A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Establishment of bioassay- and molecular marker-based acaricide resistance monitoring protocols and elucidation of additional resistance factors in the *Varroa* mite, *Varroa destructor*

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Abstract

The *Varroa* mite, *Varroa destructor*, is an ectoparasitic mite that is one of the major threats to apiculture. Acaricides, such as fluvalinate, coumaphos, and amitraz, have been used for the control of *Varroa* mites. However, the extensive use of acaricide has led to resistance development in field populations of *Varroa* mite. Resistance monitoring is essential in the management of pesticide resistance, but acaricide resistance monitoring for *Varroa* mites has rarely been conducted in Korea, perhaps due to the difficulties associated with mite collection and bioassay. In this study, bioassay (residual contact vial: RCV)- and molecular marker (mutations in voltage-gated sodium channel)-based acaricide resistance monitoring methods were developed, and acaricide resistance monitoring was conducted in Korea in two years (2021 and 2022).

To establish the RCV bioassay protocol, the LC_{50} and LC_{90} values were determined for fluvalinate and coumaphos using the 2021-SO strain as a putative susceptible strain, and used as diagnostic concentrations. In 2021, acaricide resistance monitoring was conducted using LC_{90} as the diagnosis concentration for 14 regional mite populations. The 2021-GR and 2021-UR populations showed reduced mortalities to both coumaphos and fluvalinate, whereas the 2021-SJ2, 2021-DG, and 2021-GJ strains showed reduced mortalities to only fluvalinate, suggesting the possible development of resistance to fluvalinate and coumaphos.

In 2022, acaricide resistance monitoring was conducted using LC_{50} as diagnosis concentration for 42 regional populations. *Varroa* mites from eight regions showed reduced mortalities to fluvalinate, suggesting that fluvalinate resistance began to spread in Korea.

The molecular maker-based acaricide resistance monitoring method was developed using the quantitative sequencing protocol. In 2021, molecular marker-based acaricide resistance monitoring was conducted for mites collected from 17 regions. Fluvalinate resistance mutation (L925I/M) was detected in four out of 17 regions. The resistance monitoring conducted for 90 regional populations in 2022 revealed that fluvalinate resistance mutation was detected in 83 regions out of 90 regions, showing a rapid spread of resistance allele. In the correlation analysis between the 24-h mortality obtained from RCV and fluvalinate resistance mutation frequency, no apparent correlation was observed. The knockdown bioassay followed by genotyping revealed that only mites knocked down at an early time point (< 7 h post-treatment) possessed most of the susceptible homozygous and heterozygous genotypes at the L925I/M mutation site. This finding further implicated that the early time point is desirable for discriminating susceptible individuals from resistant ones in RCV bioassay. Intriguingly, the susceptible homozygous genotype was also detected in putatively resistant *Varroa* mites knocked down after 7 h post-fluvalinate treatment, implicating the possibility of other resistance factors.

In order to identify other fluvalinate resistance factors, novel mutations were searched in the nearly entire domains of the voltage-gated sodium channel gene. As a result, ten novel mutations were identified, among which two appeared to have some potential function as a target site insensitivity factor. In addition, transcription levels of cytochrome P450 monooxygenase (P450s) were investigated to determine their possible association with resistance as metabolic factors. To this end, searching for reliable reference genes for quantitative PCR was attempted first. The *eEF1A1* and *NADHD* were recommended as reference genes for the comparison of the effects of acaricide on the whole body. Despite the lack of apparent correlation between

their expression level and the mortality obtained from RCV bioassay, two P450s (Flv-2B4 and Flv-3A19) showed a tendency of decrease in mortality as the P450 expression level increased, thus implying their possible role in fluvalinate detoxification.

Keyword: *Varroa destructor*, acaricide resistance, residual contact vial bioassay, molecular marker, target site mutation, metabolic factor

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Chapter 1. Establishment of residual contact vial bioassay protocol and survey of field acaricide resistance status of *Varroa* mites in Korea

Abstract

Varroa mite, *Varroa destructor*, is an ectoparasitic mite that is one of the major threats to apiculture. Acaricides, such as coumaphos, fluvalinate, and amitraz, have been used for the control of *Varroa* mites. However, the extensive use of acaricide has led to resistance development. Despite the importance of resistance monitoring in resistance management, it has only been conducted once in the past in Korea. The reason is that resistance monitoring methods are difficult to use. For this reason, a resistance monitoring method was developed using the RCV method, which is easy to use in the field. The LC_{50} and LC_{90} value were obtained using the potential sensitivity, 2021-SO, and used as the diagnostic concentration. In 2021, acaricide resistance monitoring was conducted using LC_{90} as diagnosis concentration in 14 regions. The 2021-GR and 2021-UR populations showed reduced mortalities to both coumaphos and fluvalinate. The 2021-SJ2, 2021-DG, and 2021-GJ strains showed reduced mortalities to only fluvalinate. *Varroa* mites from 14 regions showed reduced mortalities to coumaphos. *Varroa* mites from eight regions showed reduced mortalities to fluvalinate. In Korea, coumaphos resistance appears to be at an early stage, but resistance to fluvalinate has developed to some extent.

1. Introduction

The *Varroa* mite, *Varroa destructor*, is a honey bee ectoparasitic mite feeding on the fat body of brood and adult bees (Ramsey et al., 2019). In addition to the direct physical damage by feeding, they reduce honey bee nutrient levels and transmit several viruses such as deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), and acute bee paralysis virus (ABPV) (Bowen-Walker & Gunn, 2001; Chen & Siede, 2007; Di Prisco et al., 2011; Highfield et al., 2009). For these reasons, *Varroa* mites are considered as one of the main causes of colony collapse disorder (CCD) (Le Conte et al., 2010).

To control the *Varroa* mites, beekeepers have used synthetic acaricides such as fluvalinate, amitraz, and coumaphos (Rosenkranz et al., 2010), which are highly selective toxicity to *Varroa* mites with little harm to honey bees (Shimanuki et al., 1980; Vlogiannitis et al., 2021). However, the extensive use of these acaricides has led to resistance development against them, resulting in frequent control failure (Higes et al., 2020; Maggi et al., 2009; Pettis, 2004; Thompson et al., 2002).

In managing pesticide resistance, it is important to keep the resistance allele frequency at a manageable level in the population. Thus, it is crucial to rotate pesticides from different classes based on regular resistance monitoring (Roush & Miller, 1986). The commonly used bioassay methods for mite resistance monitoring are contact toxicity measurements using an acaricide-coated petri dish or acaricide strip put into a petri dish (Higes et al., 2020; Maggi et al., 2008). However, *Varroa* mites can escape from the acaricide strip or acaricide-treated bottom of the petri dish, making the bioassay results inconsistent.

The residual contact vial (RCV) method, which uses acaricide-coated vials,

makes target pests have a forced contact with evenly coated insecticides and is designed to use on-site in the field with greater portability. For this reason, the RCV method has been used for insecticide resistance monitoring of several pests including *Frankliniella occidentalis*, *Tetranychus urticae*, and *Thrips palmi* (Kim et al., 2019; Kwon et al., 2015; Kwon et al., 2010).

In this study, I first determined the diagnostic concentrations of two acaricides (coumaphos and fluvalinate) using the RCV method, and then established an acaricide resistance monitoring protocol based on the RCV method. Using the resistance monitoring protocol, I conducted RCV bioassay for monitoring of acaricide resistance levels in regional populations of *Varroa* mites in Korea.

2. Materials and methods

2.1. *Varroa* mite collection

The collection had been performed from June to November 2021 in the colonies of Western honey bees (*Apis mellifera*). In 2022, *Varroa* mites were collected from June 15 to 24. Phoretic mites were collected from three randomly selected hives per collection site using the sugar powder method (Macedo & Ellis, 2000). The collected *Varroa* mites were washed with running water to remove any residual sugar powder. After soaking excessive water with tissue paper, the collected *Varroa* mites were used for bioassay.

2.2. Diagnostic concentration determination

To determine the diagnostic concentration of fluvalinate and coumaphos, *Varroa* mites collected from Western honey bee colonies on a rooftop apiary at the Gwanak campus of Seoul National University (Seoul, Korea, 37º27′46.8″N, 126º57′06.9″E) were used in 2021 (2021-SO strain). The colonies had not been treated with any acaricide for a year. To confirm the susceptibility of the mites to fluvalinate, the single nucleotide polymorphisms (SNPs) on voltage-gated sodium channel (VGSC) of the mites (L925I/M/V), which are known to confer fluvalinate resistance, were inspected. Genomic DNA (gDNA) was extracted from 20 mites of the 2021-SO strain using DNeasy tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The partial gDNA fragment flanking the fluvalinate resistance mutation site was amplified with a PCR reaction using the gDNA (2021-SO) as a template and a prime set (New_Vd_925_F: 5'-CTA GCC AAG TCA TGG CCA AC-3'; New_Vd_925_R: 5'-TTG TCG AGA TAG TTC TTG

CCG-3'), and Ex Taq (Takara Korea Biomedical Inc., Seoul, Korea). The thermal program was composed of 35 cycles of 95 °C/30 sec, 55 °C/30 sec, and 72°C/30 sec. The PCR product was purified using Expin PCR SV (Geneall Biotechnology, Seoul, Korea) and sequenced in reverse directions by a primer New_Vd_925_R).

The collected live *Varroa* mites were used for RCV bioassay to calculate diagnostic concentrations. Coumaphos and *tau*-fluvalinate were purchased from Merck (Darmstadt, Germany) and dissolved in 100% acetone to make a 10,000 mg/L stock solution. For the bioassay to determine diagnostic concentrations, the acaricide stock solutions were serially diluted to the concentrations listed in Table 2 with 100% acetone. A 100 µL aliquot of each acaricide solution was transferred into a 5 mL glass vial (SamwooKurex, Seoul, Korea), and the vials were rolled on a roller to evenly coat the acaricide and dried for an hour in a fume hood to evaporate acetone. Ten live *Varroa* mites were transferred into the acaricide-coated vials. The vials were incubated at 25 °C and 60% relative humidity in the dark condition. Mortalities were checked 24 h after inoculation. *Varroa* mites that were completely immobile or barely moving when contacted with a probe were deemed dead. All bioassays were performed in triplicate.

With the bioassay results, LC_{50} (median lethal concentration) and LC_{90} (90% lethal concentration) values of coumaphos and fluvalinate were calculated using the PoloPlus probit analysis software ver. 2.0 (LeOra Software, Petaluma, CA). In 2021, the LC_{90} values of each acaricide were selected as diagnostic concentrations for field resistance monitoring. In 2022, however, the diagnostic concentrations were deduced to LC_{50} values in an attempt to expand the resistance detection limit. Freshly made acaricide-coated vials were kept in a portable refrigerator set to 25 °C during the collection trip. Collected regional mites were inoculated to the vials, and the 24-h

mortalities were checked as described above. One to three replicates (ten mites/replicate) of the bioassays were performed from each regional sample.

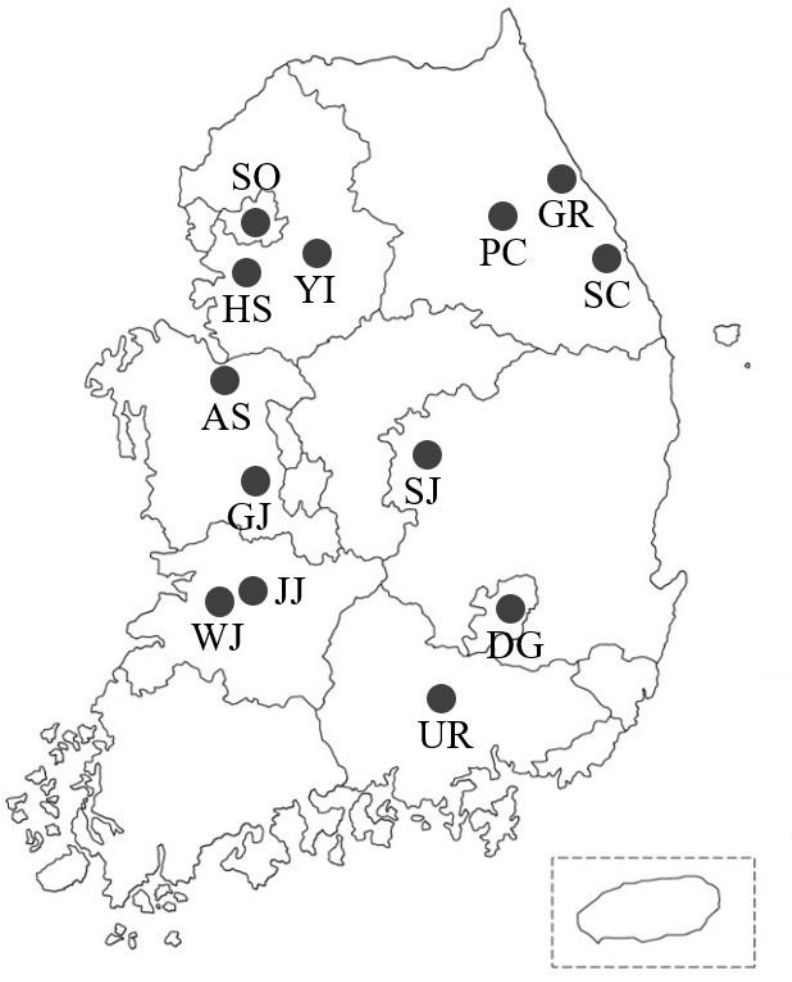

Fig. 1. Collection sites of *Varroa* mite field populations used for RCV bioassay in 2021.

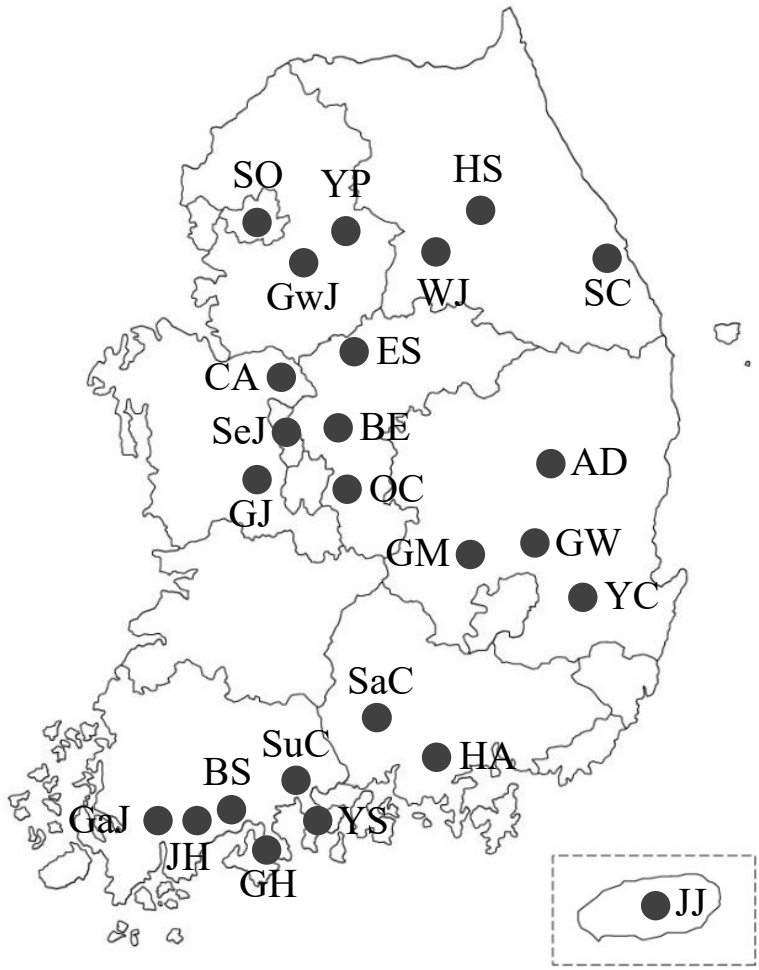

Fig. 2. Collection sites of *Varroa* mite field populations used for RCV bioassay in 2022.

Table 1. List of collection sites of *Varroa* mite field populations used for RCV bioassay in 2021.

