Experimental and Applied Acarology (2021) 84:433–444
https://doi.org/10.1007/s10493-021-00626-2

Genetic analysis and screening of pyrethroid resistance mutations in *Varroa destructor* populations from Turkey

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Received: 25 February 2021 / Accepted: 7 May 2021 / Published online: 13 May 2021

Abstract
Varroa destructor is the most common ectoparasite of the Western honey bee (*Apis mellifera* L.) worldwide and poses a serious threat to bee health. Synthetic acaricides, particularly pyrethroids, are frequently used to control *Varroa* mites. However, long-term and repeated use of synthetic pyrethroids has led to the development of resistance. In this study, we report on the presence of resistance mutations in the voltage-gated sodium channel in *V. destructor* populations from Turkish beekeeping areas. Two resistance mutations, L925V and L925I, that were previously associated with pyrethroid resistance, were found in more than 75% of the populations. A general correlation between the presence of mutations and the history of acaricide usage was observed for the sampled hives. In addition, we show there is only a low genetic distance among the sampled *V. destructor* populations, based on the analysis of three mitochondrial genes: cytochrome *b* (*cytb*), ATP synthase subunit 6 (*atp6*), and cytochrome *c* oxidase subunit III (*cox3*). Revealing the presence and geographical distribution of pyrethroid resistance mutations in *V. destructor* populations from Turkish apiaries will contribute to create more effective mite management programmes.

Keywords *Varroa* · Pyrethroids · Resistance mutation · Mitochondrial genes

Introduction

Turkey is the second largest honey-producing country across the globe, with 8 million hives and a honey production of 110 thousand tons annually (Özkırım 2018; FAOSTAT 2021). However, the average yield per hive in Turkey is far below the world average (15

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vs. 40 kg, respectively) (Terin et al. 2018). One of the main reasons for the decreased yield is the inadequate control of the pests of honey bees. *Varroa* spp. are recognised among the most important pests and drivers of honey bee colony loss throughout the world (Potts et al. 2010; Steinhauer et al. 2018; Noël et al. 2020).

Varroa destructor Anderson & Trueman (Acari: Varroidae) is the most important obligate ectoparasite of the Western honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), worldwide (Thoms et al. 2019; Traynor et al. 2020). *Varroa* spp. can cause significant damage to bee colonies by feeding on hemolymph and fat body tissues, leading to a decreased body weight and a shortened life span in honey bees (Rosenkranz et al. 2010; Ramsey et al. 2019). Besides, *Varroa* mites are known as virus vectors, transmitting various lethal pathogens to honey bees (McMenamin and Genersch, 2015). The honey bee colonies can collapse within a couple of years when the mites are not adequately controlled, resulting in dramatic decline of bee populations (Rosenkranz et al. 2010). Therefore, the management of *V. destructor* in apiaries is of great importance to maintain colony health.

Application of genetic markers to determine the genetic variation and haplotype of *Varroa* mites has been considered very important for several reasons, e.g., for species discrimination (Anderson and Trueman 2000), yet also for haplotype discrimination, as Korean haplotypes are considered more harmful than Japanese ones (Mendoza et al. 2020). As the usage of single genes is often insufficient, multiple genes should be employed to reveal genetic variation (Navajas et al. 2010; Muntaabski et al. 2020). Here, we use three mitochondrial genes, cytochrome *b* (*cytb*), ATP synthase subunit 6 (*atp6*), and cytochrome *c* oxidase subunit III (*cox3*), to assess genetic distance between various *V. destructor* populations.

Synthetic acaricides, including pyrethroids, formamidines, and organophosphates, have been the major effective tools used in the control of *Varroa* mites for years (Rosenkranz et al. 2010). Among them, the pyrethroids tau-fluvalinate and flumethrin are commonly preferred due to their in-hive selectivity, when used appropriately (Johnson et al. 2010; Blacquière et al. 2017). However, as a result of the long-term and repeated use of these pyrethroids in *Varroa* control, the beekeeping industry is facing the development of resistance and also the presence of chemical residues in bee products such as beeswax and honey (Bogdanov 2006; Rosenkranz et al. 2010; Smodiš Škerl et al. 2011).

