resistant *P. ulmi* populations was tested and is presented in Table 4. PBO, TPP, and DEM slightly enhanced the toxicity of fenpropathrin in Marand, Maraqeh, and Mianeh2 populations but SRs were lower than those observed for the Shahin Dej population. These results indicate

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that the investigated detoxifying enzymes possibly are not the main factor in the development of fenpropathrin resistance in these populations.

3.3. Enzyme activity assays

The possible role of metabolic resistance mechanisms in the fenpropathrin resistant populations (Marand, Maraqeh and Mianeh2) was further investigated by measuring the activity of three general metabolic enzyme-families (CarEs, GSTs and P450s) with model substrates and by comparing these with the values obtained for the most susceptible population (Shahin Dej population, Table 5). Enzyme activities in the resistant populations did, however, not exceed more than twofold the activities measured in the susceptible population.

3.4. Detection of mutations associated with fenpropathrin resistance

Sequencing of segment IIS6, the linker between domains II and III, and segment IIIS6 of the *vgsc* gene from pooled mites of the susceptible (Shahin Dej) and fenpropathrin resistant *P. ulmi* populations (Marand, Maraqeh and Mianeh2) revealed that a L1024V (CTT/GTT) and a F1538I (TTC/ATC) mutation is present in the resistant populations and absent in the susceptible population. In contrast, no difference was seen at the A1215 position of resistant and susceptible populations. Based on the sequence chromatographs (Fig. 3), the L1024V and F1538I mutations were not fixed in the Marand, Maraqeh, and Mianeh2 populations. To verify whether the L1024V and F1538I *vgsc* mutation co-occurred on a single VGSC haplotype, we amplified and sequenced the F1538I and L1024V region from single males of the resistant Maraqeh strain. The *vgsc* gene of two out of 15 single males from the Maraqeh strain harbored both mutations, but unexpectedly, as male mites are haploid, at least one of these mutations was not fixed in both males (data not shown). Cloning of the PCR products from these two males revealed that, based on the absence/presence of the F1538 mutation and a non-synonymous single nucleotide polymorphism located in a nearby intron, multiple haplotypes of the F1538I containing exon, and by extent possibly the complete *vgsc* gene are present in a single male (Table S1).

3.5. Discriminating concentration fenpropathrin for resistance monitoring

The discriminating concentration of fenpropathrin (80 mg a.i. L^{-1}) was based on the LC_{95} value calculated from log-dose probit-mortality relationships of the Shahin Dej

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population. The LC_{95} was selected because it allowed the detection of low resistance levels in the field-collected populations mentioned above. The bioassays showed that Shahin Dej population was susceptible and Semirom2 population was nearly susceptible when the discriminating concentration of fenpropathrin was applied (more than 60% mortality) (Fig. 4), and Ahar, Khafr, Mahabad, Urmia, Mianeh1, Semirom1 and Salmas populations showed a moderate resistance to fenpropathrin (between 30% and 60% mortality), while Marand, Maraqeh and Mianeh2 populations were highly resistant to fenpropathrin (less than 30% mortality).

4. Discussion

In Iran, nearly all apple growers rely on the application of various chemical acaricides for the control of *P. ulmi*. Several studies on spider mite species such as *P. citri*, *T. urticae* and *T. evansi* have shown that resistance to pyrethroids can develop rapidly (Ding et al., 2015; Kwon et al., 2010b; Nyoni et al., 2011; Tsagkarakou et al., 2009; Van Leeuwen et al., 2010; Ya-ning et al., 2011). Fenpropathrin and fenvalerate are two major pyrethroids that are registered to control *P. ulmi* and *L. scitella* in Iran, respectively. These pesticides have often been used in apple orchards of Iran in the last three decades (Nourbakhsh, 2017).