Year	Mon	Location	Collection site	Population	GPS
2021	Jul.	Daegu	Dong-gu	2021-DG	35°56′20.4″N 128°41′50.3″E
	Jun.	Gyeonggi-do	Namsa-eup, Cheoin-gu, Yongin-si	2021-YI	37°05′12.3″N 127°10′46.1″E
	Jun.		Bongdam-eup, Hwaseong-si	2021-HS	37°10′11.3″N 126°56′35.8″E
	Jul.		Ganam-eup, Yeoju-si	2021-YJ	37°11′35.9″N 127°33′05.1″E
	Jun.	Gangwon-do	Gujeong-myeon, Gangneung-si	2021-GR	37°41′37.9″N 128°53′37.7″E
	Jul.		Miro-myeon, Samcheok-si	2021-SC	37°22′56.0″N 129°05′25.3″E
	Jul.		Jinbu-myeon, Pyeongchang-gun	2021-PC	37°39′09.3″N 128°35′10.9″E
	Jul.	Gyeongsangbuk-do	Oeseo-myeon, Sangju-si	2021-SJ1	36°28′18.7″N 128°05′34.5″E
	Jul.		Cheongni-myeon, Sangju-si	2021-SJ2	36°22′0.4″N 128°07′01.1″E
	Jul.	Gyeongsangnam-do	Hwajeong-myeon, Uiryeong-gun	2021-UR	35°16′29.5″N 128°14′58.8″E
	Aug.	Chungcheongnam-do	Uidang-myeon, Gongju-si	2021-GJ	36°33′44.6″N 127°09′30.5″E
	Aug.		Baebang-eup, Asan-si	2021-AS	36°44′52.3″N 127°02′31.3″E
	Jul.	Jeollabuk-do	Deokjin-gu, Jeonju-si	2021-JJ	35°49′32.5″N 127°04′04.0″E
	Jul.		Iseo-myeon, Wanju_gun	2021-WJ	35°49′38.6″N 127°02′25.1″E

Table 2. List of collection sites of Varroa mite field populations used for RCV bioassay in 2022.

Year	Mon	Location	Collection site	Population	GPS
2022	May	Seoul	Gwanak-gu	2022-SO1	37°27′28.6″N 126°56′55.1″E
	Jun.		Eunpyeong-gu	2022-SO2	37°38′37.6″N 126°56′28.5″E
	Jun.	Sejong-si	Jeonui-myeon	2022-SeJ1	36°41′36.0″N 127°13′10.4″E
	Jun.		Yeondong-myeon	2022-SeJ2	36°34′13.3″N 127°20′2.8″E
	Jun.	Gyeonggi-do	Namhansanseong-myeon, Gwangju-si	2022-GwJ1	37°26′55.4″N 127°15′31.8″E
	Jun.		Toechon-myeon, Gwangju-si	2022-GwJ2	37°28′40.6″N 127°19′0.7″E
	Jun.		Jipyeong-myeon, Yangpyeong-gun	2022-YP1	37°29′2.06″N 127°37′7.31″E
	Jun.		Cheongun-myeon, Yangpyeong-gun	2022-YP2	37°35′16.55″N 127°45′13.11″E
	Jun.	Gangwon-do	Heungeop-myeon, Wonju-si	2022-WJ1	37°20′12.3″N 127°52′51.96″E
	Jun.		Hojeo-myeon, Wonju-si	2022-WJ2	37°18′55.4″N 127°54′12.37″E
	Jun.		Socho-myeon, Wonju-si	2022-WJ3	37°25′18.24″N 127°58′4″E
	Jun.		Ucheon-myeon, Hoengseong-gun	2022-HS	37°29′33.49″N 128°5′33.59″E
	Jun.		Miro-myeon, Samcheok-si	2022-SC1	37°22′55.7″N 129°5′24.7″E
	Jun.		Geundeok-myeon, Samcheok-si	2022-SC2	37°21′59″N 129°13′1.2″E
	Jun.	Chungcheongbuk-do	Iwon-myeon, Okcheon-gun	2022-OC1	36°15′11.42″N 127°39′31.13″E
	Jun.		Cheongseong-myeon, Okcheon-gun	2022-OC2	36°21′0.3″N 127°45′51.6″E
	Jun.		Gunbuk-myeon, Okcheon-gun	2022-OC3	36°20′31.36″N 127°34′57.28″E
	Jun.		Suhan-myeon, Boeun-gun	2022-BE	36°29′7.3″N 127°40′51.7″E
	Jun.		Geumwang-eup, Eumseong-gun	2022-ES	36°59′3.6″N 127°32′53.57″E
	Jun.	Chungcheongnam-do	Gwangdeok-myeon, Cheonan-si	2022-CA	36°41′39.91″N 127°4′11.55″E
	Jun.		Jeongan-myeon, Gongju-si	2022-GJ	36°35′9.95″N 127°8′54.71″E
	Jun.	Gyeongsangbuk-do	Seohu-myeon, Andong-si	2022-AD	36°36′31.46″N 128°37′55.48″E
	Jun.		Sanseong-myeon, Gunwi-gun	2022-GW	36°7′44.49″N 128°59′46.01″E
	Jun.		Cheongtong-myeon, Yeongcheon-si	2022-YC	35°59′46.01″N 128°52′54.1″E
	Jun.		Gumi-si	2022-GM1	36° 8'31.30"N

				128°23'4.52"E
Jun.	Gyeongsangbuk-do	Haepyeong-myeon, Gumi-si	2022-GM2	36°14'27.84"N 128°21'40.61"E
Jun.	Gyeongsangnam-do	Yeohang-myeon, Haman-gun	2022-HA	35°12′23.36″N 128°26′31.59″E
Jun.		Chahwang-myeon, Sancheong-gun	2022-SaC1	35°28′41.85″N 127°55′33.72″E
Jun.		Saengbiryang-myeon, Sancheong-gun	2022-SaC2	35°21′30.36″N 128°4′11.22″E
Jun.	Jeollanam-do	Jeomam-myeon, Goheung-gun	2022-GH1	34°38'1.42"N 127°28'10.87"E
Jun.		Duwon-myeon, Goheung-gun	2022-GH2	34°38'3.27"N 127°19'30.91"E
Jun.		Yeosu-si	2022-YS	34°44'34.81"N 127°38'39.36"E
Jun.		Byeollyang-myeon, Suncheon-si	2022-SuC	34°51'37.98"N 127°25'51.84"E
Jun.	Jeollanam-do	Yongsan-myeon, Jangheung-gun	2022-JH1	34°38'1.08"N 126°54'47.54"E
Jun.		Daedeok-eup, Jangheung-gun	2022-JH2	34°30'27.61"N 126°52'15.20"E
Jun.		Gundong-myeon, Gangjin-gu	2022-GaJ	34°39'2.54"N 126°50'19.92"E
Jun.		Boseong-eup, Boseong-gun	2022-BS	34°42'22.31"N 127° 5'41.74"E
Jun.	Jeju-do	Aewol-eup, Jeju-si	2022-JJ1	33°27′27.53″N 126°23′12.73″E
Jun.		Daejeong-eup, Seogwipo-si	2022-JJ2	33°16′57.41″N 127°15′2.57″E
Jun.		Daejeong-eup, Seogwipo-si	2022-JJ3	33°16′31.77″N 126°16′8.7″E

2.3. Diagnostic concentration determination

One-way ANOVA was performed to compare the average mortality of each region with Tukey's multiple comparison test as a post hoc analysis. All statistical analyses and graph designs were performed using Prism 6.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Diagnostic concentration determination

Prior to bioassay, presence of the fluvalinate-resistant mutation on VGSC in the 2021-SO population was inspected to estimate its fluvalinate susceptibility (Fig. 1). In the sequencing result of pooled 20 *Varroa* mites, only leucine (CTG) was detected at the 925 amino acid position, suggesting that the 2021- SO population is likely susceptible to fluvalinate from the perspective of target site insensitivity. Although the possibility of the 2021-SO population carrying other resistance factors, thus not being fully susceptible to fluvalinate, could not be completely excluded, it was used a tentative susceptible strain for estimating the baseline susceptibility to determine diagnostic concentrations for RCV bioassay.

The *Varroa* mites were more sensitive to coumaphos than to fluvalinate. The 24 h LC_{50} and LC_{90} values of fluvalinate were 35 mg/L and 200 mg/L, whereas the 24 h LC_{50} and LC_{90} values of coumaphos were 0.08 mg/L and 0.8 mg/L, respectively (Table 2). These 24 h LC_{50} or LC_{90} values were used as the diagnostic concentrations for acaricide resistance monitoring.

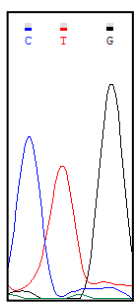

Fig. 3. Sequencing chromatogram of the 2021-SO strain of *Varroa* mite showing the susceptible allele at the fluvalinate resistance mutation (L925I/M/V) site.

Table 3. Probit mortality data of *Varroa* mites following treatment with coumaphos and fluvalinate. The RCV method was used for the bioassay and 24 h mortality was observed. The doses coated on the inner surface of a vial (ng/cm^2) are indicated in parentheses.

Acaricide	**Coumaphos**	**Fluvalinate**
Tested	$8x10^{-3}$ mg/L ($5.8x10^{-2}$ ng/cm^2)	10 mg/L (72 ng/cm^2)
range	$4x10^{-2}$ mg/L ($2.9x10^{-1}$ ng/cm^2)	2x10 mg/L (144 ng/cm^2)
	$2x10^{-1}$ mg/L (1.4 ng/cm^2)	4x10 mg/L (288 ng/cm^2)
	1 mg/L (7.2 ng/cm^2)	8x10 mg/L (576 ng/cm^2)
	5 mg/L (36 ng/cm^2)	$1.6x10^2$ mg/L (1152 ng/cm^2)
Total number[a]	150	150
LC_{50} (mg/L)	0.08	35
LC_{50} Cl 95%	lower 0.026 upper 0.225	lower 25.4 upper 48.2
LC_{90} (mg/L)	0.8	200
LC_{50} Cl 95%	lower 0.285 upper 8.99	lower 125 upper 491
Slope (± SE)	1.31 (± 0.17)	1.69 (± 0.28)

Cl, confidence level; SE, standard error

[a] The total number of *Varroa* mites used for the bioassay

3.2. Acaricide resistance monitoring of *Varroa* mites using RCV bioassay in Korea

In 2021. Acaricide resistance monitoring was conducted for 14 regions with LC_{90} values as the diagnostic concentrations. The 2021-GR (60% mortality to coumaphos and 80% mortality to fluvalinate) and 2021-UR (49% mortality to coumaphos and 43% mortality to fluvalinate) populations showed reduced mortalities to both coumaphos and fluvalinate. The 2021-SJ2 (56% mortality to fluvalinate), 2021-DG (47% mortality to fluvalinate) and 2021-GJ (67% mortality to fluvalinate) strains showed reduced mortalities to only fluvalinate. This finding suggests that the possibility of moderate resistance in some regions. Six (2021-HS, 2021-SJ1, 2021-SJ2, 2021-WJ, 2021-JJ and 2021-GJ) and five regional populations (2021-YI, 2021-YJ, 2021-SC, 2021-JJ and 2021-AS) showed 100% mortality to coumaphos and fluvalinate, respectively. Only one population (2021-JJ) exhibited 100% mortality to both coumaphos and fluvalinate. The average mortalities of all the populations examined to coumaphos and fluvalinate were 89.3% and 83.1%, respectively, suggesting that acaricide resistance, if present, is not uniform yet in Korea.

In 2022, acaricide resistance monitoring was conducted for 42 regions with more expanded detection limit by reducing the diagnosis concentration to the LC_{50} values. *Varroa* mites from 14 regions (2022-SO2: 0%, 2022-SeJ1: 30%, 2022-SeJ2: 30%, 2022-GwJ2: 0%, 2022-YP1: 10%, 2022-WJ2: 10%, 2022-OC1: 26%, 2022-BE: 25%, 2022-CA: 40%, 2022-HA: 40%, 2022-JH1: 33%, 2022-GaJ: 25%, 2022-JJ1: 20% and 2022-JJ2: 0%) showed reduced mortalities to coumaphos. *Varroa* mites from eight regions (2022-SeJ1: 40%, 2022-SC1: 30%, 2022-GM2: 25%, 2022-HA:

40%, 2022-GH1: 47%, 2022-JJ2: 25%, 2022-JJ3: 10% and 2022-JJ5: 40%) showed reduced mortalities to fluvalinate. The average mortalities to coumaphos and fluvalinate across all the regions examined were 51% and 70%, respectively.

The cross-regional comparison of average mortality revealed that mortality to coumaphos was highest in Gyeongsangnam-do (72.3%), followed by Gyeongsangbuk-do (70%), Gangwon-do (60.3%), Chungcheongbuk-do (46.5%), Jeollanam-do (44.8%), Chungcheongnam-do (42.5%), Jeju-do (30%) and Gyeonggi-do (29%) (Seoul was included in Gyeonggi-do, whereas Sejong was included in Chungcheongnam-do). The average mortality to fluvalinate was highest in Gangwon-do (82.2%), followed by Chungcheongbuk-do (80%), Jeollanam-do (78.1%), Gyeonggi-do (67.2%), Gyeongsangbuk-do (64.8%), Gyeongsangnam-do (64.3%), Chungcheongnam-do (63.3%) and Jeju-do (49%)**.** The average mortalities to coumaphos and fluvalinate across different regions were not significantly different (p = 0.16 and 0.31, respectively).

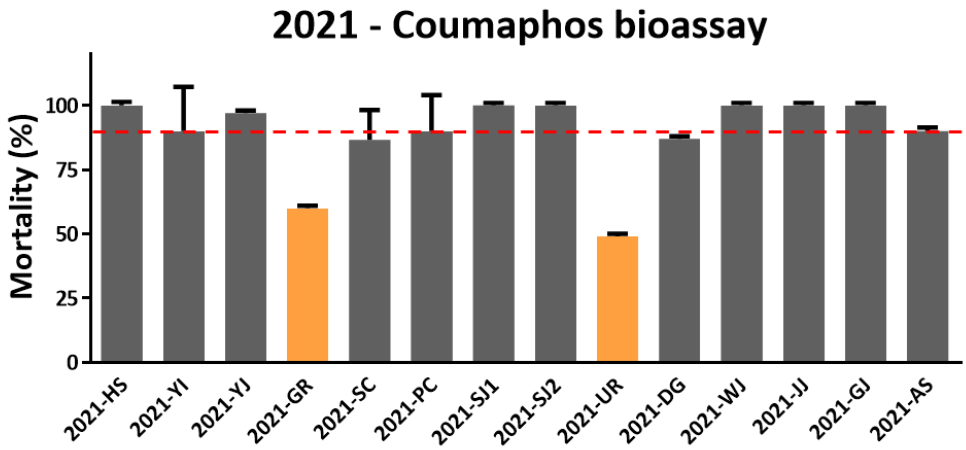

Fig. 4. Coumaphos bioassay results of *Varroa* mite field strains in 2021. The diagnostic concentration was based on the coumaphos LC_{90} value of 2021-SO strain. The strains expected to be resistant based on the mortalities are colored in orange.

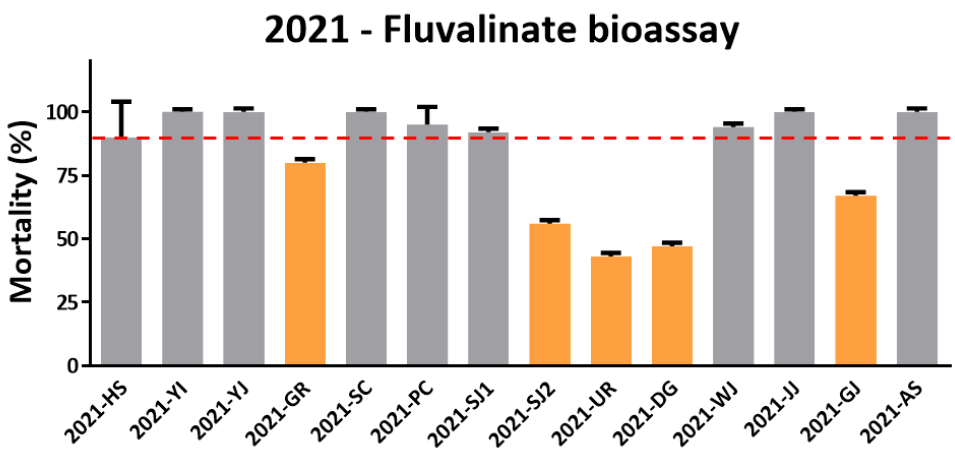

Fig. 5. Fluvalinate bioassay results of *Varroa* mite field strains in 2021. The diagnostic concentration was based on the fluvalinate LC_{90} value of 2021-SO strain. The strains expected to be resistant based on the mortalities are colored in orange.

