Association of novel mutations in a sodium channel gene with fluvalinate resistance in the mite, *Varroa destructor*

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SUMMARY

Varroa (*Varroa destructor*) has recently become resistant to Apistan, a pyrethroid pesticide with tau-fluvalinate as its active ingredient. In many insect pests, resistance to pyrethroid insecticides is due to reduced target-site (sodium channel) sensitivity to pyrethroids in the nervous system, a phenomenon called knockdown resistance (*kdr*). A number of studies showed that *kdr* and *kdr*-type resistance is a result of point mutations in the *para* family of sodium channel genes. To investigate the molecular mechanism of resistance to fluvalinate in varroa, we have cloned and sequenced a large cDNA fragment corresponding to segment 3 of domain II (IIS3) to segment 6 of domain IV (IVS6) of a *para*-homologous sodium channel gene (*VmNa*) from susceptible and resistant mite populations. The deduced amino acid sequence from this cDNA shares 71%, 60%, and 50% identity with the corresponding region of the *para*-homologous protein of the Southern cattle tick, *Boophilus microplus*, *Drosophila melanogaster* Para, and rat brain type II sodium channel α-subunit, respectively. Sequence analysis revealed that four amino acid changes, F758L in IIIS6, L826P in the linker connecting domains III and IV, I982V in IVS5 and M1055I in IVS6, were correlated with fluvalinate resistance in both Florida and Michigan populations. Interestingly, the *kdr* or super-*kdr* (which confers much higher level resistance than *kdr*) mutation previously identified in insects was not detected in the resistant mites. These results support the emerging notion that distinct sodium channel gene mutations are selected in different insects and arachnids in response to pyrethroid selection.

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INTRODUCTION

Honey bees (*Apis mellifera*) play a crucial role in US agriculture by pollinating a large number of crops. Since 1987, the beekeeping industry in the USA has been severely impacted by the mite, *Varroa destructor* (formerly *Varroa jacobsoni*) (Anderson & Trueman, 2000). Varroa is an ectoparasite of honey bees and is distributed worldwide (Matheson, 1993, 1995). Because untreated *A. mellifera* colonies almost always die within 2–3 years after varroa infestation (Ritter, 1981; De Jong *et al.*, 1982), feral bee colonies in the USA have been almost totally wiped out by this mite since its introduction around 1987 (Kraus & Page, 1995). As a result, pollination of crops depends entirely on managed honey bee colonies, which are routinely treated with Apistan (with tau-fluvalinate as its active ingredient) to control the mite population. Unfortunately, varroa recently developed resistance to tau-fluvalinate in Europe (Milani, 1995) and the USA (Baxter *et al.*, 1998; Pettis *et al.*, 1998; Elzen *et al.*, 1998; Elzen *et al.*, 2000). Resistant mites show only from 5% to 64% mortality when treated (Elzen *et al.*, 1998), virtually rendering Apistan ineffective for varroa control. It is important to understand the mechanisms underlying resistance in varroa so that we can take appropriate measures to delay resistance development, or rationally design new pesticides to specifically target the Apistan-resistant mites.

Genetic and biochemical studies have contributed immensely to our understanding of insecticide resistance. There are two major ways in which organisms can become resistant to xenobiotics such as pesticides: either by modifying the effective dose of the pesticide available at the target site or by modifying the target site itself (Feyereisen, 1995). For example, the organism can avoid the pesticide behaviorally (behavioral resistance), reduce the penetration or absorption, enhance the detoxification (metabolic resistance), or sequester pesticides to decrease the dose at the target site. Alternatively, organisms can reduce the sensitivity or the numbers of target sites to render the pesticide ineffective. It is not clear what mechanism(s) is responsible for tau-fluvalinate resistance in varroa. Studies in Europe and the Middle East suggested that resistance to tau-fluvalinate could be partly explained by the increased activity of various enzymes, such as the monooxygenases in the p450 system, or esterase (Hillesheim *et al.*, 1996, Mozes-Koch *et al.*, 2000). However data in the USA indicated that these metabolic pathways are not involved in tau-fluvalinate resistance in the varroa mite. Neither p450 nor the glutathione S-transferase detoxification (GST) pathyway seems to be involved because neither piperonyl butoxide (a p450 inhibitor) nor chlordimeform (a GST inhibitor) showed synergism with tau-fluvalinate when mites were pretreated with these chemicals (Bell *et al.*, 1999; Marion Ellis, personal communication).