Marshall and Pree (1991) reported that fenpropathrin at a concentration of 10 mg a.i.L^{-1} caused a mortality over 95% in *P. ulmi*. In our study, the LC_{50} of fenpropathrin for the most susceptible population, Shahin Dej (a field-collected population that has been subjected to various insecticide/acaricides) was determined in the same range as 20 mg a.i. L^{-1}. Three *P. ulmi* populations, Marand, Maraqeh and Mianeh2, showed 149-, 90-, and 71-fold resistance to fenpropathrin, respectively, in comparison to the Shahin Dej population. These fenpropathrin RRs exceed more than threefold the fenpropathrin RR of a Chinese *P. citri* population (RR= 23) (Ding et al., 2015) and is similar to resistance levels reported for a Greek and Korean *T. urticae* population (RR= 86 and RR> 40, respectively) (Kwon et al., 2010b; Tsagkarakou et al., 2009), although resistance levels in latter species have been reported to attain more than 10,000-fold (Van Leeuwen and Tirry, 2007). The three resistant *P. ulmi* populations also showed high resistance to fenvalerate (Fig. 2), which might partly be explained by the intensive application of fenvalerate in Iran (Nourbakhsh, 2017). Alternatively, a single resistance mechanism selected by fenpropathrin might be responsible for cross-resistance, regardless of the exerted selection pressure for fenvalerate.

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In contrast to insects, where cytochrome P450 monooxygenases are frequently reported to be involved in metabolic resistance against pyrethroids (Hemingway and Ranson, 2000; Hemingway et al., 2004), only in the case of bifenthrin resistance it was convincingly shown that detoxification can be a prime mechanism in pyrethroid resistance in spider mites (Ay and Gürkan, 2005; Van Leeuwen and Tirry, 2007). Bifenthrin resistance was shown to be esterase-mediated in a Belgian strain with very high levels of resistance, both by the detection of a 10-fold synergism ratio with DEF as well as by measuring direct bifenthrin metabolism (Van Leeuwen et al., 2004; Van Leeuwen and Tirry, 2007). Similarly, esterases were shown to be in involved in bifenthrin resistance of a Turkish mite strain (Ay and Gürkan, 2005). In our study, all SRs for the fenpropathrin resistant populations were lower than those for the susceptible population, and *in vitro* enzyme activity assays showed only slightly higher activities in the resistant populations. Thus, enhanced detoxification activities of the most frequently described multi-gene detoxification enzyme families probably do not play a significant role in the development of fenpropathrin resistance in the tested populations. Nevertheless, the TMBZ peroxidation assay for example, like any assay with model substrates, does not rule out the potential involvement of specific enzymes that might not be detectable in the setup. In addition, other players in detoxification have entered the scene for which standard assays are not yet developed (Dermauw et al., 2013).

Three main point mutations in *vgsc* have been reported in spider mite strains resistant to fenpropathrin and other pyrethroids (Ding et al., 2015; Kwon et al., 2010b; Nyoni et al., 2011; Tsagkarakou et al., 2009; Ya-ning et al., 2011). A F1538I substitution, in combination with A1215D, has been found in pyrethroid resistant populations of *T. urticae* collected from Greece (Tsagkarakou et al., 2009). In addition, a L1024V substitution in a fenpropathrin selected resistant population of *T. urticae* from Korea was subsequently detected and in this population, the A1215D substitution was also detected in combination with L1024V (Kwon et al., 2010b). The F1538I mutation was also identified in the *vgsc* gene of a fenpropathrin resistant strain of the red form of *T. urticae* from China and in the citrus red mite *P. citri* (Ding et al., 2015; Yaning et al., 2011). Our sequencing results indicated that among the previously reported possible mutations involved in pyrethroid resistance in spider mites, the L1024V and F1538I mutation were both present in three fenpropathrin resistant *P. ulmi* populations, Marand, Maraqeh and Mianeh2, but the mutations were not fixed when assessed by PCR on pooled individuals. We could not identify an amino acid substitution at position 1215 of the VGSC (*M. domestica* numbering) of Marand, Maraqeh and Mianeh2

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populations. Similarly, mutations could not be detected at this position in the *vgsc* gene of a fenpropathrin resistant strain of *P. citri* (Ding et al., 2015; Tan et al., 2005), and the A1215D substitution seems present only in *T. urticae* haplotypes (Riga et al. 2017).