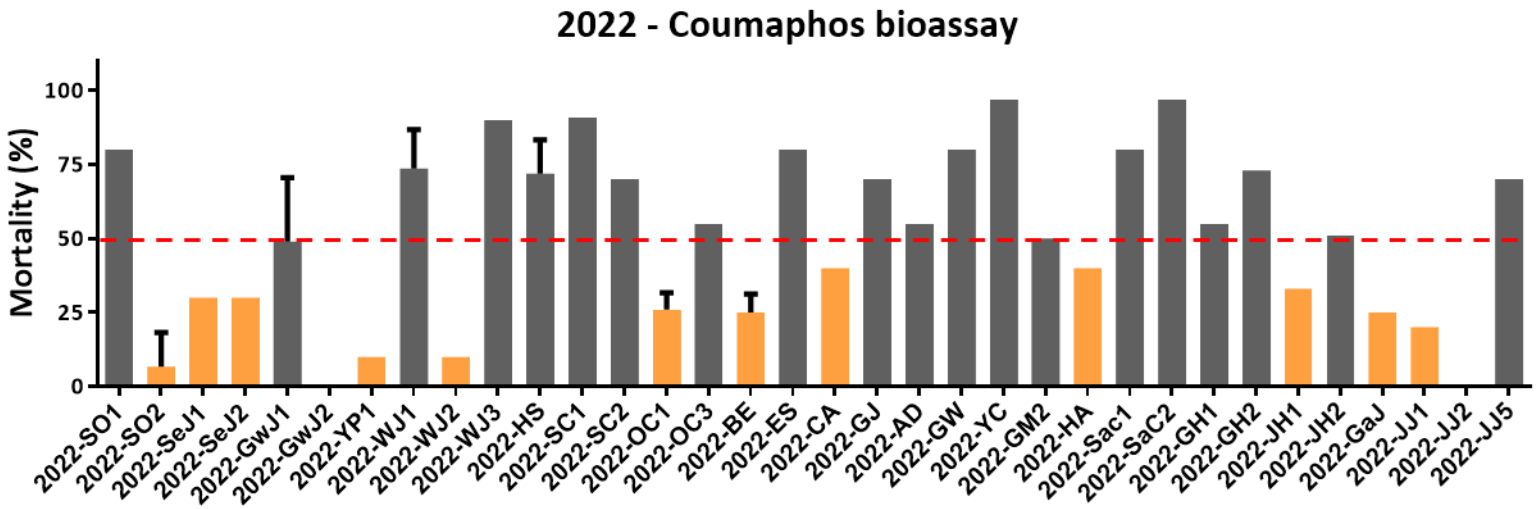

Fig. 6. Coumaphos bioassay results of *Varroa* mite field strains in 2022. The diagnostic concentration was based on the coumaphos LC_{50} value of 2021-SO strain. The strains expected to be resistant based on the mortalities are colored in orange.

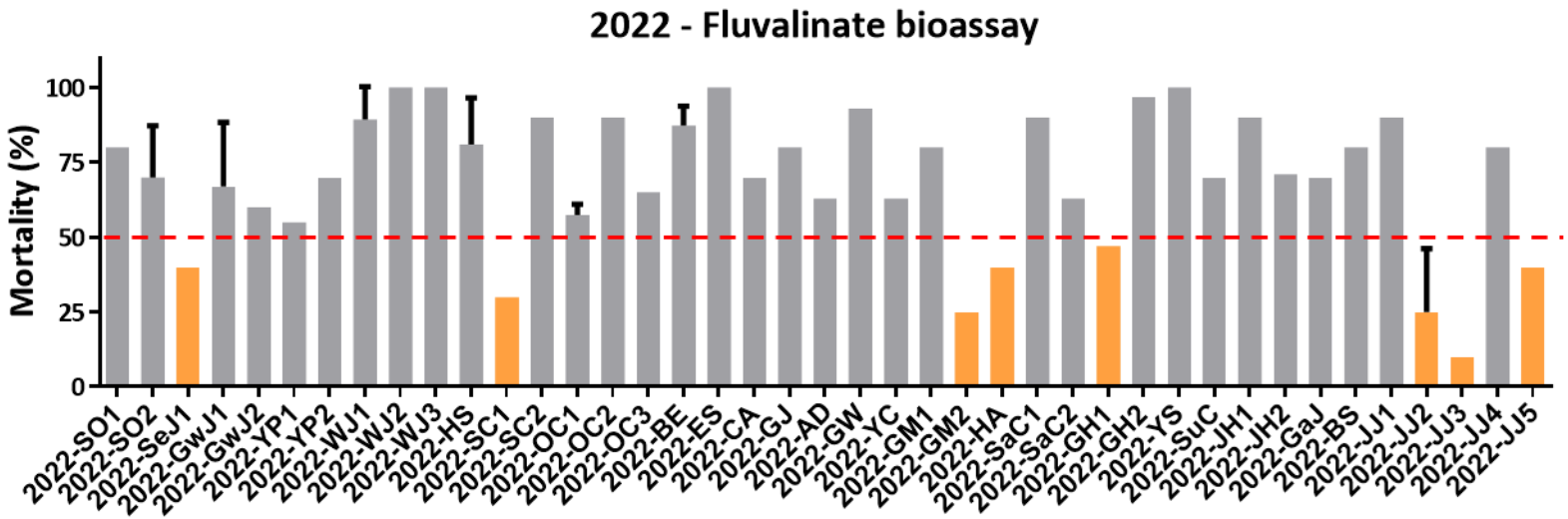

Fig. 7. Fluvalinate bioassay results of *Varroa* mite field strains in 2022. The diagnostic concentration was based on the fluvalinate LC_{50} value of 2021-SO strain. The strains expected to be resistant based on the mortalities are colored in orange.

4. Discussion

The acaricide resistance development of *Varroa* mites has been reported worldwide. A *Varroa* mite population with a 14480-fold resistance to fluvalinate was reported from Spain (Gracia-Salinas et al., 2006). In addition, *Varroa* mite populations showing 197-559 fold higher resistance to coumaphos than the susceptible strain was found in Argentina (Maggi et al., 2009), and a *Varroa* mite population with 16-fold resistance to coumaphos was reported in Italy (Spreafico et al., 2001). In order to prevent or suppress the acaricide resistance development, essential is the efficient management of resistance (Roush & Miller, 1986), which primarily requires constant resistant monitoring. In case of the conventional resistance monitoring of *Varroa* mites, however, there have been inconveniences both in the sampling of parasitic stage mites and in the conventional Petri dish bioassay itself, thus impairing overall monitoring efficiency. With this in mind, employment of the RCV-based resistance monitoring protocol in conjunction with the phoretic mite collection using sugar powder would facilitate the monitoring efficiency significantly.

The RCV method has many advantages over the Petri dish bioassay conventionally used. The bioassay method using Petri dishes does not force *Varroa* mite into contact with acaricides and requires larger amount (1 ml) of acaricide solution when producing the acaricide-coated Petri dishes (Higes et al., 2020; Maggi et al., 2008). In contrast, the RCV method makes *Varroa* mites have a forced contact with acaricides, thereby enabling the acquisition of more consistent bioassay results. In addition, the RCV method requires only 100 µl acaricide solution for coating the whole vial, thus saving the amounts of toxic compounds used. In the bioassay

conducted in previous studies, since the parasitic stage *Varroa* mites collected from brood cells were mostly were used (Hernández-Rodríguez et al., 2021; Maggi et al., 2009; Maggi et al., 2010), mixed specimens of females with two different generations (i.e., old mother and new daughter), which likely differ in their acaricide susceptibility, can pose negative impacts on overall bioassay quality. In the current RCV bioassay, however, since only the phoretic stage *Varroa* mites collected by the sugar powder method were used, the toxic response variation in-between specimens would be minimized, improving bioassay results. Additional advantages of RCV method include its less technique-dependency and the capability of mass production and long-term storage. The vial-coated acaricides were stable at least for 1 year when stored at -20 °C (Kwon et al., 2010).

Among the many haplogroups of *Varroa* mites, the major type found in Western honey bees is K1 haplotype, which is thought to switch from *A. cerana* to *A. mellifera* near Vladivostok, the north end of Korean peninsula (Traynor et al., 2020). The Asian honey bee, the original host of the *Varroa* mites, was introduced to Korea around 2,000 years ago (Oh et al., 2016). Considering its endemic nature, the genetic pool of the *Varroa* mites in Korea is expected to be more diverse, which can accelerate the selection of acaricide resistance trait under a heavy exposure scenario. Although there is no official record on the use pattern of synthetic acaricides for *Varroa* mite control in Korea, a recent survey indicated that fluvalinate is be the most commonly used, followed by amitraz and coumaphos. In my private interviews with beekeepers, most of them treated fluvalinate strip to the colonies, whereas coumaphos has barely used because it has not been available in the market perhaps at least during last decade. Thus, it was expected that fluvalinate resistance has been already developed in regional populations of *Varroa* mites in Korea but coumaphos

resistance is less likely.

In the results from RCV conducted in 2021, reduced fluvalinate mortalities observed in several populations are likely due to resistance development. Among them, the 2021-UR population showed a similar level of resistance to coumaphos as well (43% mortality to fluvalinate vs. 49% mortality to coumaphos). This suggests that 2021-UR might have developed cross-resistance to both acaricides, possibly via altered detoxification systems as in other pests (Wang et al., 2021; Zhang et al., 2017). Carboxylesterase and cytochrome P450 are likely involved in the putative cross-resistance (Berenbaum & Johnson, 2015; Mao et al., 2011).

As the average of mortality rates over the different regions was close to 90% in 2021 survey, the diagnostic concentrations were adjusted in 2022. In the RCV-based monitoring in 2022, which covered more regions than in 2021 using LC_{50} values, the coumaphos average mortality (51.0) was significantly lower than that of 2021 (89.3%), suggesting that switching to LC_{50} values sacrificed the discrimination power to an unacceptable level. This is particularly true when considering that the likelihood of coumaphos resistance is substantially lower compared to fluvalinate in Korea. Thus, the coumaphos mortalities of lower than 50%, which were observed in many regional populations of 2022, does not necessarily mean that they have developed coumaphos resistance, and further mandating the re-adjustment of diagnostic concentration. Increasing the dose again along with the reduction of observation time before the saturation of intoxication response would provide a more enhanced resolution in bioassay.

In case of the fluvalinate bioassay, the average mortality increased to 70% compared to the results of 2021. This finding that a large number of mite populations rather showed higher fluvalinate susceptibility compared to the reference 2021-SO

strain suggested that the putative susceptible population likely possesses some resistance factors other than target site insensitivity mediated by the L925I/M/V mutations. If this is the case, further lowering the diagnostic concentration below the LC_{50} level or reducing the observation time would be a feasible option to achieve a more field-relevant resistance monitoring. Despite the increased average mortality across different geographical regions, some populations still showed mortalities significantly lower than 50%, suggesting they have developed fluvalinate resistance and further requiring precise resistance monitoring to suppress this trend.

The cross-regional comparison of average mortality to coumaphos and fluvalinate revealed that there is no apparent region-dependent tendency in average mortality**.** The possible reasons for this result are that the Korea is relatively small in area and that mobile beekeeping is common during the blooming season of acacia and chestnut flowers, which occupies most of honey production in Korea (Kohsaka et al., 2017), facilitating the rapid introgression between mite populations.

In the two-year monitoring of *Varroa* mite acaricide resistance levels in Korea, putative resistant strains against either coumaphos and fluvalinate or both were found. Although resolution of the monitoring was not clear enough due to lack of an absolutely susceptible mite strain and to the less optimized bioassay conditions including the diagnostic concentration and observation, fluvalinate resistance appears to begin spreading, whereas coumaphos resistance is locally limited, if exists. With this in mind, use of alternative acaricides including coumaphos and amitraz along with other natural compounds is recommended instead of fluvalinate. Given that the relative efficacy of coumaphos is much greater (~438 fold) than fluvalinate as judged by LC_{50} values, coumaphos seems a feasible option to control fluvalinate-resistant *Varroa* mite. Nevertheless, precise monitoring of coumaphos and amitraz

resistance level in the field is essential prior to the introduction of these alternatives. As there is a precedent that indiscriminate use of coumaphos eventually had resulted in the banning from the market (Hernández-Rodríguez et al., 2021), a judicial acaricide rotation following regular resistance monitoring based on a susceptible strain should be performed together.

Chapter 2. Development and application of molecular diagnosis protocol for fluvalinate resistance monitoring

Abstract

A molecular marker-based resistance monitoring method was developed using the quantitative sequencing (QS) protocol that can estimate the L925I/M mutation frequency. To this end, the nucleotide signal ratios generated from sequencing chromatogram and the corresponding resistance allele frequencies at the mutation site (L925) were plotted and fitted to a quadratic prediction equation. In 2021, QS-based resistance monitoring was conducted for mites collected from 17 regions. Fluvalinate resistance mutation (L925I/M) was detected in four out of 17 regions. The resistance monitoring conducted for 90 regional populations in 2022 revealed that fluvalinate resistance mutation was detected in 83 regions out of 90 regions, showing a rapid spread of resistance allele. In the correlation analysis between the 24-h mortality obtained from RCV and fluvalinate resistance mutation frequency, no apparent correlation was observed. The knockdown bioassay followed by genotyping revealed that only mites knocked down at early time point (< 7 h post-treatment) possessed most of susceptible homozygous and heterozygous genotypes at the L925I/M mutation site. This finding further implicated that the early time point is desirable for discriminating susceptible individuals from resistant ones in RCV bioassay. Intriguingly, the susceptible homozygous genotype was also detected in

putatively resistant *Varroa* mites knocked down after 7 h post-fluvalinate treatment, implicating the possibility of other resistance factors.

1. Introduction

The *Varroa* mite, *Varroa destructor*, is one of the major threats to apiculture (Rosenkranz et al., 2010). Fluvalinate, a pyrethroid pesticide, disturbs the inactivation kinetics of voltage-gated sodium channel (VGSC), leading to the death of target organisms (Silver et al., 2014). With its high selective toxicity to *Varroa* mites and easy use as a strip, fluvalinate has been widely used for the in-hive treatment for past several decades (Elzen et al., 1998; Shimanuki et al., 1980). However, extensive use of acaricide has led to the resistance development in *Varroa* mite populations over the world (Elzen 2002, Maggi 2009, Thompson 2002). The mutation scanning of VGSC gene in resistant mite populations revealed that three single nucleotide polymorphisms (SNP) at a single amino acid position (L925V/M/I) confer fluvalinate resistance (Gonzalez-Cabrera et al., 2013; González-Cabrera et al., 2016). The L925V/M/I mutations were detected from fluvalinate-resistant populations of *Varroa* mites worldwide (Benito-Murcia et al., 2022; González-Cabrera et al., 2016; Koç et al., 2021). The L925V/M/I mutations are located in the VGSC domain II S5 that was expected to be the major binding site of pyrethroid (O'Reilly et al., 2006), thus functioning as the knockdown resistance (*kdr*) trait to pyrethroid.

Bioassay-based resistance monitoring, as performed in Chapter I, can measure the overall resistance level of pests. However, it demands a large numbers of live pest specimens, and different results can be drawn depending on inconsistent experimental conditions. On the other hand, molecular marker-based resistance monitoring has the advantages that allow the use of ethanol-stored or frozen samples and the results are consistent regardless of different experimental conditions. DNA

pooling method further reduces the cost and time for determining the frequencies of resistance mutations (Sham et al., 2002). However, since the SNPs conferring target site insensitivity are most largely used as molecular markers, the SNP-based resistance monitoring may reflect only the partial resistance level contributed by the target site SNPs particularly when multiple factors are involved in resistance.

In this study, a quantitative sequencing (QS) method was developed to predict the fluvalinate-resistant VGSC allele frequency in pooled *Varroa* mite specimens. With the QS method, fluvalinate resistance levels of regional *Varroa* mite populations in Korea were monitored. The correlation coefficient was calculated from the molecular marker-based result and the fluvalinate RCV bioassay result from Chapter I. By tracking resistance allele frequencies in mite groups knocked down at sequential time points in the RCV assay, the optimum mortality observation time that can distinguish susceptible and resistant individuals more precisely in the RCV assay was proposed.

2. Materials and methods

2.1. *Varroa* mite sample collection and cDNA preparation

Varroa mite samples were obtained in two ways. First, *Varroa* mites were collected using sugar powder method in several regions (Table. 1), stored in RNAlater solution (Invitrogen, Carlsbad, CA, USA) during transport, and kept at a -70 °C deep freezer until use (Macedo & Ellis, 2000). Second, mite samples collected by beekeepers in various regions (Table. 2) using the sugar powder method were immediately stored in 75% ethanol and sent to my laboratory. The ethanol-stored mite samples were directly stored at a deep freezer as they arrived. Total RNA, instead of genomic DNA (gDNA), was extracted from the mite specimens for the SNP analysis after considering further analysis for gene expression profiles of putative metabolic factors conferring resistance as well. RNA was extracted from the whole bodies of 20 *Varroa* mites collected in various regions using the TRI reagent (MRC, Cincinnati, OH, USA). The extracted RNA was treated with DNase I (Takara Korea biomedical Inc., Seoul, Korea) to remove gDNA. DNase I-treated RNA (2.5 μg) was used to synthesize cDNA with Superscript IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 55 °C for 10 min. The synthesized cDNA samples were diluted to 5 ng/μl with double-distilled water.