Similar to other arthropods, mites can develop resistance to pesticides via pharmacokinetic and pharmacodynamic mechanisms (Feyereisen et al. 2015; Van Leeuwen and Dermauw 2016). The former often occurs by increased activity of major detoxification enzymes such as cytochrome P450-monooxygenases, glutathione *S*-transferases and carboxyl/cholinesterases (Surlis et al. 2016; Panini et al. 2019), whereas mutations that alter the target-site structure or expression are most frequent in the latter (Feyereisen et al. 2015). Recently, decreased activation of coumaphos to its toxic oxon form, mediated by decreased expression of CYP4EP4, has been uncovered as a rare but evolutionarily powerful solution to achieve resistance in *V. destructor* (Vlogiannitis et al. 2021).

Resistance to pyrethroids is well documented for many arthropods (Dong et al. 2014; Feyereisen et al. 2015) and has been firstly reported in the early 1990s in European *V. destructor* populations (Martin 2004). Although pyrethroid resistance has been associated with increased P450 monooxygenase and esterase activity (Hillesheim et al. 1996; Mozes-Koch et al. 2000), the L925V/I/M and M918L mutations in the voltage-gated sodium channel have been reported as major resistance mechanism in *V. destructor* populations from different continents (González-Cabrera et al. 2013, 2016, 2018; Millán-Leiva et al. 2021a, b).

The presence of *Varroa* has been documented in Turkey since the 1980s (Çakmak et al. 2003) and increasing bee health problems caused by these mites have been reported (Warrit et al. 2004; Çakmak and Sevençakmak 2016). However, the resistance status and genetic population structure have been rarely investigated in beekeeping areas of Turkey. In this study, we determined the genetic variation among *V. destructor* populations from Turkey and the presence of pyrethroid resistance mutations.

Materials and methods

Sampling of *Varroa destructor* populations

Using the powdered sugar method (Gregorc et al. 2017), a total of 22 *Varroa* populations were collected from 17 locations in Turkey during 2020 (Fig. 1). Mite samples were transferred to the laboratory in 90% ethanol for further processing. Detailed information about pesticide usage history, beekeeping practice and the location of the apiaries is presented in Table 1 and Fig. 1.

Total DNA isolation

Total DNA was extracted from pools of ten adult female mites per population using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. At the final step, DNA was dissolved in 100 µl elution buffer. The purity and quantity of genomic DNA were checked using agarose gel electrophoresis (1.5%) and a NanoDrop 2000 (Thermo Scientific) spectrophotometer. DNA extracts were stored at − 20 °C until used.

PCR amplification conditions

The mtDNA sequences of the *cytb*, *atp6* and *cox3* genes of *Varroa* mites were used to determine the genetic distance among populations from different geographical origins. PCR conditions for above-mentioned genes were as follows: 4 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 51 °C (55 °C for *cytb*) and 75 s at 72 °C and a final extension of 8 min at 72 °C. Screening of the resistance mutations located at the IIS4–IIS5 region of the voltage-gated sodium channel (VGSC) gene was performed as previously described (Alissandrakis et al. 2017).

All primers used in this study are given in Table S1. Each PCR reaction was performed in a total volume of 30 µl containing 3 µl of mite DNA (30–50 ng/µL), 0.5 µl each of forward and reverse primer (10 µM stock), 20 µl of PCR-grade water and 6 µl of FIREPol Master Mix (Solis Biodyne). The presence of PCR products was confirmed by gel electrophoresis on a 1.5% agarose gel in 0.5× TAE buffer at 100 V for 40 min, stained with SAFE-T-STAIN (BioShop, Canada), and visualised with a UV transilluminator.