F1538 is an aromatic residue that possibly interacts with double ringed cyclic pyrethroids (O'Reilly et al., 2006). This mutation, when functionally expressed in an *in vitro* assay system, has been shown to confer strong resistance to several pyrethroids (O'Reilly et al., 2006) and previous studies indicated that the F1538I mutation in combination with A1215D is associated with high pyrethroid resistance levels in mites (Ding et al., 2015; Tsagkarakou et al., 2009; Yaning et al., 2011). In contrast, the A1215D mutation alone has no effect on pyrethroid toxicity (Riga et al., 2017). Based on computer modeling, the domain II segment 6 of the VGSC is another important region for binding of pyrethroids (O'Reilly et al., 2006) and the L1024V mutation is located at this segment (O'Reilly et al., 2006, Riga et al., 2017). Kwon et al. (2010b) has concluded that the L1024V mutation combined with the A1215D mutation leads to a high fenpropathrin resistance in *T. urticae*. In a recent study, a number of spider mite target-site mutations was crossed into a susceptible genetic background by marker assisted back-crosses. This allowed to uncouple mutations from other genetic factors contributing to resistance and assess the strength of the phenotype associated with the mutations. Based on these backcrossing experiments, Riga et al. (2017) reported that the combination of the A1215D and F1538I mutation leads to high fenpropathrin, bifenthrin and fluvalinate resistance levels. Similarly, the A1215D + L1024V mutations, when introgressed in a susceptible background, conferred high resistance levels to bifenthrin, fenpropathrin and fluvalinate. The L1024V mutation alone has only been observed in a laboratory selected strain but its frequency in field-collected populations has not yet been investigated in pyrethroid resistant spider mites (Kwon et al., 2010b).

In contrast to vertebrates, which have 8-10 sodium channel genes of the Nav1 family, invertebrates usually have only one gene of the Nav1 family (also known as *para* in *Drosophila*) (Zakon, 2012). In insects and mites, it is known that alternative splicing increases the functional diversity of the sodium channel, including the susceptibility to pyrethroid insecticides (Davies et al., 2007; Dong et al., 2014). In the mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*, on the other hand, it was shown that the *vgsc* gene is duplicated (Martins et al., 2013; Martins et al., 2017). In line with the observations made for the latter two species, cloning of the F1538I and L1024V regions from a single *P. ulmi* male showed that multiple copies of these regions are present in the resistant Maraqeh strain. This

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strongly suggest that this region, and by extent the complete *vgsc* gene, is amplified. However, as we did not use a high-fidelity polymerase, the possibility remains that some polymorphisms revealed by sequencing after cloning are artefacts, although it seems very unlikely that the polymerase would introduce errors specifically at both *kdr* sites. Whether copy number differs between strains, and how many of the copies carry resistance mutations, needs further study. In hindsight, gene amplification does explain why these mutations are not fixed in the population (Fig. 3), as mutations are most likely spread over multiple copies (Table S1). Copy number variation related to resistance has been previously documented by Kwon et al. (2010a) for *T. urticae* acetylcholinesterase, where it was suggested that copy number variation can be a compensatory mechanism for potential fitness costs of resistance carrying copies. Furthermore, fitness costs have been reported for fly and mosquito strains that carry *vgsc* resistance mutations (Brito et al., 2013: Rinkevich et al., 2013). However, for the spider mite *T. urticae*, it was recently shown that at least the L1024V mutation did not carry any (measured) fitness cost (Bajda et al., 2018). Finally, at present we cannot exclude that both mutations occur in a single copy, as they are assessed by PCR on different gene fragments, and therefore cannot be linked to a single gene. Long PCR, encompassing both regions of the *vgsc* gene, is needed in future study. To conclude, we showed that the L1024V and F1538I mutations are associated with fenpropathrin resistance of *P. ulmi* populations and that the VGSC is present in multiple copies. Based on the presence of these mutations, a marker can be developed for molecular diagnostics of pyrethroid resistance.