Target site (sodium channel) insensitivity has emerged as one of the most important pyrethroid resistance mechanisms in many insect species. Multiple point mutations in the *para* family of proteins are responsible for pyrethroid resistance in insects (Smith *et al.*, 1997; Lee *et al.*, 1999a; Zhao *et al.*, 2000; Vais *et al.*, 2000; Tan *et al.*, 2002; Liu *et al.*, 2002). A point mutation (leucine (L) to phenylalanine (F)) in IIS6 of the *para*-homologous proteins is responsible for *kdr* to pyrethroids in many insect pest species (Taylor, 1993; Williamson *et al.*, 1996; Miyazaki *et al.*, 1996; Park *et al.*, 1997; Dong, 1997; Guerrero *et al.*, 1997; Martinez-Torres *et al.*, 1997; 1998; 1999a; 1999b; Lee *et al.*, 1999b). An L to H (histidine) mutation at the same position was found in pyrethroid-resistant tobacco budworm (*Heliothis virescens*) (Park & Taylor, 1997). In the house fly, a second mutation (methionine (M) to threonine (T)) is found together with the L to F mutation to enhance pyrethroid resistance in strains carrying the super-*kdr* trait (Williamson *et al.*, 1996; Lee *et al.*, 1999a). In the German cockroach, four unique point mutations (D to G in the N-terminus, E to K and C to R in the linker between IS6 and IIS1, and P to L in the C-terminus), together with the *kdr* mutation (L to F in IIS6), are associated with high levels of resistance (Liu *et al.*, 2000). Three of these cockroach mutations (E to K, C to R, and L to F) have already been proven to be involved in pyrethroid resistance (Tan *et al.*, 2002). Two mutations in the linker connecting domains II and IV, D to V and E to G, are associated with pyrethroid resistance in *H. virescens* and the cotton bollworm (*Helicoverpa armigera*) (Head *et al.*, 1998). An F to I mutation in IIIS6 was recently identified to be associated with pyrethroid resistance in the Southern cattle tick (*Boophilus microplus*) (He *et al.*, 1999a). To examine whether tau-fluvalinate-resistant varroa mites have sodium channel mutations, we cloned and sequenced cDNA fragments encoding IIS3 to IVS6 of a *para*-homologous sodium channel gene, because most of the identified insect and arachnid sodium channel mutations are located in IIS3 to IVS6. Sequence comparison between susceptible and tau-fluvalinate-resistant varroa populations in Florida and Michigan revealed four amino acid changes that are associated with tau-fluvalinate resistance.

MATERIALS AND METHODS

Sampling varroa

Varroa were collected from two locations in the USA. The first population of mites was obtained from Texas, which was originally collected from Florida. This population showed 27% mortality when treated with tau-fluvalinate. Resistant and susceptible varroa were separated using a vial assay (Elzen *et al.*, 1998). Briefly, 0.5 ml acetone (spectral grade) containing 2.4 μg of technical grade tau-fluvalinate was pipetted into a 20-ml glass scintillation vial. Vials were rolled slowly until all of the acetone dried. Mites were collected from bee larvae or pupae by opening larva or pupa cells using forceps, and transferred into vials. Each vial containing three mites was incubated at 25°C for 24 h. A total of

180 mites were tested. Varroa that died within 24 h were designated as susceptible (Florida-S) and the survivors as resistant (Florida-R) and both groups were shipped on dry ice to Michigan State University.

The second population of resistant and susceptible varroa mites was collected at Michigan State University using the following method. Two strips of Apistan were placed in a 10-frame honey bee colony. Varroa knocked down by tau-fluvalinate were collected on a sheet of aluminum foil on top of dry ice in a Styrofoam box placed directly under the hive. Mites collected during the first three days were designated as susceptible (Michigan-S), those that survived the treatment were then collected using coumaphos strips and designated as resistant (Michigan-R).