The PCR products were purified using the HighPrep PCR clean-up system (MagBio Genomics) and subsequent sequencing of PCR products was performed by Macrogen (Seoul, Korea).

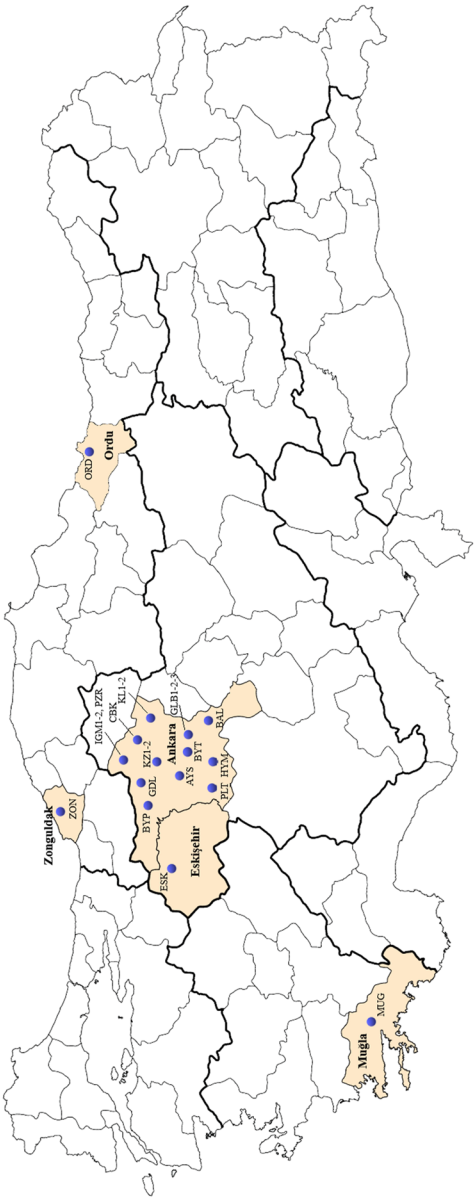

Fig. 1 Map of sampling locations of *Varroa destructor* populations from beekeeping areas (map created using mapchart.net)

Table 1 Sampled *Varroa destructor* populations: beekeeping type, amino acid at position L925, insecticide usage history and collection dates

Population	Beekeeping type	L925*	Pesticide usage history	Collection date
KL1 (Kalecik 1)	Migratory	V/L	Flumethrin, amitraz, essential oils	July 2020
KL2 (Kalecik 2)	Stationary	L/I	Amitraz	July 2020
BYP (Beypazarı)	Stationary	L/V	Flumethrin, amitraz	July 2020
KZ1 (Kazan 1)	Migratory	I	Flumethrin	July 2020
KZ2 (Kazan 2)	Migratory	I/L	Flumethrin	July 2020
PLT (Polatlı)	Migratory	L	Flumethrin, amitraz	Sept 2020
IGM1 (İğmir 1)	Migratory	L/V	Flumethrin, amitraz, organic acids	July 2020
IGM2 (İğmir 2)	Stationary	V/L	Flumethrin, organic acids	July 2020
AYS (Ayaş)	Stationary	V/I/L	Flumethrin, organic acids	Sept 2020
GLB1 (Gölbaşı 1)	Migratory	V/L	Flumethrin, amitraz	Sept 2020
GLB2 (Gölbaşı 2)	Migratory	L/V	Flumethrin, amitraz	Sept 2020
GLB3 (Gölbaşı 3)	Migratory	L/V/I	Flumethrin, amitraz	Sept 2020
PZR (Pazar)	Migratory	I/V/L	Flumethrin, organic acids	July 2020
BAL (Bala)	Migratory	L/V	Amitraz, organic acids	Sept 2020
BYT (Beytepe)	Stationary	V/L	Flumethrin, essential oils	Sept 2020
CBK (Çubuk)	Migratory	L/V	Amitraz, organic acids	Sept 2020
GDL (Güdül)	Stationary	L/V	Amitraz	July 2020
ESK (Eskişehir)	Stationary	V/L	Amitraz, essential oils, organic acids	Sept 2020
MUG (Muğla)	Stationary	V/L	Flumethrin, organic acids	Sept 2020
ZON (Zonguldak)	Stationary	L/I	Flumethrin, essential oils	August 2020
HYM (Haymana)	Stationary	L/V	Flumethrin, amitraz	Sept 2020
ORD (Ordu)	Stationary	V/I/L	Flumethrin, organic acids	Nov 2020