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Figure legends

Fig. 1 - Map of collection sites of *P. ulmi* populations from major apple growing regions of Iran

Fig. 2 - Correlation between LC50 values of *P. ulmi* populations to fenpropathrin and fenvalerate

Fig. 3 - Sequencing chromatograms of the partial nucleotide sequences of PCR-amplified DNA fragments corresponding to the mutation sites in VGSC of different populations of *P. ulmi* (Shahin Dej, Marand, Maraqeh and Mianeh2). The positions of nucleotide substitutions leading to mutations (L1024V or F1538I) are marked with arrows.

Fig. 4 - Responses of field populations of *P. ulmi* to a discriminating concentration of fenpropathrin (LC95= 80 mg a.i. L^{-1}). All data are mean values ± SE (n= 4)

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Tables

Table 1.
Primers used for the detection of resistance associated mutations in the VGSC gene of *P. ulmi* (position of mutations according to *M. domestica*).

Primers	Mutation	Fragment size	Sequence (5' – 3')
PulmiSC6-F1	F1538I	492	GGACCATGCGATTGATTCTC
PulmiSC6-R1			AAGCCTGGTAGTGGTCAAGG
Pu.L1024V-F	L1024V	403	TCCGTGTTCTTTGTGGTGAA
Pu.L1024V-R			CCTCCTCCCAGTGTTAGAGT
Pu.A1215D-F	A1215D	481	CGTCCTTAAATGATCCCAATGGT
Pu.A1215D-R			CCCATCACATCCTCTCCCAG

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Table 2.

Log-dose probit-mortality data for fenpropathrin tested against female adults of *P. ulmi* field populations.

Population	n*	LC_{50} mg a.i.L^{-1} (CI 95%)	Slope±SE	χ^2(df)	RR** (CI 95%)***
Shahin Dej	267	20.03 (12.27-27.76)	2.67±0.61	1.06 (3)	
Mahabad	233	110.43 (86.00-142.6)	1.98±0.30	0.39 (3)	5.45 (3.32-8.92)
Mianeh1	316	57.75 (42.83-74.52)	1.68±0.23	2.30 (3)	2.88 (1.68-4.93)
Ahar	210	65.45 (36.75-107.45)	2.00±0.27	4.58 (3)	4.47 (2.00-6.02)
Semirom1	236	90.43 (64.59-153.77)	2.16±0.58	1.61 (3)	4.75 (2.88-7.82)
Semirom2	257	77.49 (58.12-98.50)	2.01±0.30	0.83 (3)	3.80 (2.35-6.14)
Urimia	254	88.99 (55.84-113.46)	1.59±0.28	3.49 (3)	4.29 (2.70-6.81)
Salmas	221	105.72 (71.88-147.77)	1.89±0.29	2.76 (3)	5.62 (3.01-10.49)
Marand	350	2556.33 (849.66-4362.89)	2.21±0.27	9.27 (3)	149.37 (87.21-255.84)
Maraqeh	219	1805.14 (785.49-2620.37)	2.35±0.59	1.64 (3)	90.11 (48.75-166.57)
Mianeh2	359	1340.44 (884.18-2227.76)	0.86±0.13	3.98 (6)	71.82 (32.89-156.84)
Khafr	247	68.91 (51.01-89.81)	1.65±0.24	0.28 (3)	3.40 (2.00-5.78)

*Number of mites tested.

** Resistance ratio = LC_{50}/LC_{50} Shahin Dej.

***Confidence interval.

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Table 3.
Log-dose probit-mortality data for fenvalerate tested against the female adults of *P. ulmi* field populations.