2.2. Development of QS protocol for fluvalinate resistance mutation (L925I/M) detection

gDNA was extracted from *Varroa* mites using DNeasy tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. To obtain susceptible and resistant genotypes, partial VGSC sequence containing the resistant SNP position was cloned. DNA samples of 2021-DG and 2021-UR collected in 2021 (Chapter I, Table. 1) were amplified using primer sets (New_Vd_925_F: 5'-CTA GCC AAG TCA TGG CCA AC-3'; New_Vd_925_R: 5'-TTG TCG AGA TAG TTC TTG CCG-3') and Ex Taq () with 35 cycles of 95 °C/30 sec, 55 °C/30 sec, 72 °C/30 sec. The amplified products were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The plasmids were sequenced to identify the susceptible and resistant genotypes at the SNP position. The susceptible and resistant plasmids were mixed together in the following ratios: 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 10:0. PCR of the VGSC was conducted using plasmid DNA template mixtures and primer sets (New_Vd_925_F and New_Vd_925_R), and plasmid DNA template mixtures were sequenced using primer (New_Vd_925_R). Following sequencing, the signal ratios between susceptible vs. resistant nucleotides were obtained from the sequence chromatogram (Chromas, Technelysium, South Brisbane, Australia). Plotting the nucleotide signal ratios against actual genotype frequencies was conducted using Prism 6.0 (GraphPad, San Diego, CA, USA), and regression equations were deduced for the prediction of resistance allele frequency.

2.3. Fluvalinate resistance monitoring using QS protocol

PCR was conducted using cDNAs from the pooled *Varroa* mites as templates with a primer set (Vd_L925_cDNA_F: 5'- CAT GGA CCA TCA CGA CAT GG-3'; Vd_L925_cDNA_R: 5'- CAG GTT GCC AAT AAC GAC GG-3') and Ex Taq (Takara Korea Biomedical Inc., Seoul, Korea). The thermal program was conducted with 35 cycles of 95 °C/30 sec, 59 °C/30 sec, and 72°C/30 sec. PCR products were purified using Expin PCR SV (Geneall Biotechnology, Seoul, Korea) and sequenced by primer (Vd_L925_cDNA_F), and fluvalinate resistance mutation frequencies were calculated by substituting the nucleotide signal ratios obtained from sequencing results for the standard prediction equation.

2.4. Optimization of observation time for mortality check in RCV assay

Bioassays were performed using the residual contact vial (RCV) method. Vials coated with fluvalinate (35 ppm: 24 h LC_{50}, 200 ppm: 24 h LC_{90}, and 400 ppm: two-fold of 24 h LC_{90}) were prepared as described in chapter I. Ten live *Varroa* mites were transferred into fluvalinate-coated vials. The vials were incubated at 25 °C and 60% relative humidity. Knockdown (or mortality) of *Varroa* mites was checked at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 12 h, and 24 h post-treatment. Knockdown *Varroa* mites were collected at each time point. Collected *Varroa* mites were flash frozen by liquid nitrogen and stored at -80 °C until use. Total RNA was extracted from individual mites. The protocols for RNA extraction and cDNA synthesis were the same as described in section 2-1. PCR was conducted using cDNAs of the individual *Varroa* mites as templates with a primer set (Vd_L925_cDNA_F: 5'- CAT GGA CCA TCA CGA CAT GG-3'; Vd_L925_cDNA_R: 5'- CAG GTT GCC AAT AAC GAC GG-

3') and Ex Taq (Takara Korea Biomedical Inc.). The thermal program was conducted with 35 cycles of 95 °C/30 sec, 59 °C/30 sec, and 72°C/30 sec. PCR products were purified using Expin PCR SV (Geneall Biotechnology) and sequenced by a primer (Vd_L925_cDNA_F).

Table 4. Collection sites of regional *Varroa* mite samples.

Num.	Population	Location		Num.	Population	Location	
1	2022-WJ1	Wonju	Gangwon-do	20	2022-BE	Boeun	Chungcheongbuk-do
2	2022-WJ2			21	2022-ES	Eumseong	
3	2022-WJ3			22	2022-OC1	Okcheon	
4	2022-HS	Hoengseong		23	2022-OC3		
5	2022-SC2	Samcheok		24	2022-CA	Cheonan	Chungcheongnam-do
6	2022-GW	Gunwi	Gyeongsangbuk-do	25	2022-GJ	Gongju	
7	2022-YC	Yeongcheon		26	2022-GC	Gochang	Jeollabuk-do
8	2022-AD	Andong		27	2022-GiJ	Gimje	
9	2022-UJ1	Uljin		28	2022-NW	Namwon	
10	2022-UJ2			29	2022-GH1	Goheung	Jeollanam-do
11	2022-UJ3			30	2022-GH2		
12	2022-GM1	Gumi		31	2022-SuC	Suncheon	
13	2022-GM2			32	2022-BS	Boseong	
14	2022-HA	Haman	Gyeongsangnam-do	33	2022-JH1	Jangheung	
15	2022-SO1	Seoul	Gyeonggi-do	34	2022-JH2		
16	2022-SO2			35	2022-GaJ	Gangjin	
Jeju-do	2022-GwJ1	Gwangju		36	2022-JJ1	Jeju	Jeju-do
	2022-GwJ2			37	2022-JJ3		
19	2022-YP2	Yangpyeong		38	2022-JJ5		

Table 5. Collection sites of regional *Varroa* mite samples from beekeepers.

Num.	Population	Location	
1	2022P-SC	Samcheok	Gangwon-do
2	2022P-JuS	Jeongseon	
3	2022P-HC	Hongcheon	
4	2022P-NY	Namyangju	Gyeonggi-do
5	2022P-SO	Seoul	
6	2022P-SW	Suwon	
7	2022P-AnS	Anseong	
8	2022P-IC1	Icheon	
9	2022P-IC2		
10	2022P-JaS	Jsngsu	Jeollabuk-do
11	2022P-JJ	Jeonju	
12	2022P-GJ	Gwangju	Jeollanam-do
13	2022P-HS	Hwasun	
14	2022P-HN1	Haenam	
15	2022P-HN2		
16	2022P-GR	Gyeryong	Chungcheong nam-do
17	2022P-GS	Geumsan	
18	2022P-DJ1	Dangjin	
19	2022P-DJ2		
20	2022P-DJ3		
21	2022P-SJ	Sejong	
22	2022P-AS1	Asan	
23	2022P-AS2		
24	2022P-YS	Yesan	
25	2022P-BE	Boeun	Chungcheong buk-do
26	2022P-ES	Eumseong	

Num.	Population	Location	
27	2022P-GM	Gumi	Gyeongsang buk-do
28	2022P-GC1	Gimcheon	
29	2022P-GC2		
30	2022P-DG	Daegu	
31	2022P-SaJ	Sangju	
32	2022P-SeJ1	Seongju	
33	2022P-SeJ2		
34	2022P-YJ1	Yeongju	
35	2022P-YJ2		
36	2022P-YJ3		
37	2022P-UJ1	Uljin	
38	2022P-UJ2		
39	2022P-UJ3		
40	2022P-CD	Cheongdo	
41	2022P-CS	Cheongsong	
42	2022P-CG	Chilgok	
43	2022P-NH1	Namhae	Gyeongsang nam-do
44	2022P-NH2		
45	2022P-MY	Miryang	
46	2022P-US	Ulsan	
47	2022P-CW1	Changwon	
48	2022P-CW2		
49	2022P-HA	Haman	
50	2022P-HC	Hapcheon	
51	2022P-CJ	Chungju	Chungcheongbuk-do
52	2022P-CB	Chungcheongbuk-do	

3. Results

3.1. Establishment of regression equations for the prediction of resistance allele frequency

A set of plasmid DNA template mixtures with increasing frequencies of resistance alleles were sequenced. The resistance nucleotide signal intensity increased as the resistance allele frequency increased in the template DNA mixture (Fig. 8). The nucleotide signal ratios and the corresponding resistance allele frequencies at the mutation site (L925) were plotted and fitted to a quadratic equation (Fig. 9). Both of the two resulting regression lines showed high correlation coefficients (R^2 = 0.9984 and 0.9928), demonstrating that the nucleotide signal ratio is highly proportional to the resistance allele frequency.

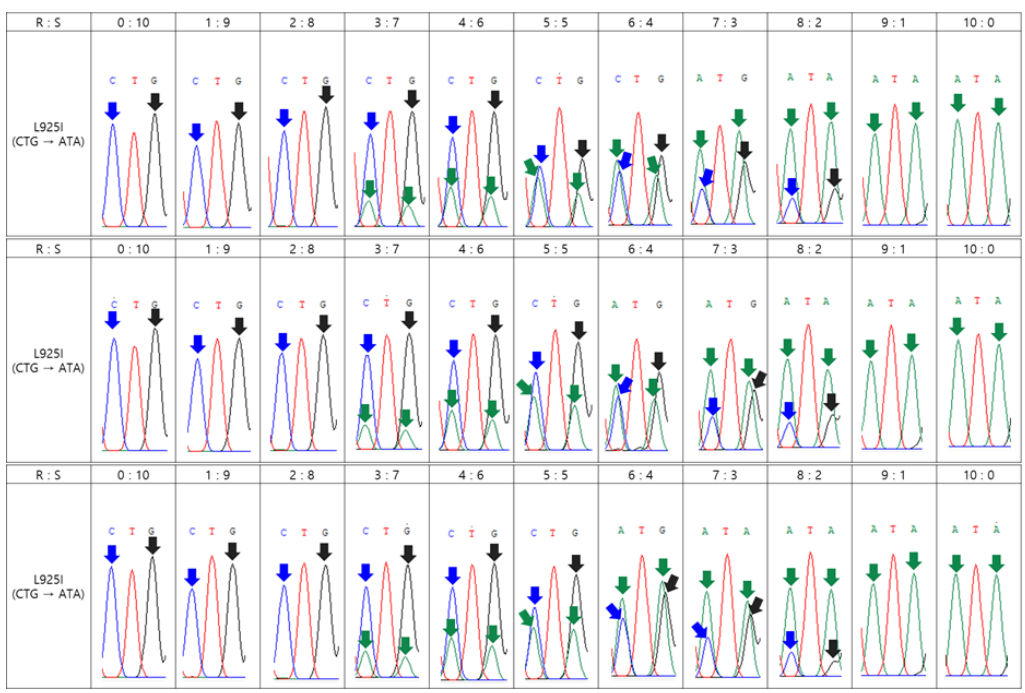

Fig. 8. Sequencing chromatogram of the standard template DNA mixtures with different ratio of resistant and susceptible alleles at the L925 mutation site.

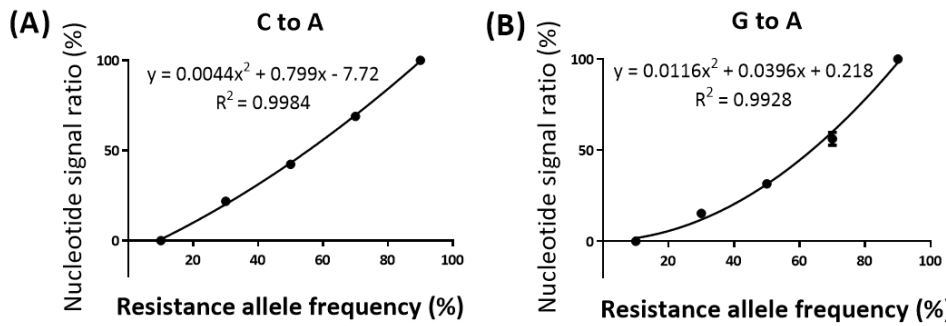

Fig. 9. Generation of regression equations for frequency prediction of the L925M and L925I resistance alleles. Resistance nucleotide signal ratios obtained from sequencing were plotted against corresponding resistance allele frequencies at the L925M (CTG → ATG) and L925I (CTG → ATA) mutation site.

3.2. Prediction of fluvalinate resistance allele frequency in regional populations of *Varroa* mites in Korea using QS

In 2021, molecular marker-based resistance monitoring was conducted for 17 *Varroa* mite populations. Only the L925I and L925M mutations were detected, whereas the L925V mutation was not detected in Korea. The L925I mutation was predominantly found, but the L925M mutation was only detected at a frequency of less than 5%. Fluvalinate resistance mutations were not detected in 13 populations. Three populations (2021-PC, 2021-IS) showed 42.8% and 29.1% of fluvalinate resistance mutations (L925I and L925M), and 2021-AS showed 44.5% of fluvalinate resistance mutation (L925I) frequency, respectively. Only the 2021-UR population showed 100% frequency of L925I mutation (Table 5.).

In 2022, molecular marker-based resistance monitoring was conducted for 90 *Varroa* mite populations. As in 2021, the L925I mutation was predominantly detected, whereas the L925V mutation was not found in Korea. The L925M mutation was observed in some regions (2022-GM1, 2022P-HN1, and 2022P-HN2) at frequencies higher than 20%. In particular, the 2022P-HN2 population possessed only the L925M mutation at 30.5% frequency (Table 6). Contrast to 2021, however, either the L925I or L925M mutation was detected in significantly more regions (84 out of 90 sites examined) in 2022, indicating that the fluvalinate resistance allele had spread very rapidly during one year.

Gyeongsangnam-do (100%) showed the highest average frequency of L925I/M mutation, followed by Gyeongsangbuk-do (87.7%), Chungcheongnam-do (86.1%), Chungcheongbuk-do (76.4%), Jeollanam-do (737%), Gyeonggi-do (72.2%), Jeju-do (66.7%), Gangwon-do (61.2%), and Jeollabuk-do (50.9%). The frequencies of

fluvalinate resistance mutations (L925I/M) were over than 75% in most *Varroa* mite populations from Gyeonggi-do (100% in three regions and 77.3~81.4% in three regions), Gyeongsangbuk-do (100% in 17 regions and 76.4% in one region), Chungcheongbuk-do (100% in 3 regions and 73.6~77.7% in three regions) and Chungcheongnam-do (100% in eight regions) (Table 6 and Fig. 10). In particular, the *Varroa* mite populations from Gyeongsangnam-do showed 100% mutation frequency in every region examined. These results showed that the fluvalinate resistance mutations (L925I/M) are widespread in Korea.

When the mortality data from RCV bioassay were plotted against the fluvalinate resistance mutation frequency data, little correlation was observed (Fig. 11). This finding indicates that the 24-h mortality data obtained by exposure to a single diagnostic dose of fluvalinate is not sensitive enough to be represented by the L925I/M mutation frequency data, further demanding the adjustment of RCV bioassay protocol to be more sensitive.

Table 6. Fluvalinate resistance mutation (L925M/I) frequency obtained from *Varroa* mites collected in the Korea (2021). Gray region is the location where the fluvalinate resistance allele frequency exceeds 75%.

Num.	Location	Population	L925M frequency (%)	L925I frequency (%)	Fluvalinate resistance mutation frequency (%)
1	Gangwon-do	2021-PC	3.1	39.7	42.8
2		2021-SC	0	0	0
3	Gyeonggi-do	2021-SO	0	0	0
4		2021-HS	0	0	0
5		2021-YI	0	0	0
6		2021-IS	4.9	24.2	29.1
7		2021-PJ	0	0	0
8	Gyeongsangbuk-do	2021-SJ1	0	0	0
9		2021-AD	0	0	0
10		2021-DG	0	0	0
11		2021-CD	0	0	0
12	Gyeongsangnam-do	2021-UR	0	100	100
13	Chungcheongbuk-do	2021-ES	0	0	0
14		2021-CJ	0	0	0
15	Chungcheongnam-do	2021-AS	0	44.5	44.5
16	Jeollabuk-do	2021-DJ	0	0	0
17	Jeollanam-do	2021-DY	0	0	0

Table 7. Fluvalinate resistance mutation (L925M/I) frequency obtained from *Varroa* mites collected in 2022. Gray area is the location where the fluvalinate resistance allele frequency exceeds 75%.