*In case there were multiple alleles in the sample, amino acids are given in decreasing order of estimated frequency

Data analysis and screening for resistance mutations

Multiple sequence alignment was performed using MAFFT v.7 with 'Auto' strategy (Katoh et al. 2019) and edited using Bioedit v.7.0.5 software (Hall 1999). The incidence of mutations was determined by inspecting the sequencing chromatographs as previously described (İnak et al. 2019).

To investigate the genetic variation in different *Varroa* species, sequences obtained in this study were analysed together with sequences retrieved from the public GenBank database (accession numbers are provided in Table 2). A maximum likelihood phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA X) using the T92 model (identified to be the best-fit model by MEGA X) and with 1000 bootstraps (Kumar et al. 2018) based on *atp6* sequences that showed the highest mean genetic variation among *Varroa* species.

Table 2 Mean genetic distance (%) (min–max) within and among *Varroa* species

Gene	Genetic distance				Accession numbers
	Between *V. destructor* populations			Within the genus *Varroa*	
	From Turkey	From Turkey and other countries	And other species within the genus *Varroa*		
cytb	0.06 (0–0.27)	0.34 (0–0.81)	–	0.29 (0–0.81)	MW553985–MW554006
atp6	0	0.45 (0–1.2)	10 (7.78–11.36)	4.4 (0–11.23)	MW553941–MW553962
cox3	0.55 (0–1.48)	1.26 (0–2.76)	8.36 (4.6–11.6)	2.44 (0–13.7)	MW553963–MW553984

Results and discussion

Varroa spp. are among the most destructive pests in apiculture and are considered to be among the main drivers of colony collapse disorder (Le Conte et al. 2010; Gajic et al. 2013; Steinhauer et al. 2018; Noël et al. 2020). The occurrence of *Varroa* mites in Turkey has been first documented in 1977 (Çakmak and Sevençakmak 2016) and only 40% *Varroa* infestation rate has been reported in Turkish apiaries in 2003 (Çakmak et al. 2003). However, although we did not systematically record the exact number of mites, all sampled apiaries in the present study were infested with *V. destructor*, similar to the findings of Yalçınkaya and Keskin (2010) that reported 100% *Varroa* infestation in apiaries from southern Turkey. Rapid spread of *Varroa* mites in Turkey may be associated with the geographical location of the country, honey bee importation and migratory beekeeping practices (Warrit et al. 2004). Here, we investigated the genetic variation among *V. destructor* populations from Turkey and compared them with other populations as well as with other *Varroa* species.