Population	n*	LC_{50} mg a.i. L^{-1} (CI 95%)	Slope±SE	χ^2(df)	RR** (CI 95%)***
Shahin DeJ	121	99.41 (57.74-193.89)	1.53±0.49	2.49 (4)	
Mahabad	125	223.70 (159.55-320.68)	2.20±0.50	2.90 (3)	2.25 (1.14-4.46)
Ahar	130	435.17 (296.89-970.93)	1.36±0.38	0.60 (3)	4.37 (2.01-9.52)
Urmia	108	113.59 (78.62-163.98)	1.88±0.39	1.42 (3)	1.14 (0.57-2.28)
Marand	255	2148.90 (1651.10-3057.11)	1.76±0.25	3.00 (3)	21.61 (11.46-40.77)
Maraqeh	180	>2000			>20
Mianeh2	241	1933.80 (1491.51-2734.00)	1.65±0.32	2.90 (3)	19.87 (9.68-40.81)
Khafr	103	226.80 (145.23-452.50)	1.32±0.37	1.65 (3)	2.28 (1.04-4.99)

*Number of mites tested.
** Resistance ratio = LC_{50}/LC_{50} Shahin Dej.
***Confidence interval.

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Table 4.

Synergistic effect of PBO (1000 mg L^{-1}), TPP (1500 mg L^{-1}) and DEM (1000 mg L^{-1}) on fenpropathrin resistance in *P. ulmi* populations, compared to the susceptible population of Shahin Dej.

Treatment	n*	LC_{50} mg a.i. L^{-1} (CI 95%)	Slope±SE	χ^2 (df)	RR** (CI 95%)***	SR**** (CI 95%)
Shahin Dej						
fenpropathrin	267	20.03 (12.27-27.76)	2.67±0.61	1.06 (3)		
+PBO	163	6.22 (3.74-9.98)	1.56±0.22	1.11(3)		3.21 (1.80-5.74)
+TPP	123	7.61 (3.69-11.29)	1.56±0.34	1.31 (3)		2.62 (1.39-4.93)
+DEM	267	8.21 (4.74-18.37)	1.12±0.30	2.87 (3)		2.43 (1.23-4.81)
Marand						
fenpropathrin	350	2556.33 (849.66-4362.89)	2.21±0.27	9.27 (3)	149.37 (87.21-255.84)	
+PBO	170	1898.53 (726.44-3828.57)	1.12±0.29	8.13 (4)	457.60 (145.17-451.37)	1.34 (0.94-1.92)
+TPP	197	1783.72 (1440.12-2122.01)	3.71±0.64	2.38 (3)	255.98 (145.17-451.37)	1.43(1.09-1.88)
+DEM	267	2197.95 (1269.81-4945.38)	0.99±0.24	3.85 (4)	274.37 (97.58-771.43)	1.16 (0.62-2.18)
Maraqeh						
fenpropathrin	219	1805.14 (785.49-2620.37)	2.35±0.59	1.64 (3)	90.11 (48.75-166.57)	
+PBO	126	1447.25 (59.98-2223.84)	2.33±0.57	2.47 (3)	232.49 (113.87-474.65)	1.24 (0.59-2.61)
+TPP	114	1198.63 (442.93-1934.63)	1.95±0.58	1.57 (3)	157.31 (72.99-339.03)	1.50 (0.70-3.20)
+DEM	109	1143.94 (479.30-1844.09)	1.80±0.51	1.31 (3)	139.19 (63.04-307.31)	1.57 (0.75-3.29)
Mianeh2						
fenpropathrin	359	1340.44 (884.18-2227.76)	0.86±0.13	3.98 (6)	71.82 (32.89-156.84)	
+PBO	178	914.35 (538.60-1282.27)	2.14±0.39	0.71 (3)	146.88 (80.22-266.92)	1.57 (0.70-3.50)
+TPP	157	910.23 (616.64-1213.96)	2.04±0.34	1.67 (3)	120.10 (61.79-233.43)	1.47 (0.84-2.55)
+DEM	165	859.28 (552.62-1179.13)	2.11±0.35	1.16 (3)	104.55 (53.07-205.96)	1.67 (0.76-3.64)

*Number of mites tested.
** Resistance ratio = LC_{50}/LC_{50} Shahin Dej
***Confidence interval.
****Synergistic ratio.