Num.	Location	Population	L925M frequency (%)	L925I frequency (%)	Fluvalinate resistance mutation frequency (%)
1	Gangwon-do	2022P-SC	9.7	31.9	48.8
2		2022P-JuS	0	77.7	77.7
3		2022P-HC	0	100	100
4		2022-WJ1	0	100	100
5		2022-WJ2	0	0	0
6		2022-WJ3	0	0	0
7		2022-HS	0	62.9	62.9
8		2022-SC2	0	100	100
9	Gyeonggi-do	2022P-NY	0	100	100
10		2022P-SO	0	100	100
11		2022P-SW	1.5	69.5	71
12		2022P-AnS	4.2	50	54.2
13		2022P-IC1	0	100	100
14		2022P-IC2	0	72.7	72.7
15		2022-SO1	0	59.7	59.7
16		2022-SO2	0	0	0
17		2022-GwJ1	0	77.3	77.3
18		2022-GwJ2	0	81.4	81.4
19		2022-YP2	0	78.1	78.1
20	Gyeongsangbuk-do	2022P-GM	0	100	100
21		2022P-GC1	0	100	100
22		2022P-GC2	0	100	100
23		2022P-DG	0	100	100
24		2022P-SaJ	0	100	100
25		2022P-SeJ1	0	100	100
26		2022P-SeJ2	0	100	100
27		2022P-YJ1	0	100	100
28		2022P-YJ2	0	100	100
29		2022P-YJ3	0	100	100
30		2022P-UJ1	10	44.8	54.8
31		2022P-UJ2	0	100	100

32		2022P-UJ3	0	100	100
33		2022P-CD	5	68.6	73.6
34		2022P-CS	0	100	100
35		2022P-CG	0	100	100
36		2022-GW	0	45.9	45.9
37		2022-YC	0	48	48.0
38		2022-AD	13.8	51.3	65.1
39		2022-UJ1	0	100	100
40		2022-UJ2	0	100	100
41		2022-UJ3	13.1	26.7	39.8
42		2022-GM1	20.8	79.2	100
43		2022-GM2	0	76.4	76.4
44	Gyeongsangnam-do	2022P-NH1	0	100	100
45		2022P-NH2	0	100	100
46		2022P-MY	0	100	100
47		2022P-US	0	100	100
48		2022P-CW1	0	100	100
49		2022P-CW2	0	100	100
50		2022P-HA	0	100	100
51		2022P-HC	0	100	100
52		2022-HA	0	100	100
53	Chungcheongbuk-do	2022P-CJ	0.5	32.6	33.1
54		2022P-BE	0	75.3	75.3
55		2022P-ES	0	100	100
56		2022-BE	0.6	50.8	51.4
57		2022-ES	0	77.7	77.7
58		2022-CB	0	100	100
59		2022-OC1	0	73.6	73.6
60		2022-OC3	0	100	100
61	Chungcheongnam-do	2022P-GR	0	100	100
62		2022P-GS	0	100	100
63		2022P-DJ1	0	100	100
64		2022P-DJ2	0.4	34.4	34.8
65		2022P-DJ3	0	100	100
66		2022P-SJ	0	100	100
67		2022P-AS1	0	65.8	65.8

68		2022P-AS2	1.1	45.3	46.4
69		2022P-YS	0	100	100
70		2022-CA	0	100	100
71		2022-GJ	0	100	100
72		2022P-JaS	0	100	100
73		2022P-JJ	0	100	100
74	Jeollabuk-do	2022-GC	13.4	41.3	54.7
75		2022-GiJ	0	0	0
76		2022-NW	0	0	0
77		2022P-GJ	3.3	36	39.3
78		2022P-HS	0	100	100
79		2022P-HN1	21.7	45.5	67.2
80		2022P-HN2	30.5	0	30.5
81		2022-GH1	0	100	100
82	Jeollanam-do	2022-GH2	0	100	100
83		2022-SuC	0	100	100
84		2022-BS	17.6	54.6	72.2
85		2022-JH1	0	100	100
86		2022-JH2	15.2	45.3	60.5
87		2022-GaJ	4.9	31.6	36.5
88		2022-JJ1	0	0	0
89	Jeju-do	2022-JJ3	0	100	100
90		2022-JJ5	0	100	100

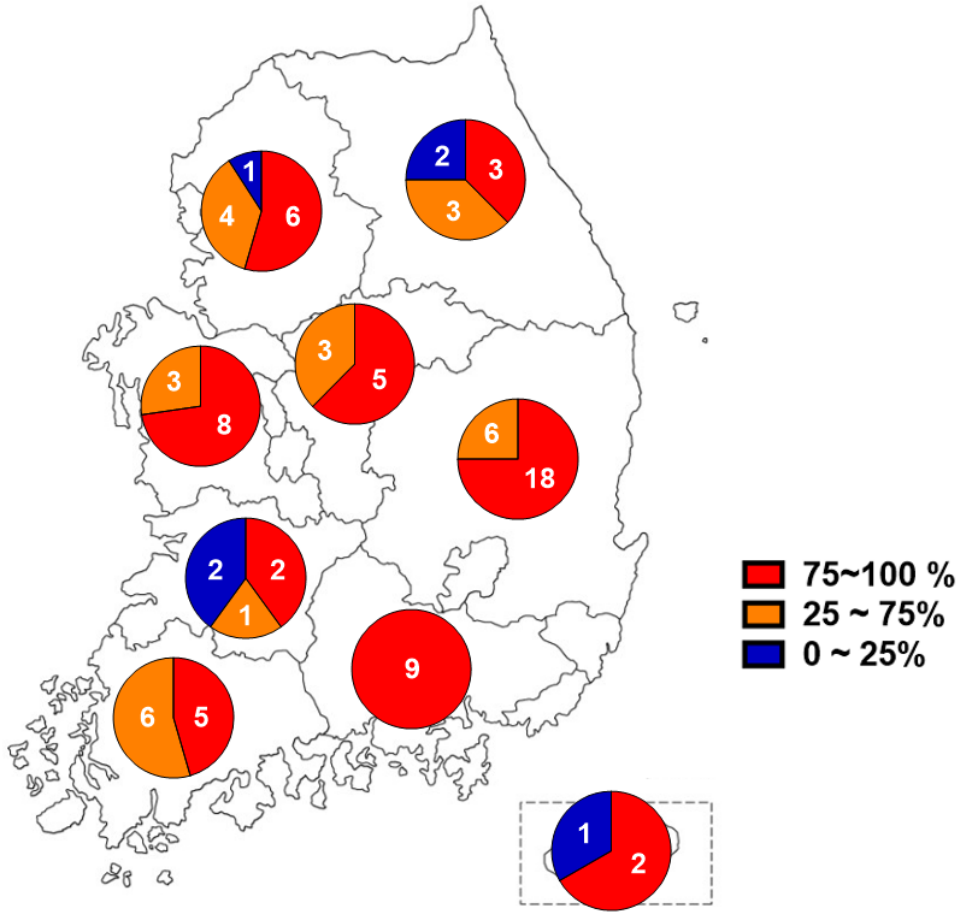

Fig. 10. A pie graph of the fluvalinate resistance mutation frequency on the map. Blue, orange, and red colors in the pie graphs represent the locations where the fluvalinate resistance allele frequency is less than 25%, between 25% and 75%, and higher than 75%, respectively. Numbers in the pie graphs are the number of mite populations investigated.

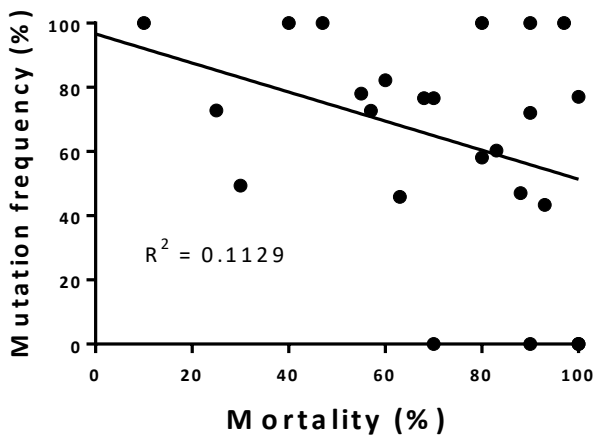

Fig. 11. Correlation between the 24-h mortality data obtained from RCV bioassay and the fluvalinate resistance mutation frequency data.

3.2. Genotype analysis for the mites with differential knockdown response

To investigate the time-dependent knockdown response, *Varroa* mites were treated with three different doses of fluvalinate via RCV method and their knockdown rate was monitored at a series of time points. The overall time-dependent knockdown pattern was biphasic: The number of knockdown mites rapidly increased until 4 h, reached plateaus around 5-6 h, and slowly increased again after 7 h until 24 h post-treatment in all the fluvalinate doses tested (Fig. 12).

When the fluvalinate resistance genotypes were analyzed for three groups of mites treated with 35 ppm fluvalinate (mites knocked down before 7 h post-treatment; mites knocked down between 12 h and 24 h post-treatment; and mites survived after 24 h post treatment), majority of early knockdown group possessed homozygous susceptible (SS, 63.6%) and heterozygous (SR, 31.9%) genotypes although a small portion (4.5%) of mites carried resistant homozygous (RR) genotype (Fig. 13). The

12-24 h knockdown group carried 38.5% SS, 15.4% SR, and 46.2% RR genotypes, whereas the 24 h survived group carried only RR genotypes. This finding revealed that mites knocked down early (<7 h) are mostly susceptible, and thus the <7 h post-treatment time is suitable for distinguishing susceptible individuals (SS and SR) from resistant individuals (RR) when 35 ppm fluvalinate was treated via RCV. Since a large portion of RR genotypes were observed in the 12-24 h knockdown group, the 12-24 h post-treatment time was determined as an inappropriate time point for the evaluation of knockdown or mortality in the 35 ppm fluvalinate-treated RCV.

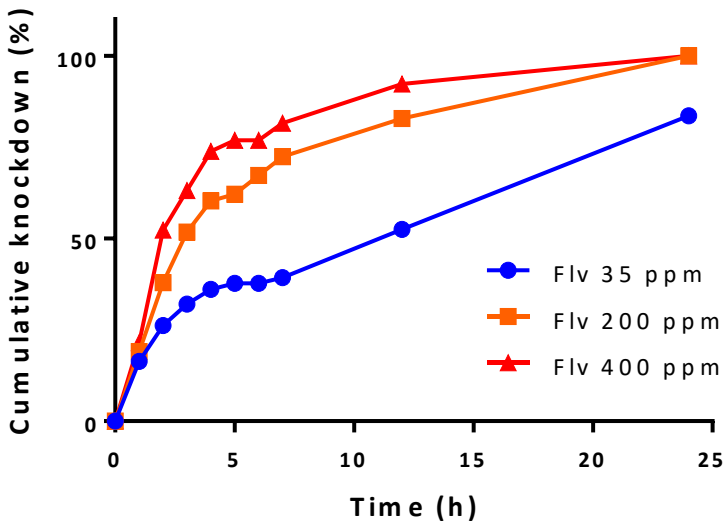

Fig. 12. Cumulative knockdown ratio of *Varroa* mites following 35, 200, and 400 ppm fluvalinate treatment using RCV method. The observation time points were 1, 2, 3, 4, 5, 6, 7, 12, and 24 h.

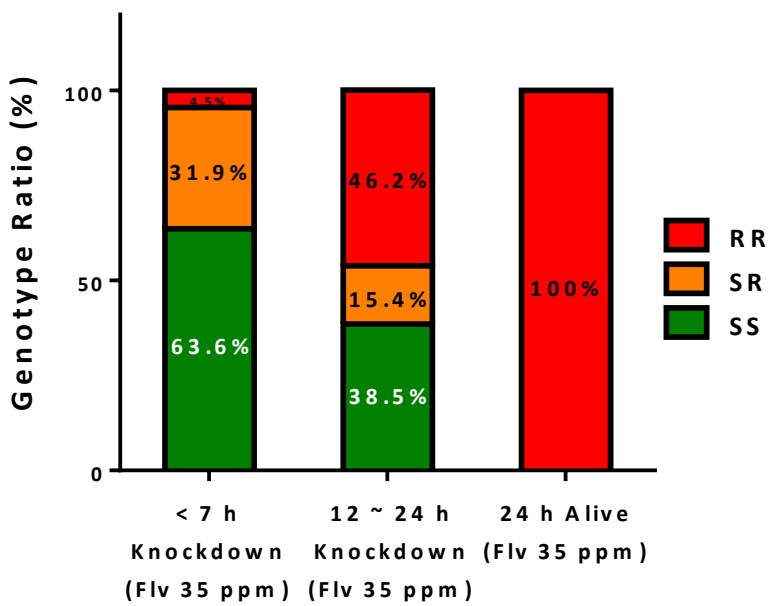

Fig. 13. Genotype ratio between each group. SS type is homozygous susceptible, SR type is heterozygous, and RR type is homozygous resistant.

4. Discussion

The QS protocol-based resistance monitoring method was developed and used for the monitoring of *Varroa* mite fluvalinate resistance in this study. The QS protocol is a population genotyping method and has been used as a resistance monitoring tool for several insect pests (Kwon et al., 2008; Seong et al., 2010). The DNA pooling methods is an effective way of reducing the cost and time of genotyping when analyzing large amounts of samples (Sham et al., 2002). For this reason, the QS-based resistance monitoring method is suitable for resistance monitoring of *Varroa* mite resistance for the specimens collected from multiple geographical regions. As demonstrated in this study, rapid population genotypings of more than 107 regional mite specimens were possible within a relatively short time with a low cost ($5 per one regional specimen). If assuming that the target site insensitivity mediated by the L929I/M mutations is a primary resistance mechanism in *Varroa* mites, the QS-based estimation of mutation frequency should facilitate the rapid monitoring of actual fluvalinate resistance status in a large-scale survey. If other resistance factors, such as cytochrome P450 (P450)-mediated metabolism, are involved in fluvalinate resistance, the caution should be excised in the fact that the actual level of resistance may not be fully represented by the resistance allele frequency data.

In Korea, the fluvalinate resistance mutations were not detected in the survey conducted in 2009 (Kim et al., 2009), however, 23.5% of regions examined (four out of 17 regions) showed to carry the resistance mutation. Moreover, the 2022 survey revealed that the resistance mutations were found in almost all the region examined (93.3%, 84 out of 90 regions). Such a rapid spread of fluvalinate resistance allele,

particularly during one year, to many regions in Korea can be attributed to several factors. Firstly, fluvalinate has been the most widely used varroacide during past several decades in Korea, thus posing a high selection pressure and accelerating resistance development. Secondly, the conventional beekeeping in Korea is migratory from the southern to northern regions by chasing the blooming season of acacia, thereby likely facilitating the spread of resistant mite populations in-between apiaries. Thirdly, the rapid spread of resistance trait can be also contributed by the forced brother-daughter mating system of *Varroa* mite inside the honey bee comb cells (Carrière, 2003; Conlon et al., 2018), in which resistant females always produce resistant offspring. Considering the rapid expansion of resistance mutation over many geographical regions in Korea, the resistance mediated by the L925I/M mutation appears to be stable. Therefore, if not managed properly, the fluvalinate resistance trait is likely saturated within a short period of time in Korea. To suppress further spread of resistance, constant monitoring of resistance allele frequency based on QS and timely introduction of alternative varroacides are very crucial (Cho et al., 2020; Kwon et al., 2004).

Fluvalinate resistance mutations have been detected in *Varroa* mite populations from many countries. Only the L925V mutation has been found in fluvalinate-resistant *Varroa* mite populations in Europe (UK, Czech Republic, Italy, Germany, and Spain) (Benito-Murcia et al., 2022; González-Cabrera et al., 2018; Gonzalez-Cabrera et al., 2013). In contrast, either the L925I or L925M mutation has been reported in fluvalinate-resistant mite populations in the US (González-Cabrera et al., 2016). Since the L925I mutation has been recently found with the L925M being a minor mutation, the resistance trait may have been driven from the US mite populations instead of being locally selected. Considering that most *Varroa* mite

populations over the world is the K1 haplogroup that was originated from Korea, it would be intriguing to trace the migration of genetic traits of *Varroa* mite populations between different geographical regions using molecular markers such as mitochondrial cytochrome oxidase subunit I and microsatellites (Traynor et al., 2020).

Despite the well-known function of L925I/M mutations in fluvalinate resistance, the L925I/M mutation frequency data obtained from QS did not match the 24-h mortality data from RCV bioassay. Given that the L925I/M mutations confer *kdr* trait against fluvalinate, a fast-acting pyrethroid, at an early stage of intoxication, the mortality evaluation at 24 h post-treatment in RCV bioassay appears to be too late to distinguish susceptible vs. resistant individuals. As demonstrated in the biphasic time-dependent knockdown response in conjunction with genotyping, most of susceptible mites (SS and SR) were knocked down within 7 h post-treatment (the first knockdown phase), whereas even resistant mites showed knockdown response when the evaluation time increased to 12-24 h post-treatment (the second knockdown phase). In the RCV treated with 35 ppm fluvalinate, the *kdr* trait mediated by the L925I/M mutations appears to function at the first knockdown phase (<7 h post-treatment), whereas other detoxification/resistance factor is likely involved in the second knockdown phase (12-24 h post-treatment). This finding further indicates that the 24-h mortality evaluation protocol in RCV cannot efficiently distinguish phenotypically susceptible mites from resistant mites, thus impairing the resolution of bioassay in terms of resistance detection. Based on current findings, the optimum time point for mortality (or knockdown) evaluation appears to be around 7 h post-treatment in the 35 ppm fluvalinate-treated RCV protocol. Since the time-dependent intoxication response is also affected by test dose,

the optimum evaluation time would change depending on the test dose used for RCV. Nevertheless, since the knockdown responses at the first phase were similar each other within a large range of test doses (35, 200, and 400 ppm), the 7-h mortality evaluation time appears to be robust at least within the range of 35-400 ppm fluvalinate.

It is also worth to note that a large portion (53.8%) of mites knocked down within 12-24 h post-treatment was either susceptible or homozygous at the L925I/M genotype although they are phenotypically more resistant than the mites knocked down within 7 h post-treatment. This finding may suggest that the mites with susceptible or homozygous genotypes likely possess other resistance traits including other VGSC mutations or metabolic resistance factors yet unknown. Discovery of novel VGSC mutations and elucidation of P450-mediated resistance factor would be beneficial for complete understanding of fluvalinate resistance mechanism in *Varroa* mites and for establishing more elaborated resistance monitoring system.