Application of mitochondrial gene sequences in *Varroa* species allows to identify different haplotypes and to discriminate between species (Navajas et al. 2010; Muntaabski et al. 2020). In the present study, three mitochondrial markers (*atp6*, *cox3*, *cytb*) were used to determine the haplotypes and genetic variation among *V. destructor* populations. Similar to other reports from Turkey (Warrit et al. 2004; Ayan et al. 2017; Ayan and Aldemir 2018) and other closely located countries (Solignac et al. 2005; Farjamfar et al. 2018; Traynor et al. 2020), all sampled populations belonged to the Korean haplotype according to the discrimination method suggested by Navajas et al. (2010). The average genetic distance among Turkish *V. destructor* populations was 0.06 and 0.55% for *cytb* and *cox3*, respectively. Genetic distance based on mt-markers between Turkish *V. destructor* populations was very low (even no distance for *atp6*) (Table 2), which is in line with previous studies (Solignac et al. 2005; Navajas et al. 2010; Gajic et al. 2013; Farjamfar et al. 2018). The low genetic distance among *V. destructor* populations is probably caused by the shift from its original host, *Apis cerana*, to *A. mellifera* and subsequent spread over wide areas resulting in a 'genetic bottleneck' (Solignac et al. 2005; Navajas et al. 2010). As a result, low genetic structuring between *V. destructor* and *V. jacobsoni* colonies from similar locations has been reported (Dietemann et al. 2019).

The highest genetic distance between *V. destructor* and other *Varroa* species was determined in the sequences of *atp6*. Similarly, *atp6* sequences had the highest average genetic distance within the genus *Varroa* (Table 2). In addition, the phylogenetic tree based on *atp6* has successfully discriminated all *Varroa* species herein considered (Fig. 2). Recently, a mitochondrial gene, *ND4*, has been reported as a sensitive marker to assess genetic variability since the variation significantly correlates with geographic distance among various *V. destructor* populations (Muntaabski et al. 2020). The sequences of *ND4* could be analyzed in future studies to get a better understanding of the population genetics of *Varroa* mites.

Together with organic acids and essential oils, the control of *Varroa* mites is mainly based on acaricides, in particular synthetic pyrethroids. However, development of resistance, causing failure in chemical control of *Varroa* mites, has been reported from many countries (Trouiller 1998; Martin 2004; Tutun et al. 2018; Higes et al. 2020; Millán-Leiva et al. 2021a; Hernández-Rodríguez et al. 2021). Synthetic pyrethroids are able to bind to voltage-gated sodium channels and cause prolonged channel opening (Soderlund 2012; Rinkevich et al. 2013) and are recognized as the second most widely used insecticide/acaricide group, corresponding to 15% of the total insecticide/acaricide market share (Sparks et al. 2020). Due to the low mammalian toxicity, relatively rapid degradation (Tang et al. 2018) and arthropod selectivity of pyrethroids (Khambay and Jewess 2005), this pesticide group is commonly used to control *Varroa* mites in apiaries. However, the limited number of active ingredients has resulted in repeated selection pressure and resistance development (Van Leeuwen and Dermauw 2016).

The development of pyrethroid resistance in *Varroa* mites has been observed since the early 1990s in Europe (Martin 2004). Thus far, different resistance mechanisms such as detoxification and point mutations have been associated with pyrethroid resistance in *V. destructor* (Wang et al. 2002; Liu et al. 2006). A leucine to valine substitution at position 925 (*Musca domestica* numbering) in the transmembrane segment 5 of domain II of the VGSC has been reported to confer pyrethroid resistance in *V. destructor* from UK and Czech Republic (González-Cabrera et al. 2013; Hubert et al. 2014). More recently, the presence of the L925V (CTG leucine to GTG valine) mutation in *V. destructor* populations has been reported throughout Europe and is considered to be a driving factor of pyrethroid resistance (González-Cabrera et al. 2018; Panini et al. 2019; Stara et al. 2019a; Hernández-Rodríguez et al. 2021). Other mutations in the same position, L925M and L925I, have been found in *V. destructor* populations from USA and Greece (González-Cabrera et al. 2016; Alissandrakis et al. 2017; Millán-Leiva et al. 2021a). On the contrary, no mutation has been found in *V. destructor* populations collected from 28 different locations in Iran (Farjamfar et al. 2018). Just recently, the *kdr-like* mutation M918L in combination with L925V, associated with pyrethroid resistance in other arthropod species, has been detected in *V. destructor* for the first time (Millán-Leiva et al. 2021b). All *Varroa* populations from Turkey, except the PLT population, harboured either one or a combination of L925V and L925I mutations (Fig. 3), whereas the L925M substitution was not detected. More than 75% of sampled *Varroa* populations contained the L925V mutation (yet never in a fixed state, see Figure S1) that reflects common usage of pyrethroids in beekeeping areas of Turkey (18 out of 22 sampled apiaries of sampled apiaries, see Table 1). In the KZ1 population, based on the mites analysed, it was concluded that the L925I mutation was fixed, probably caused by usage of flumethrin without any rotation in last treatments. The only population that did not contain any known resistance mutations in the target-site, was PLT, even though treated with flumethrin. Last, none of the populations harboured the M918L substitution.