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Table 5.

Detoxification enzyme activities in different populations of *P. ulmi* (mean ± SEM).

Population	P450s TMBZ*	Ratio	GSTs CDNB conjugation**	Ratio	Esterases α-naphthyl acetate***	Ratio
Shahin Dej	8.35±0.42^{ab}		459.05±18^{c}		3766.11±197^{b}	
Marand	6.80±0.18^{b}	0.81	391.10±30^{c}	0.85	5274.84±315^{a}	1.40
Maraqeh	8.64±0.27^{a}	1.03	689.82±38^{b}	1.50	4579.73±187^{ab}	1.21
Mianeh2	8.30±0.38^{ab}	0.99	932.47±51^{a}	2.03	4642.16±238^{ab}	1.23

* Equivalent units of cytochrome P450 mg^{-1} protein (± SEM).
**nmol glutathione conjugated min^{-1} mg^{-1} protein (±SEM).
***nmol 1-naphthol min^{-1} mg^{-1} protein (± SEM).
Significant differences are indicated by a different letter (one-way ANOVA, P < 0. 05; LSD test).

Graphical abstract:

Highlights

- several Iranian populations of *P. ulmi* were highly resistant to pyrethroids
- detoxification played a secondary role in resistance to pyrethroids
- L1024V and F1538I mutations of VGSC were detected in resistant populations
- multiple copies of *vgsc* gene were found in a resistant population

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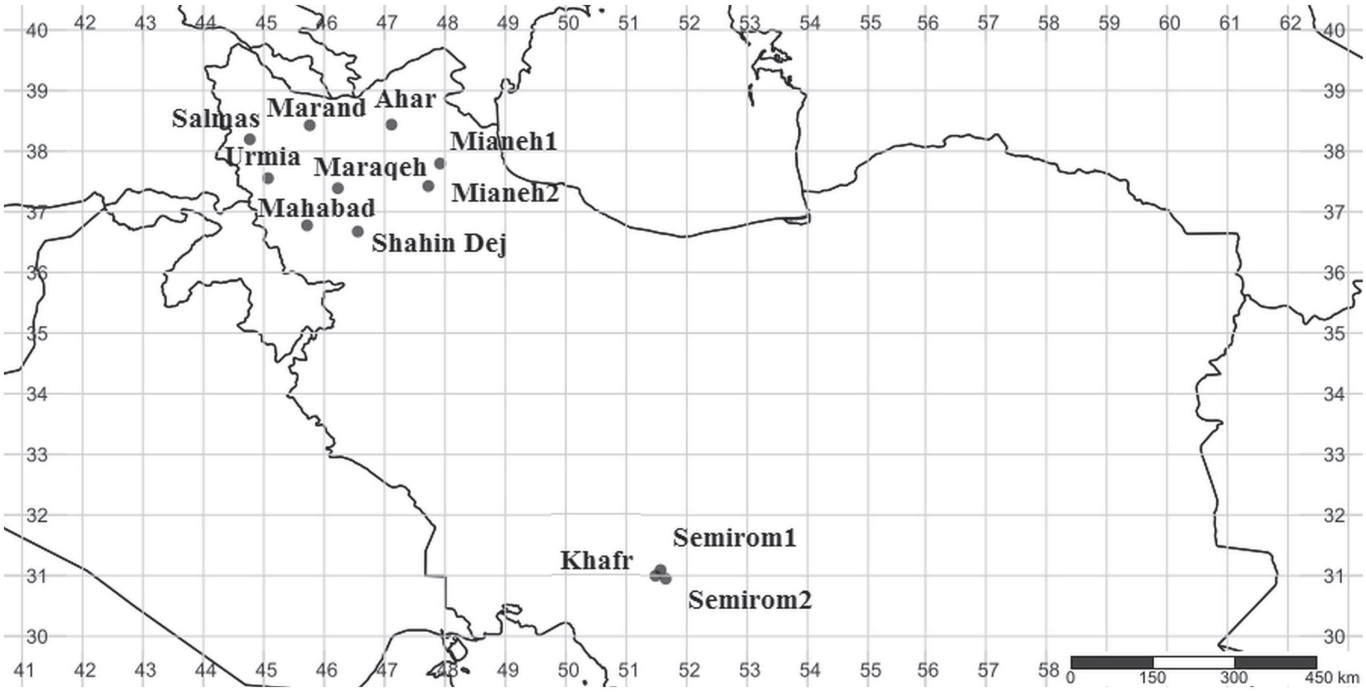
Salmas
Marand
Ahar
Mianeh1
Urmia
Maraqeh
Mianeh2
Mahabad
Shahin Dej
Khafr
Semirom1
Semirom2
0
150
300
450 km