Chapter 3. Searching for novel target site mutations and metabolic factors for fluvalinate resistance

Abstract

In order to identify other fluvalinate resistance factors, I searched novel mutations in the near entire domains of the voltage-gated sodium channel gene. As a result, ten novel mutations were identified, among which two appeared to have some potential function as a target site insensitivity factor. In addition, transcription levels of cytochrome P450 monooxygenase (P450s) as metabolic factors were investigated to determine their possible association with resistance. To this end, searching for reliable reference genes for quantitative PCR was attempted first. The *eEF1A1* and *NADHD* were recommended as reference genes for the comparison of the effects of acaricide on the whole body. Despite the lack of apparent correlation between their expression level and the mortality obtained from RCV bioassay, two P450s (Flv-2B4 and Flv-3A19) showed a tendency of decrease in mortality as the P450 expression level increased, thus implying their possible role in fluvalinate detoxification and their potential as novel candidate markers for resistance de

1. Introduction

Pyrethroid acaricides such as fluvalinate and flumethrin prevent the closure of voltage-gated sodium channel (VGSC), which is essential for the initiation and transmission of the electrical signal in neurons. With their selective toxicity to *Varroa* mites and convenient usage as a strip, fluvalinate and flumethrin have been extensively used for in-hive treatment worldwide for past several decades (Elzen et al., 1998; Shimanuki et al., 1980), leading to resistance development against them (Elzen & Westervelt, 2002; Gracia-Salinas et al., 2006; Maggi et al., 2009; Mozes-Koch et al., 2000; Spreafico et al., 2001). Three single nucleotide polymorphisms (SNP) at a single amino acid position (L925V/M/I) have been identified to be responsible for fluvalinate resistance (Gonzalez-Cabrera et al., 2013; González-Cabrera et al., 2016). The site of these mutations in the VGSC domain II S5 is close to the major binding site of pyrethroid (O'Reilly et al., 2006), thus affecting the affinity of pyrethroids to the channel.

In the fluvalinate RCV bioassay using the LC_{50} diagnostic concentration conducted in 2022 (Chapter I), the average mortality was around 70% compared to the results of 2021, suggesting that the putative susceptible 2021-SO strain is rather more resistant compared to a large number of regional mite populations perhaps due to the presence of other resistance factors. The lack of correlation between the mortality from RCV bioassay and the fluvalinate resistance mutation frequency (Chapter II) suggested that the 24-h mortality data is not sensitive enough to be represented by the L925I/M mutation frequency data, thus resulting in the adjustment of the mortality observation time in RCV to less than 7 h. In addition, some mites that appear as phenotypically resistant in the RCV bioassay were

determined to possess either the susceptible homozygous or heterozygous genotype at the L925I/M mutation site. Taken together, these findings suggest that *Varroa* mites likely have additional resistance traits, including the target site insensitivity mediated by VGSC mutations yet unknown other than the L925I/M/V mutations and/or metabolic resistance factors likely mediated by cytochrome P450 monooxygenases (P450s).

Knockdown resistance (*kdr*) mediated by the reduced sensitivity of VGSC is one of the major pyrethroid resistance mechanisms (Soderlund & Bloomquist, 1990). Various VGSC mutations responsible for the *kdr* or *kdr*-like trait have been identified in several arthropod species, and most of them are located at the transmembrane segments in the homology domain IIS4-IIS6, followed by the homology domains I and III (Du et al., 2016; González-Cabrera et al., 2016; Haddi et al., 2012; Tsagkarakou et al., 2009). Some mutations were confirmed to reduce the pyrethroid sensitivity of VGSC via the *Xenopus* oocyte expression in conjunction with electrophysiological recording (Dong et al., 2014). These mutations have been used as molecular markers in molecular detection of pyrethroid resistance in filed populations (Knipple et al., 1994; Williamson et al., 1993). In *Varroa* mites, although only the two mutations (L925I/M) have been reported in Korea, additional mutations responsible for fluvalinate resistance can be present as suggested earlier.

The P450s play an important role in detoxification and bioactivatin of xenobiotics. P450s increase enzymatic detoxification and, unlike target site resistance, have the potential to confer cross-resistance to other insecticide groups with different mode of actions as far as they share common metabolophores. For this reason, P450s are one of the main metabolic factors conferring pesticide resistance. The metabolic resistance against pesticides can be achieved by either up or

downregulation of P450 expressions. Several insect developed resistance to insecticide by upregulation of P450 (Komagata et al., 2010; Wondji et al., 2009; Yang et al., 2006), and *Varroa* mites developed resistance to coumaphos by downregulation of some P450s involved in the bioactivation of coumaphos (Vlogiannitis et al., 2021). However, no P450-mediated resistance mechanism has been reported to be associated with fluvalinate resistance in *Varroa* mites so far. Nevertheless, considering that there are a total of 26 P450 genes in the *Varroa* mite genome, out of which 7 belong to the Clan 3 P450 that are mostly involved in detoxification of xenobiotics, *Varroa* mites have sufficient biological potential to develop the P450-mediated resistance to fluvalinate.

In this study, almost entire regions of the *Varroa* mite VGSC gene in various regional populations of mites were scanned to identify any novel mutations potentially associated with fluvalinate resistance. In addition, transcription profiles of several representative *Varroa* mite P450 genes were analyzed for some regional populations of mites, and their transcription levels were compared with corresponding mortality data to determine their potential as a detoxification factor.

2. Materials and methods

2.1. Identification of novel voltage-gated sodium channel mutations

Several regional populations of *Varroa* mite showing distinct properties in the mortality response and mutation frequency (>80% mortality and >80% mutation frequency, <50% mortality and 0% mutation frequency) (2021-UR, 2022-SO, 2022-WJ1, 2022-WJ2, 2022-WJ3, 2022-HS, 2022-SC2, 2022-JJ3, and 2022-JJ5) were selected and used for the analysis of near-full length sequence of VGSC. PCR was conducted using the cDNA samples previously prepared (Chapter II) as template, primer sets (Table 7.) and Ex Taq (Takara Korea Biomedical Inc., Seoul, Korea). The thermal program was composed of 35 cycles of 95 °C/30 sec, 61 °C/30 sec, and 72°C/ 1 min 30 sec. The PCR products were purified using Expin PCR SV (Geneall Biotechnology, Seoul, Korea) and sequenced by primer walking with a series of primers (Table 7.). The resulting sequences were aligned with those of reference *Varroa* mite VGSC (LOC555743) and house fly Musca domestica VGSC (LOC101898332) in NCBI to identify any novel mutations.

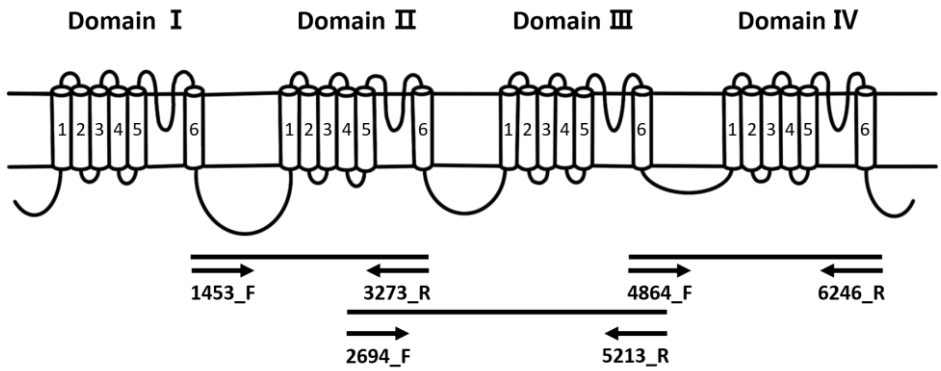

Fig. 14. Voltage-gated sodium channel was divided into three sections and sequence analysis was performed. Primer information for amplification of sodium channel is shown in Table 7.

Table 8. Information of the primer sets used for PCR.

Primer name	Purpose	Primer Sequence (5'-3')	Tm (ºC)	PCR Produce size (bp)
Vd_SC_1453_F	PCR Sequencing	CGATTGGAGGAAGAAGCTCG	60.5	1821
Vd_SC_3273_R		CAGGTTGCAATAACGACGG	60.5	
Vd_SC_2694_F	PCR Sequencing	CATGGACCATCACGACATGG	60.5	2520
Vd_SC_5213_R		ATTTCCAGGGATCCTCCAGC	60.5	
Vd_SC_3981_F	Sequencing	TCAACACGAGAACAACCCCC	60.5	-
Vd_SC_4202_R		TCCTTGTCTTCTRTCAGCCCC	60.5	
Vd_SC_4864_F	PCR Sequencing	GTAGACGCCAACAACTCCCA	60.5	1383
Vd_SC_6246_R		GGGTTTTGGTATCTGCAGCG	60.5	

2.2. Acaricide treatment

All treatments and bioassays were performed using the residual contact vial (RCV) method. Prior to acaricide treatment, a preliminary bioassay was conducted to determine the treatment concentration of each acaricide (24 h LC_{10}). Coumaphos, tau-fluvalinate, and amitraz were purchased from Merck (Darmstadt, Germany) and dissolved in 100% acetone to obtain 10,000 mg/L stock. For the preliminary bioassay, acaricide stocks were serially diluted with 100% acetone (Table 1). RCV were prepared as described in chapter I. And, bioassay conducted as described in chapter I.

2.3. Putative reference gene selection from transcriptome data

Eight candidate genes for reference genes were selected from the transcriptome data of *Varroa* mites. RNA was extracted from the whole bodies of 8 h acaricide-treated *Varroa* mites using the TRI reagent (MRC, Cincinnati, OH, USA). Twelve transcriptomes and 20 *Varroa* mites per group (control and coumaphos-, fluvalinate-, and amitraz-treated groups) with three biological replicates were analyzed. Transcriptome analysis was performed following the protocol of Kim et al. (2022) with *V. destructor* Vdes_3.0, as a reference genome (Techer et al., 2019). Gene expression was normalized, and fold changes were calculated between the control and each group. The genes, whose fold changes (FC) were in the range of 0.8–1.2 for all treatments (coumaphos, fluvalinate, and amitraz relative to control), were listed in a descending order of the base mean (Supplementary File). In the list of 120 genes, seven housekeeping genes (GAPDH, eEF1A1, eEF2, RpL5, Actin, α-tubulin, and Rab1) that have been used as reference genes in other organisms were selected

as putative reference genes for *Varroa* mites along with NADHD, which showed good performance in a previous study (Ewan M Campbell et al., 2016).

2.4. Primer design and qPCR

NADHD, *GAPDH*, *eEF1A1*, *eEF2*, *RpL5*, *Actin*, *α-tubulin*, and *Rab1* were selected as the reference genes. The mRNA sequences of the eight putative reference genes were retrieved from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Primers were designed from the coding sequences of the genes using Primer3Plus (Untergasser et al., 2007), except NADHD, whose primer sequences were obtained from E. M. Campbell et al. (2016). The melting temperature of all primers was set to 60.5 ºC, and the amplicon sizes of each primer set were in the range of 72–145 bp. Primer information for the reference and target genes is shown in Table 8. RNA was extracted from whole bodies of *Varroa* mites treated with each acaricide (8 and 24 h exposure/three replications). The protocols for RNA extraction and cDNA synthesis were the same as described in chapter II. For qPCR primer efficiency evaluation, cDNA samples were serially diluted (1, 0.2, 0.04, 0.008, and 0.0016 ng/μL) and used as templates for each reference gene test. qPCR reactions were conducted using the SYBR Premix Ex Taq (Takara Korea Biomedical Inc.) in a final volume of 10 μL containing 5 μL of SYBR Premix Ex Taq, 3.2 μL of cDNA (3.2, 0.64, 0.128, 0.0256, and 0.00512 ng), and 1.8 μL of 5 μM forward and reverse primer mix. The cycling process consisted of an initial polymerase activation step at 95 °C for 30 s, followed by 35 cycles of 95 °C for 30 sec, 56 °C for 15 sec and 72 °C for 30 sec with one cycle of melting curve analysis (95 °C for 5 sec, 60 °C for 60 sec and 95 °C for 1 sec). The PCR products were

analyzed by 1.5% agarose gel electrophoresis to confirm the amplification specificities. When conducting qPCR for the evaluation of reference gene stabilities, 16 ng of cDNA was used in a 10 μL total reaction under the same conditions described above. qPCR efficiency and R^2 values were calculated from the standard curve of the qPCR results using a qPCR efficiency calculator (Thermo Fisher Scientific, Massachusetts, USA).

2.5. Ranking the stabilities of the reference genes

Transcription levels of the reference genes in acaricide-treated *Varroa* mites were divided into four pairwise groups (control-coumaphos, control-fluvalinate, control-amitraz, and pooling of the four groups) to compare the effects of individual acaricide treatments on the reference gene expression levels. Four reference gene validation programs, BestKeeper, geNorm, NormFinder, and RefFinder, were used to digitize the stabilities of the eight putative reference genes. Raw Cq values of the reference genes were used as input for BestKeeper and RefFinder, whereas the log-transformed Cq values, the relative transcription levels, were used for geNorm and NormFinder for the calibration. Reference genes were ranked based on the standard deviation values in BestKeeper, the expression stability values in geNorm and NormFinder, and the comprehensive gene stability values in RefFinder. The stability ranks were averaged within each program to obtain the average stability ranks. The number of reference genes for proper normalization was estimated from the pairwise variations obtained from geNorm. The pairwise variation ($V_n/_{n+1}$) was estimated between the normalization factors, NF_n and NF_{n+1}, and the cut-off value was set to 0.15 (Vandesompele et al., 2002).

2.6. Establishment of regression equations for the prediction of resistance allele frequency

Quantitative PCR (qPCR) were conducted to measure transcription levels of P450 genes in cDNA extracted from the *Varroa* mite regional samples in Chapter II. The test P450 genes were selected from candidate P450s that appear involved in metabolism of xenobiotics in *Varroa* mites: 4V2 and 3012A6 P450s from the Clan 3, Clan 4, and Clan 2, respectively. Final PCR volume was 10 μL, which contained 5 μL of SYBR Premix Ex Taq (Takara Korea Biomedical Inc), 3.2 μL of cDNA diluted 5 ng and 1.8 μL of 5 μM forward and reverse primer mix (Table 2.). The cycling process consisted of an initial polymerase activation step at 95 °C for 30 s, followed by 35 cycles of 95 °C for 30 sec, 56 °C for 15 sec, and 72 °C for 30 sec with one cycle of melting curve analysis (95 °C for 5 sec, 60 °C for 60 sec, and 95 °C for 1 sec). *eEF1A1* and *NADHD* were used as reference gene. Correlation of mortality data with transcription levels of P450 genes normalized by reference genes and graph design were conducted using Prism 6.0 (GraphPad, San Diego, CA, USA).

Table 9. Information of the primer sets used for quantitative real-time PCR (qPCR).

Primer name	Gene ID	Primer Sequence (5'-3')	Tm (°C)	PCR Produce size (bp)
Vd_cyp_2B4_F	111247968	TTATCAGCGCACACCGATCC	60.5	94
Vd_cyp_2B4_R		TGTCTTTGCGAAAGGTGCCG	60.5	
Vd_cyp_2H2_F	111251940	CCAATGCCGTCATCCCTTTC	60.5	107
Vd_cyp_2H2_R		CCGAAATCGCTGCAGAATGG	60.5	
Vd_cyp_2J6_F	111244479	TACCGCATTCCCAAGGGATC	60.5	103
Vd_cyp_2J6_R		GAAAGCGTTCGGGACGGTAT	60.5	
Vd_cyp_4c1_F	111252664	TATCGCTTACCAGCTGGAGG	60.5	177
Vd_cyp_4c1_R		ACCTATGCAGTTCCTTGGCC	60.5	
Vd_cyp_4V2_F	111248731	GAAGGCAGAGCATTTGACCG	60.5	101
Vd_cyp_4V2_R		CCCACACTCCGATCTAGAAG	60.5	
Vd_cyp_3A13_F	111244198	ATTGCTAACGCCTGCTGCCT	60.5	98
Vd_cyp_3A13_R		GTGACGTCTTCAGGTCCACT	60.5	
Vd_cyp_3A14_F	111246682	AATCCCCACGAATTCCGACC	60.5	100
Vd_cyp_3A14_R		TCATTCCGAGACAGTTGCGG	60.5	
Vd_cyp_3A19_F	111251253	TTCGGTTCACACTTTGCGCC	60.5	102
Vd_cyp_3A19_R		TTAGTCACCAACGACGGACG	60.5	
Vd_cyp_3A56_F	111247963	TGGCGGCATTGAAACAAGCG	60.5	98
Vd_cyp_3A56_R		GGTTTACGACGAGGTGAAGC	60.5	
Vd_cyp_307a1_F	111246028	CCAGAACTTTGGGACAAGCC	60.5	92
Vd_cyp_307a1_R		CCAAAGGGCAAGAAGTGGTC	60.5	
Vd_cyp_3012A6_F	111247963	ACAGAGTTGACCGAGGGATG	60.5	107
Vd_cyp_3012A6_R		AAGTACCGCCAGAACGGTAG	60.5	
Vd_cyp_503_R	111245503	AAGACAAAGCGCTCACTGGC	60.5	132
Vd_cyp_503_R		CGACACGAACGATGTTTCCC	60.5	
Vd_cyp_735_F	111243735	CACTTCGAATGTACCCACCC	60.5	128
Vd_cyp_735_R		GTCGTTGTGTAACTGGCAGG	60.5	

3. Results

3.1. Identification of novel mutations on VGSC

The PCR and sequencing of VGSC were conducted by dividing the domain II S1 to domain IV S6 of the *Varroa* mite VGSC into three regions. Total 10 amino polymorphisms (T912A, I9225M, F975L, L1375I, F417C, N1552S, K1556R/N, S1575N, D1610N, and M1614I) were identified when compared to the reference sequence of *Varroa* mite VGSC in NCBI. Three mutations (T912, I922M, F975L) were located nearby the domain II S5 forming the binding site of fluvalinate. Five mutations (N1552S, K1556R/N, S1575N, D1610N and M1614I) were located nearby the linker connecting the domain III and IV that contribute the movement of an inactivation gate. The F1528C substitution was identified same site where the F1528L substitution was found (Wang et al., 2002). The S1575N substitution was found at the same site of the previously known N1575Y substitution (Dong et al., 2014).