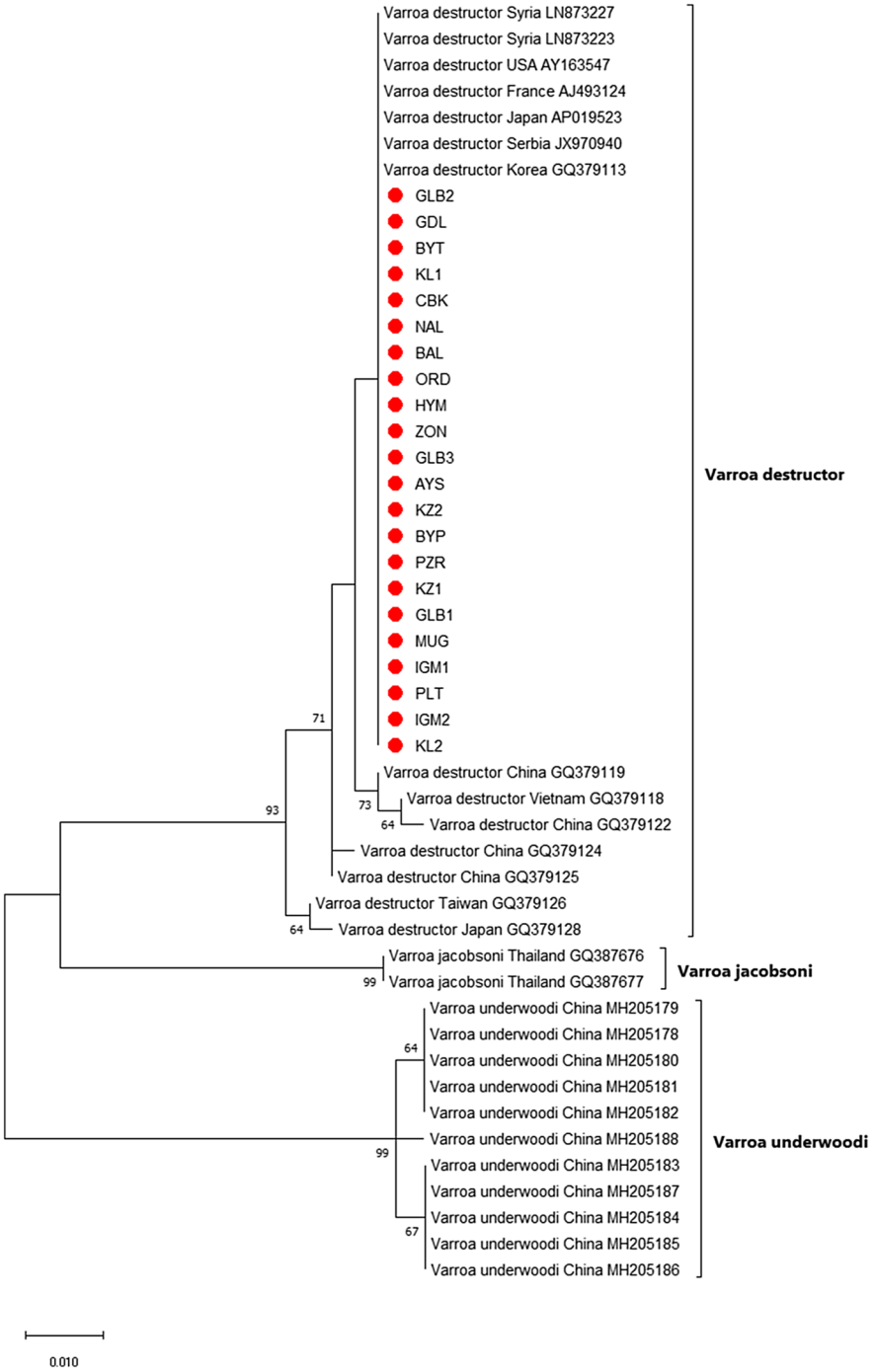

Fig. 2 Phylogenetic tree of *Varroa* species based on the *atp6* gene. Bootstrap values lower than 60% are not shown. Sequences of the specimens obtained in the present study are indicated with red dots. (Color figure online)

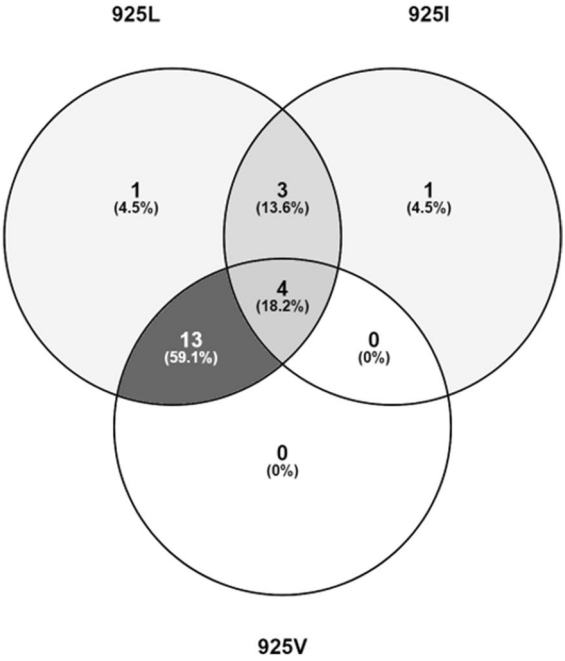

Fig. 3 Venn diagram of the amino-acid substitutions at position 925 of the voltage-gated sodium channel in *Varroa destructor* populations (VENNY, Oliveros 2007)

As a putative fitness cost associated with the L925V mutation has been suggested (González-Cabrera et al. 2018), the use of pyrethroids in rotation with other chemical control options such as organic acids and amitraz in *Varroa* control will result in decreasing the frequency of the resistance mutations and allow for more sustainable chemical control. The populations that were not exposed to flumethrin (KL2, BAL, CBK, GDL) also contained mutant individuals; however, susceptible alleles were determined as more frequent (L/V or L/I) based on visual inspection of sequencing chromatographs (Figure S1). This may reflect the decreasing resistance allel frequency in the absence of selection pressure. In addition, a vial test and a PCR–RFLP method have been reported to be fast and cost-effective alternatives to detect resistance and L925V/M/I mutations, respectively, allowing to monitor resistance widely and to design effective *Varroa* control management (Millán-Leiva et al. 2018; Stara et al. 2019b).

Considering the economic importance of beekeeping, the knowledge about genetic variation and pyrethroid resistance mutations in *V. destructor* populations in Turkey was very limited. In the current study, we revealed the presence and geographical distribution of pyrethroid resistance mutations (L925V and L925I) in Turkey. The results indicated that pyrethroids should include only a small fraction of the chemical treatments used within a rotation sheme, in order to decrease the frequency of pyrethroid resistance in apiaries of Turkey.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10493-021-00626-2.

Acknowledgements We would like to thank the beekeepers of the research area for their kind cooperation.

Data availability All relevant data are within the manuscript.

Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

Ethical approval Not applicable.

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