Figure 1

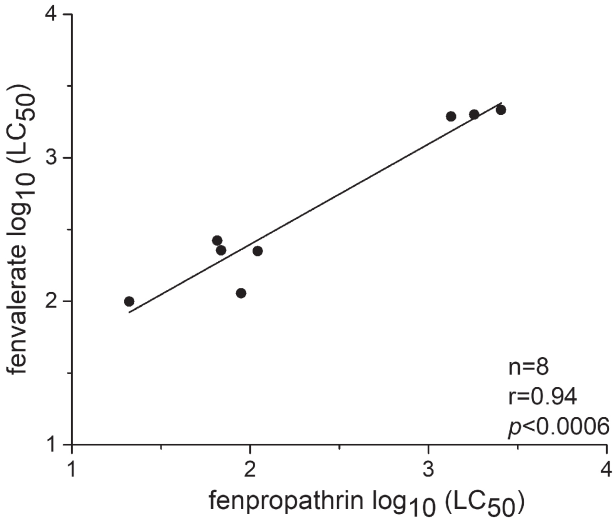
fenvalerate log10 (LC50)
fenpropathrin log10 (LC50)
n=8
r=0.94
p<0.0006
1
2
3
4

Figure 2

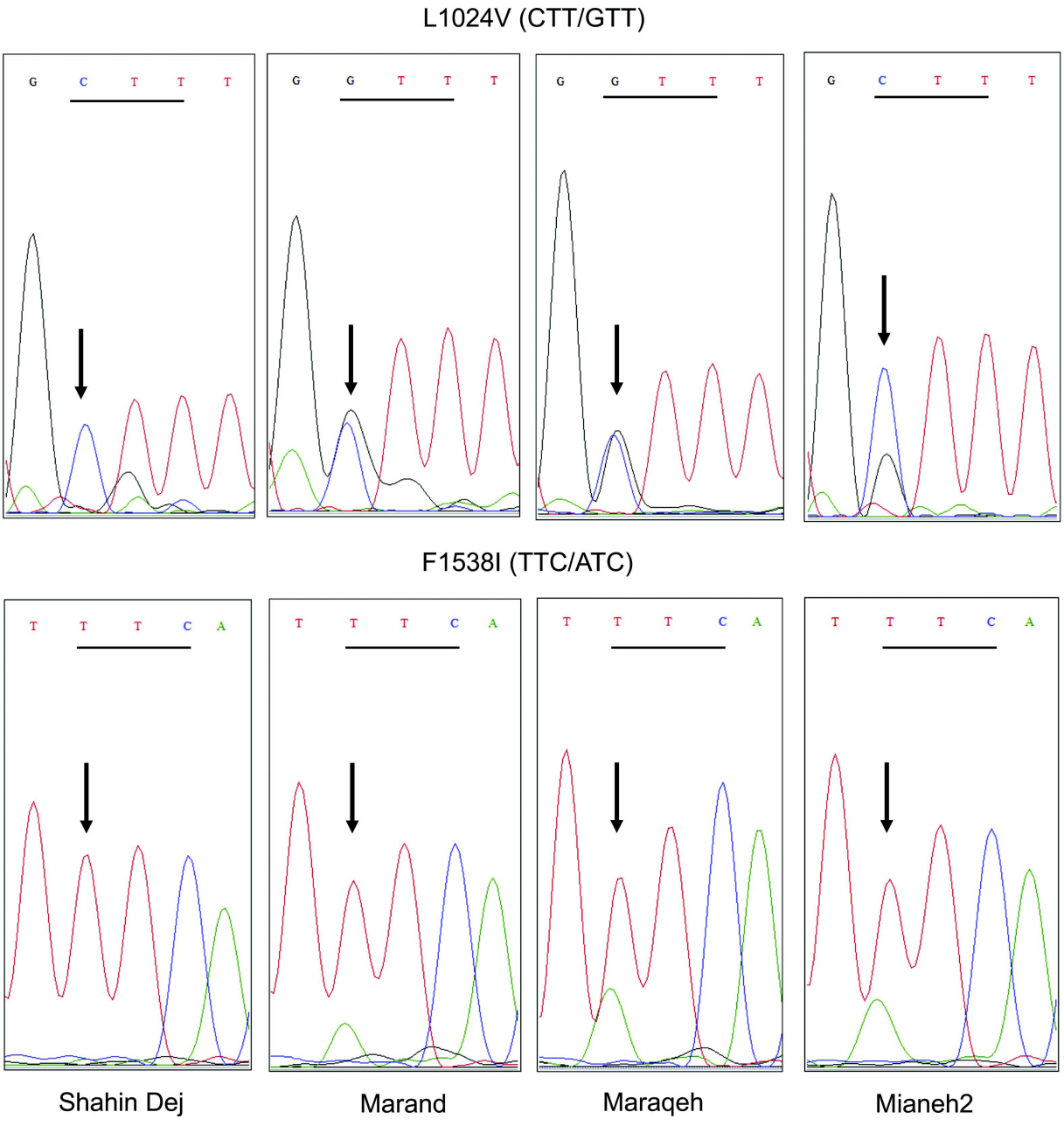
L1024V (CTT/GTT)
G C T T T
G G T T T
G G T T T
G C T T T
F1538I (TTC/ATC)