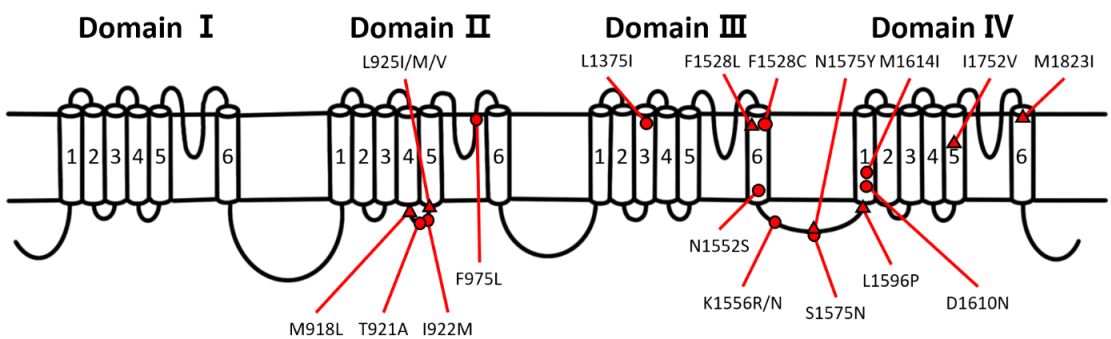

Fig. 15. Mutations in sodium channels. Circles are mutations discovered this study and triangles are previously discovered mutations.

3.2. LC_{10} values of the three acaricides

After the estimation of acaricide toxicity from the RCV bioassay data, fluvalinate was found to show the highest 24 h LC_{50} value (5.8 mg/L) followed by coumaphos (0.008 mg/L) and amitraz (0.0035 mg/L) (Table 9). The 24 h LC_{10} values were also calculated as sublethal concentrations (Table 9) and used as the single treatment concentration for each acaricide in the mite sample preparation for the gene stability studies.

Table 10. Probit mortality data of *Varroa* mites following treatment with coumaphos, fluvalinate, and amitraz. Residual contact vial method was used for the bioassay and 24 h mortality rate was observed. The 10% lethal concentration (LC_{10}) values represent the concentration of 100 µL acaricide-acetone solution used for coating the vial. The doses coated on the inner surface of vial (ng/cm^2) are indicated in parentheses.

Acaricide	Coumaphos	Fluvalinate	Amitraz
Tested range	$8x10^{-3}$ mg/L ($5.8x10^{-2}$ ng/cm^2) $4x10^{-2}$ mg/L ($2.9x10^{-1}$ ng/cm^2) $2x10^{-1}$ mg/L (1.4 ng/cm^2) 1 mg/L (7.2 ng/cm^2) 5 mg/L (36 ng/cm^2)	1.25 mg/L (9 ng/cm^2) 2.5 mg/L (18 ng/cm^2) 5 mg/L (36 ng/cm^2) 10 mg/L (72 ng/cm^2) 20 mg/L (144 ng/cm^2)	$3.2x10^{-4}$ mg/L ($2.3x10^{-3}$ ng/cm^2) $1.6x10^{-3}$ µg/L ($1.15x10^{-2}$ ng/cm^2) $8x10^{-3}$ mg/L ($5.8x10^{-2}$ ng/cm^2) $4x10^{-2}$ mg/L ($2.9x10^{-2}$ ng/cm^2) $2x10^{-1}$ mg/L (1.1 ng/cm^2)
Total number[a]	150	150	150
LC_{10} (mg/L)	0.008	2	0.0034
LC_{10} Cl 95%	lower 0.000 upper 0.036	lower 0.578 upper 3.389	lower 0.000247 upper 0.0091
LC_{50} (mg/L)	0.084	5.8	0.02
LC_{50} Cl 95%	lower 0.013 upper 0.34	lower 3.7 upper 9.4	lower 7.1 upper 59
Slope (± SE)	1.31 (± 0.17)	2.85 (± 0.39)	1.70 (± 0.23)

3.3. PCR efficiency and raw Cq value comparison

PCR efficiencies of the eight putative reference genes and two target genes were calculated (Table 10). The efficiencies of all genes were in the range of 95–110 % with $R^2 \geq 0.99$, indicating that all primer sets used for PCR amplification were acceptable. In addition, the melting curves of all the genes showed one clear peak (Fig. 17), and the agarose gel electrophoresis of PCR products showed clear single bands (Fig. 18). The average Cq values of all samples were highest for *eEF1A1* (15.0), followed by *Actin* (16.4), *α-tubulin* (16.9), *GAPDH* (17.1), *RpL5* (18.2), *eEF2* (18.5), *Rab1* (18.8), and *NADHD* (20.2) (Fig. 16). No significant differences were detected among the eight putative reference genes.

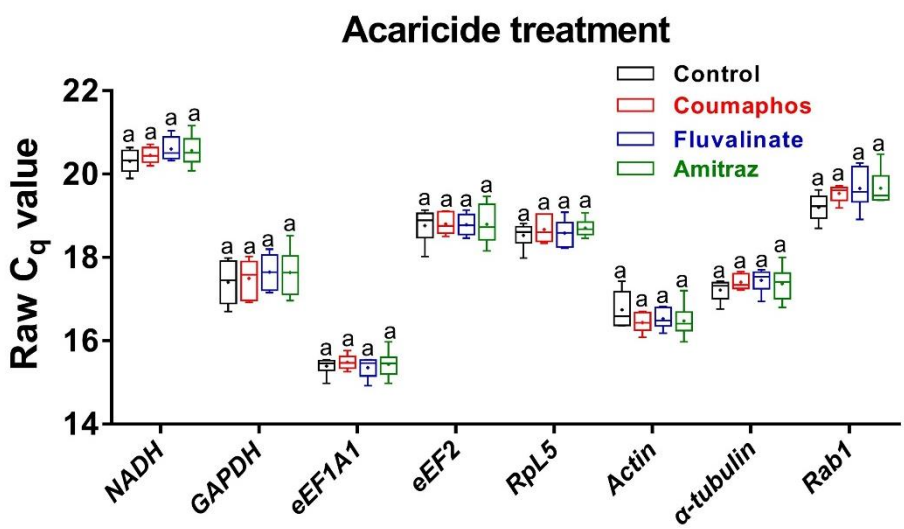

Fig. 16. Raw Cq value comparisons of the acaricide-treated groups. Box and whisker plots represent min to max with a cross (+) and a line which indicate average and median values, respectively. Significant differences ($p < .05$) calculated by one-way analysis of variance (ANOVA) and Tukey's post-hoc test within each reference gene is marked with different letters. Error bars represent the standard deviation.

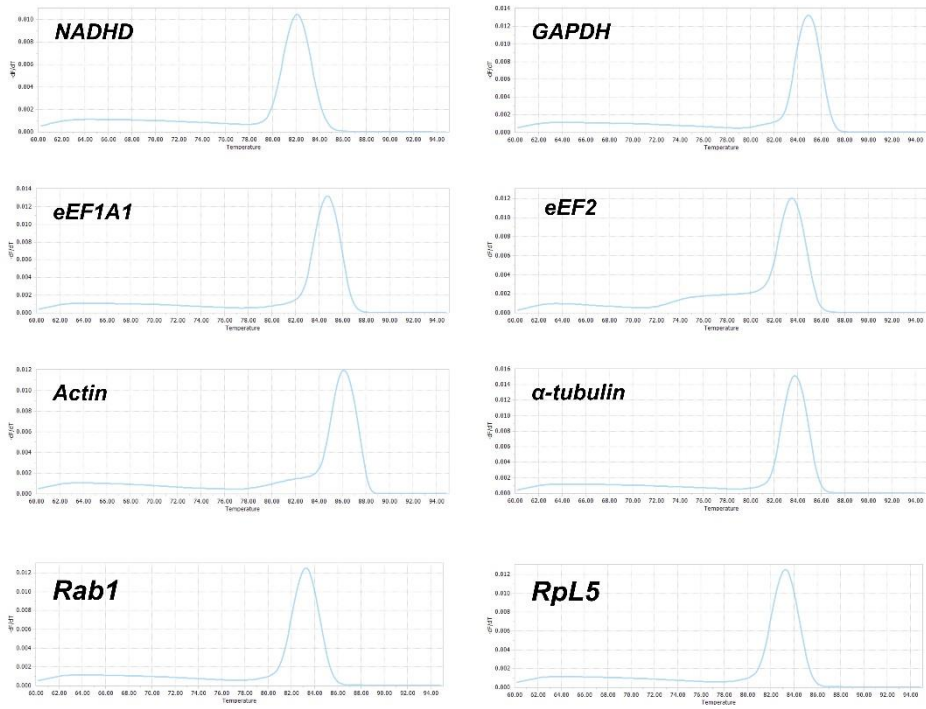

Fig. 17. Melting curves of the eight reference genes.

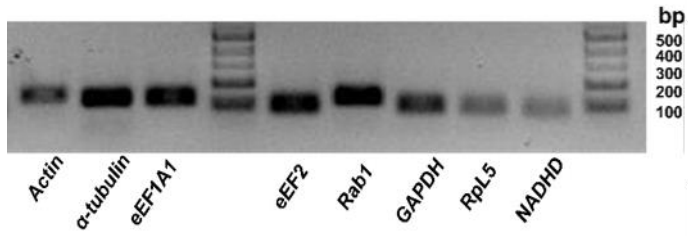

Fig. 18. Gel electrophoresis of the eight putative reference genes.

Table 10. Information of the primer sets used for the reference genes in quantitative real-time PCR (qPCR).

Symbol	Full name	Accession no.		Primer Sequence (5′–3′)	Tm (°C)	Amplicon size (bp)	qPCR efficiency (%)	R^2
NADHD	NADH dehydrogenase	LOC111249888	F	TCCGCTTAAGGAGCTTATCG	60.5	72	95.6%	0.9996
			R	ATCACGCACAGCAGGTTATC	60.5			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	LOC111247883	F	CGGACGTCTTGTCTTACGTG	60.5	128	96.2%	0.999
			R	CCTTGAAACGACCGTGAGTC	60.5			
eEF1A1	Eukaryotic translation elongation factor 1 alpha 1	LOC111248738	F	CGAGGCCTTCACAGAGTTTC	60.5	124	97.6%	0.9998
			R	CTTTGTCACCTTCCCACCAG	60.5			
eEF2	Eukaryotic translation elongation factor 2	LOC111251454	F	GTCTTAAGCGTCTCGCCAAG	60.5	88	97.3%	0.9999
			R	AGTTCACCAGCTCCAGCAAC	60.5			
RpL5	Ribosomal protein L5-A	LOC111244403	F	CTACGCTCGTAAGCGTTTGG	60.5	91	95.6%	0.9999
			R	GATATCGCGGTTGGTCACAC	60.5			
Actin	Actin	LOC111246368	F	TGTACCCCGGTATTGCTGAC	60.5	145	99.4%	0.9996
			R	TGGAAGGTGGACAGAGAAGC	60.5			
α-tubulin	Tubulin alpha-1D chain	LOC111254752	F	ACTCATTCGGAGGAGGAACC	60.5	127	98.4%	0.9999
			R	ACGGCAGTCGATACTTGAGG	60.5			
Rab1	Ras-related protein ORAB-1-like	LOC111243381	F	TGCGTACGACGTAACTGACC	60.5	85	103.5%	0.9995
			R	TACGTTCTCGCAGGCATAGC	60.5			

3.4. Stability ranking calculated by the four programs

The stability ranking of the refence genes was calculated using the four programs (Table 11). *eER1A1* showed the highest stability in all the programs used (1.5, 1.0, 1.3, and 1.5 of average stability ranks from geNorm, BestKeeper, NormFinder, and RefFinder respectively), followed by *α-tubulin*, *RpL5*/*NADHD*, *eEF2*, *Rab1*, *GAPDH*, and *Actin* based on the average of all the ranks from the four programs.

The number of reference genes required for reliable normalization was determined by pairwise variations calculated from geNorm (Fig. 19). In the acaricide treatment groups, all subgroups showed lower pairwise variations (V2/3, V3/4, V4/5, V5/6, V6/7, and V7/8) than the cut-off value (0.15), indicating that two genes are sufficient for accurate normalization in acaricide-treated groups (eEF1A1 and RpL5 for coumaphos and fluvalinate, *eEF1A1* and *α-tubulin* for amitraz, and *eEF1A1* and *NADHD* for the pooled group). The pairwise variation V4/5 in every subgroup showed the lowest value, indicating that the application of the four reference genes was the most reliable for normalization.

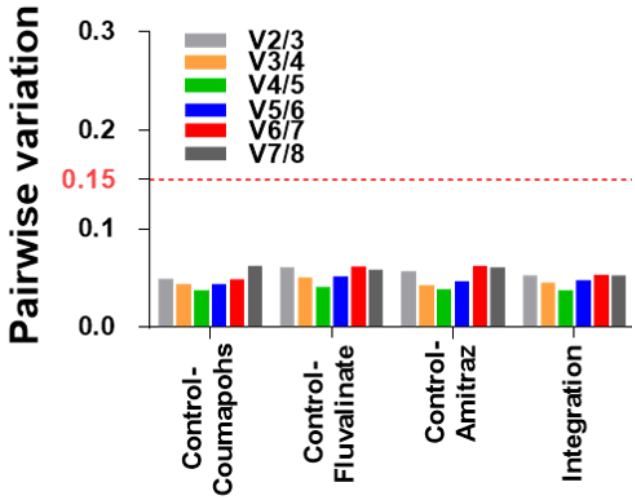

Fig. 19. Pairwise variation result from geNorm. Red dotted line indicates the cut-off value (0.15).

Table 11. Stability ranking of the reference genes calculated by BestKeeper, geNorm, NormFinder, and RefFinder. Total rank was calculated based on the average ranks of the four programs. The most stable genes in each subgroup are marked with an asterisk (*).

Group		Ref. gene	geNorm Stability[a]	geNorm Rank	BestKeeper Stability[b]	BestKeeper Rank	NormFinder Stability[c]	NormFinder Rank	RefFinder rank	Total rank
Acaricide	Control-Coumaphos	*NADHD*	0.257	3	0.18	3	0.080	2	7	3
		GAPDH	0.409	7	0.42	8	0.257	7	4	7
		*eEF1A1**	0.250	1	0.13	1	0.079	1	3	1
		eEF2	0.275	4	0.25	6	0.131	5	2	5
		RpL5	0.256	2	0.23	4	0.092	3	6	3
		Actin	0.533	8	0.29	7	0.302	8	8	8
		α-tubulin	0.276	5	0.16	2	0.114	4	1	2
		Rab1	0.349	6	0.24	5	0.181	6	5	6
	Control-Fluvalinate	*NADHD*	0.312	5	0.23	4	0.137	5	3	5
		GAPDH	0.426	6	0.40	8	0.254	7	7	7
		*eEF1A1**	0.287	2	0.16	1	0.074	1	1	1
		eEF2	0.297	4	0.25	5	0.131	4	2	4
		RpL5	0.273	1	0.22	3	0.092	2	4	2
		Actin	0.514	8	0.29	6	0.276	8	5	6
		α-tubulin	0.295	3	0.21	2	0.096	3	6	3
		Rab1	0.471	7	0.35	7	0.228	6	8	7
	Control-Amitraz	*NADHD*	0.279	3	0.25	4	0.094	3	3	3
		GAPDH	0.403	6	0.45	8	0.244	7	7	8
		*eEF1A1**	0.264	2	0.17	1	0.046	2	1	1
		eEF2	0.312	5	0.34	7	0.150	5	6	5
		RpL5	0.296	4	0.18	2	0.112	4	4	4
		Actin	0.522	8	0.34	6	0.275	8	5	7
		α-tubulin	0.259	1	0.24	3	0.045	1	2	2
		Rab1	0.485	7	0.28	5	0.238	6	8	6
	Integration	*NADHD*	0.270	2	0.22	4	0.088	2	2	2
		GAPDH	0.389	6	0.40	8	0.229	7	7	7
		*eEF1A1**	0.264	1	0.16	1	0.074	1	1	1
		eEF2	0.283	5	0.28	6	0.129	5	5	5
		RpL5	0.281	3	0.21	3	0.112	4	3	4
		Actin	0.424	7	0.26	5	0.189	6	6	6
		α-tubulin	0.281	3	0.21	2	0.100	3	4	3
		Rab1	0.463	8	0.28	7	0.239	8	8	8

3.5. P450 basal transcription level and correlation between fluvalinate mortality rate

The average transcription levels of P450s are presented in Fig. 20. The Flv-3A14 showed the highest transcription level, then followed by Flv-3A56, and Flv-4V2. The P450s showed the lowest transcription level included Flv-2B4, Flv-3A19, and Flv-3012A6. Flv-3A14 showed the largest standard deviation, followed by Flv-4V2, and Flv-3A56, indicating their transcription levels varied more across different regional samples. Correlation analysis revealed that no apparent correlation is present between the basal transcription levels of any P450s and the mortality rates of regional *Varroa* mites obtained from the 24-h RCV bioassay. A total of six P450s (2B4, 4c1, 3A13, 3A19, 3A56, and 503) showed a tendency of decrease in mortality as the P450 expression level increased. Among them, two P450s (2B4 and 3A19) showed somewhat higher slope values than remaining P450s despite their correlation coefficients (R^2 = 0.08057 in 2B4 and R^2 = 0.04153 in 3A19).