T T T C A
T T T C A
T T T C A
T T T C A
Shahin Dej
Marand
Maraqeh
Mianeh2

Figure 3

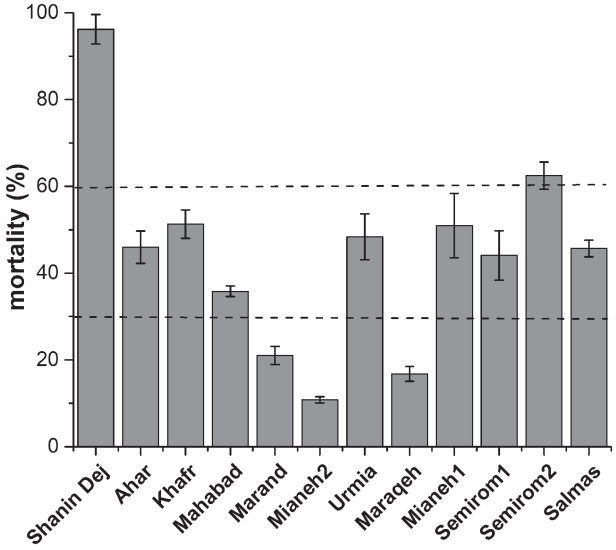
mortality (%)
100
80
60
40
20
0
Shanin Dej
Ahar
Khafr
Mahabad
Marand
Mianeh2
Urmia
Maraqeh
Mianeh1
Semirom1
Semirom2
Salmas

Figure 4