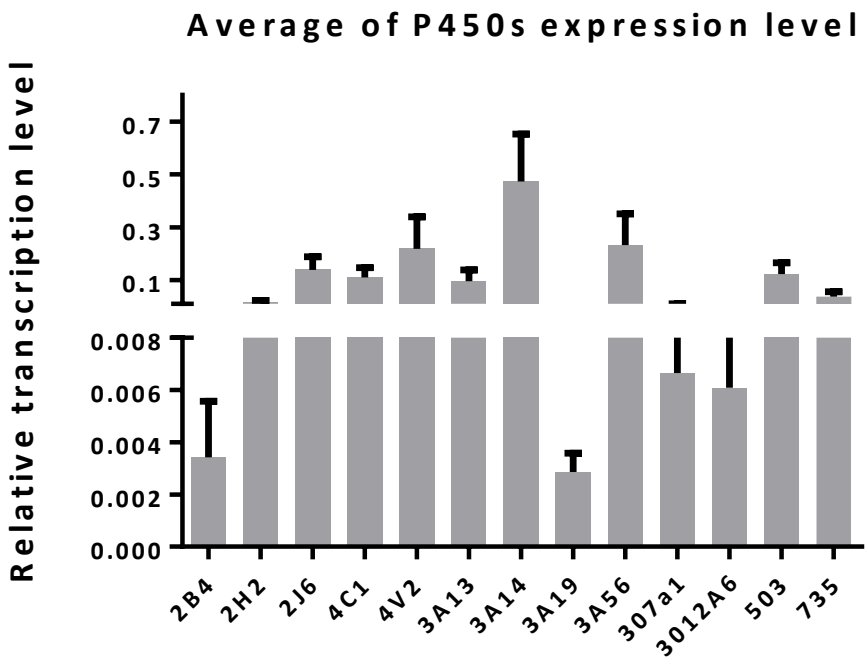

Fig. 20. Mean expression levels of P450s across regional populations of *Varroa* mite. The Flv-3A14 showed the highest transcription level, and the Flv-2B4 showed the lowest transcription level.

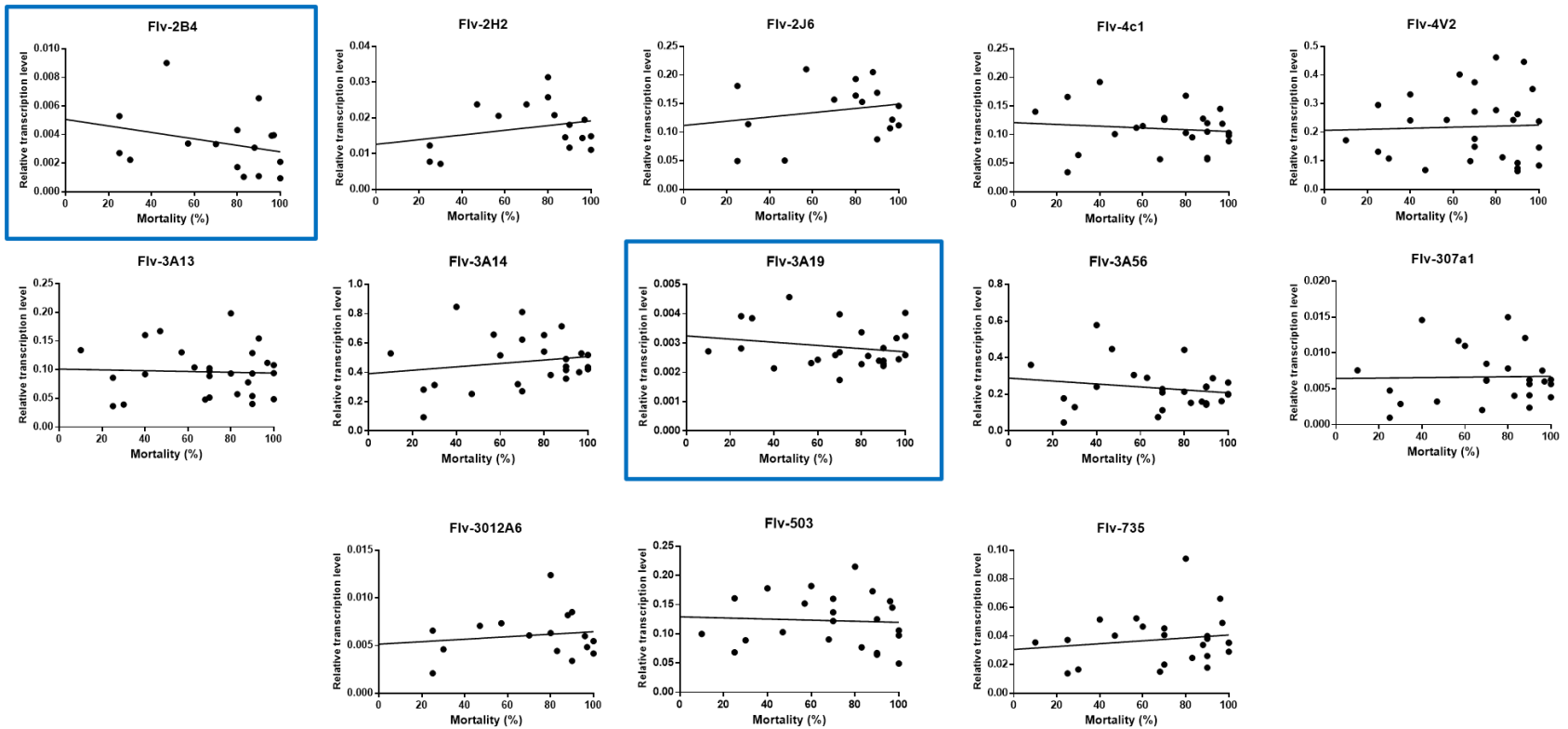

Fig. 21. Correlation between fluvalinate mortality data and relative transcription levels of P450s. Graph framed with blue border are P450s that showed tendency to decrease mortality as the P450 expression level increased.

4. Discussion

Several new nucleotide polymorphisms resulting in non-silent amino acid substitution were discovered in the *Varroa* mite VGSC, some of which were previously known in other arthropod species. It seems highly likely that the novel mutations such as F1528C and N1552S located in the transmembrane III S6, forming the pyrethroid binding site (O'Reilly et al., 2006), affect fluvalinate sensitivity, thus having some role in resistance to fluvalinate. Remaining polymorphisms appear to have relatively less critical roles in pyrethroid sensitive if considering their location in either extracellular loop or in the large intracellular loop between domains II and IV, where high degrees of amino acid sequence variations have been reported. To determine the roles of novel mutations in fluvalinate resistance, a larger-scale association study should be performed based on more sophisticated bioassay method as the current 24-h RCV bioassay is not sensitive enough to separate mite individuals with different genotypes at the L925I/M mutation site. To this end, evaluation of time-dependent knockdown responses following the topical application, which is more controllable than RCV bioassay, would be beneficial in discriminating susceptible vs. resistant individual mites. Nevertheless, in order to confirm the roles of novel mutations in fluvalinate resistance, it is essential to conduct the functional analysis of mutated VGSC variants based on the Xenopus oocyte expression in conjunction with the two-electrode voltage clamp analysis (Burton et al., 2011; Lee et al., 1999).

Thus far, only one paper has been published for the reference validation of *Varroa* mites. E. M. Campbell et al. (2016) investigated the stabilities of ten candidate reference genes following different stages and deformed wing virus loads.

Owing to increasing acaricide resistance development in *Varroa* mites (Higes et al., 2020; Maggi et al., 2011; Pettis, 2004), it is important to investigate the physiological responses of these mites to acaricides. Thus, in this study, the stabilities of eight putative reference genes were compared with and without acaricide treatment. *eEF1A1* and *NADHD* were determined to be the most stable among the eight candidate genes in the pooled group, whereas *Rab1* and *GAPDH* showed the least stability. The stability of *eEF1A1* ranked 1st among all three acaricides. *eEF1A1* codes for the alpha subunit of the elongation factor 1, which has multiple functions in translation (one of the basic housekeeping processes) (Ejiri, 2002). *eEF1A1* has been proven to be a stable reference gene in pig tissues (Svobodova et al., 2008), human vascular stromal cells (Gentile et al., 2016), and cervical tissues (Shen et al., 2010). *eEF1A1* also showed the highest expression levels among the eight genes, which is advantageous for analysis if there is a limited amount of sample. *NADHD*, as previously shown in differential stages (E. M. Campbell et al., 2016), showed good performance as a reference gene in acaricide treatment despite its low expression level.

When the basal transcription level of P450s was correlated with mortality data, no apparent correlation was observed. This lack of correlation appears to mainly due to the limit of mortality data with a finite scale of 0-100%, particularly when the mortality data were obtained at a time point beyond the saturated mortality response (i.e., 24 h post-treatment). Use of LC_{50} values obtained from different mite populations for the correlation analysis would increase the power of analysis significantly, but estimating the LC_{50} values for a large number of mite populations is practically very difficult though not impossible. As the second best option, therefore, more refined mortality data obtained from the linear time-response section

(i.e., < 7 h) in the RCV bioassay can be used for the correlation to improve resolution. Nevertheless, two P450 (2B4 and 3A19), despite their extremely low coefficients, showed some tendency of negative correlation. As both 2B4 and 3A19 belong to the clan 3 P450s that are mostly involved in xenobiotic resistance (Baldwin et al., 2009), if overexpressed, they likely confer metabolic resistance in *Varroa* mites, thereby being feasible candidates for metabolic resistance factors. Although their overall expression levels are relatively low compared to other P450s, if they have neuronal tissue-specific expression pattern, they still can provide effective protection from the action of fluvalinate in the neuronal tissues. Although there are conflicting studies on the role of P450s in fluvalinate resistance in *Varroa* mites (Hillesheim et al., 1996; Johnson et al., 2010; Mozes-Koch et al., 2000), overexpression of P450s has been reported to be associated with resistance in several other arthropod species (Komagata et al., 2010; Wondji et al., 2009; Zimmer & Nauen, 2011). Since P450s induce oxidative stress as a side effect, P450s expression level is usually kept low (Barouki & Morel, 2001; Gonzalez, 2005; Zangar et al., 2004) but their expression is inducted upon exposure to xenobiotics, such as plant toxins and insecticides (Waxman, 1999). Therefore, in future studies, it is worth to investigate the induction profiles of not only the two P450s (2B4 and 3A19) but also other P450s after treating with fluvalinate. Identification of P450-mediated resistance factor should facilitate the discovery of additional novel molecular markers for the detection and quantification of metabolic resistance in *Varroa* mites.

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초 록

꿀벌응애는 꿀벌에 기생하는 외부 기생성 해충으로서 양봉 산업에 큰 피해를 입힌다. 꿀벌응애로 인한 피해를 줄이기 위해서 다양한 방제 방법이 사용되는데, 그 중에서 편의성과 방제효율을 고려하여 합성 살비제 스트립 사용이 선호되고 있다. 하지만 이러한 합성 살비제의 과도한 사용은 저항성 문제를 야기하여 전세계적으로 꿀벌응애의 합성 살비제 저항성 발달에 따른 방제실패가 보고되고 있다. 저항성 발달을 관리하기 위해서는 지속적인 저항성 모니터링이 필요하나 국내에서는 지금까지 한 차례의 저항성 모니터링만이 진행되었다. 이와 같이 꿀벌응애 저항성 모니터링이 빈번하게 진행되지 않는 이유로는 꿀벌응애의 채집과 생물검정 상의 어려움을 들 수 있다. 따라서 본 연구에서는 생물검정(잔류접촉바이알법)과 분자마커(정량적 염기서열 분석 방법)을 이용한 꿀벌응애 저항성 모니터링 방법을 개발하였고 이를 이용하여 전국에서 꿀벌응애 살비제 저항성 모니터링을 진행하였다.

잔류접촉바이알법을 이용한 저항성 모니터링 방법을 개발하기 앞서 감수성으로 추정되는 계통인 2021-SO 계통을 이용하여 LC 값을 구하였고 이를 진단농도로 사용하였다. 2021년에는 17지역에서 저항성 모니터링을 수행하였다. 17지역 중 2지역(2021-GR, 2021-UR)에서 쿠마포스와 플루발리네이트에 대한 사충률 감소가 관찰되었고, 3지역(2021-SJ2, 2021-DG, 그리고 2021-GJ)에서 플루발리네이트에 대한 사충률 감소가 관찰되었다. 2022년에는 42지역에서 저항성 모니터링을 수행하였는데, 이중 14지역에서 쿠마포스 저항성이 8지역에서 플루발리네이트에 대한 사충률 감소가 관찰되었다. 플루발리네이트 저항성의 경우 2021년보다 2022년에 관찰된 곳이 더 많았기 때문에 플루발리네이트 저항성이 한국에서 확산되고 있다고 생각할 수 있다.

정량적 염기서열 분석 방법을 이용하여 저항성 모니터링을 실시하기 전 실제 염기 비율과 염기서열 분석 결과를 보정할 예측식을 구하였다. 이렇게 구한 예측식을 이후에 수행한 저항성 모니터링 방법에 사용하였

다. 2021년 17지역에서 분자마커 기반 저항성 모니터링을 실시하였고 4지역에서 플루발리네이트 저항성 돌연변이(L925I/M)가 검출되었다. 2022년에는 90지역에서 분자마커 기반 저항성 모니터링을 실시하였는데, 이중 83지역에서 플루발리네이트 저항성 돌연변이(L925I/M)가 검출되었다. 위 결과를 보면 한국에 플루발리네이트 저항성 돌연변이가 빠른 속도로 확산되고 있는 것을 알 수 있다. 또한 플루발리네이트에 의한 사충률과 저항성 돌연변이 빈도 사이의 연관성을 확인하였지만 연관이 없는 것으로 확인되었다. 플루발리네이트를 처리한 후 매시간마다 기절(knockdown)한 꿀벌응애에서 플루발리네이트 저항성 돌연변이 유전형 빈도를 비교하였다. 그 결과 7시간 이후에 기절한 꿀벌응애에서 저항성 동형접합 유전자형이 급격히 증가하였다. 이를 보아 RCV를 이용한 생물검정에서 감수성 개체와 저항성 개체를 구별하는데 초기 시점이 바람직하다는 것을 알 수 있었다. 또한 플루발리네이트 처리 후 7시간 뒤에 기절한 꿀벌응애에서도 감수성 동협접합 꿀벌응애가 검출되었는데, 이는 다른 저항성 인자의 존재 가능성을 암시한다.

따라서 플루발리네이트의 작용점인 전압개폐나트륨 채널에서 새로운 돌연변이를 확인한 결과, 총 10개의 새로운 돌연변이가 발견되었다. 또한 cytochrome P450(P450s)의 발현량을 확인하여 대사 인자로서 저항성과의 연관성을 확인하였다. 이를 위하여 qPCR을 위한 적합한 대조유전자(reference gene)를 먼저 조사한 결과, *eEF1A1*과 *NADHD*가 꿀벌응애에 있어서 살비제가 미치는 영향 비교 시 적합한 대조유전자로 선발되었다. P450의 발현량과 RCV를 통해 얻은 사충률 사이의 명백한 상관 관계가 없음에도 불구하고 2종의 P450s(Flv-2B4 및 Flv-3A19)는 발현량이 증가함에 따라 사충률이 감소하는 경향을 보여 플루발리네이트 저항성에 기여할 가능성을 암시하였다.