RNA isolation and cDNA cloning

Amplification and sequencing of the *VmNa* cDNA were first carried out using a susceptible varroa population (Michigan-S). Total RNA was isolated from about 50 mites, using RNA isolation kits from Gibco-BRL (Bethesda, MD) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using Superscript II RNase H$^-$ reverse transcriptase (Gibco-BRL, Bethesda, MD) in the presence of oligo(dT)12–18 at 42°C, or from 5 μg of total RNA using ThermoScript RT (Gibco-BRL) in the presence of a gene-specific primer at 50°C. Polymerase chain reaction (PCR), which contained 1 μl cDNA, 5 μl 10 × PCR buffer, 0.2 μM of each gene-specific primer or 0.4 μM of each degenerate primer, 200 μM dNTPs, 1.5 mM $MgCl_2$ and 2.5 U *Taq* polymerase, was carried out in a total volume of 50 μl. PCR was started on a GeneAmp 2400 (Applied Biosystems) with an initial denaturation at 94°C for 2 min followed by 35 thermal cycles (30 sec at 94°C, 30 sec at 58°C for gene-specific primers or 53°C for degenerate primers and 1 or 2.5 min at 72°C) and a final extension for 7 min at 72°C.

Amplified products were isolated by agarose gel electrophoresis and purified using the Prep-A-Gene kit (Bio-Rad, Hercules, CA), then cloned into pCR-TOPO using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Competent Top 10 Cells (Invitrogen) was used for transformation. Plasmid DNA was isolated using the Wizard Plus Minipreps DNA Purification System

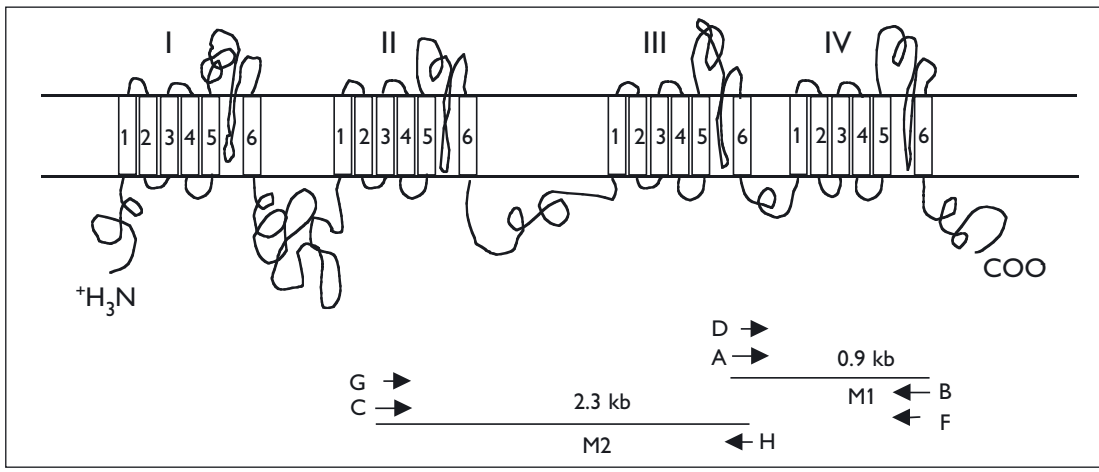

FIG. 1. Cloning of partial *VmNa* cDNA. At the top is a diagram of the proposed organization of *VmNa* , showing the four homologous domains, each divided into six transmembrane segments (S1-S6). Degenerate primers A, B, and C and gene-specific primers D, F, G, and H were used in PCR to amplify the 0.9-kb (clone M1) and 2.3-kb (clone M2) cDNA fragments. Gene-specific primer F was used to make the first-strand cDNA. The primer sequences (and corresponding amino acid number and primer position) are given below:

A: 5'-GTIATDATDGAYAAYTTAA (V766 in IIIS6)

B: 5'-ATNACIGCDATRTACATRTT (N1073 in IVS6)

C: 5'-GARGGNTGGAAYATHTTYGA (E4 in IIS3)

D: 5'-CTTCCAAGGCATGAGGGTCGTAG (F631 in IIIS4-IIIS5)

F: 5'-CGGCAATGTACATGTTGATGATC (I1071 in IVS6)

G: 5'-ATTGTCGTTACTCGAGTTGGGTC (L16 in IIS3-IIS4)

H: 5'-CCTCCAGCTTTCTTCTTTTGTTC (E783 in IIIS6-IVS1)

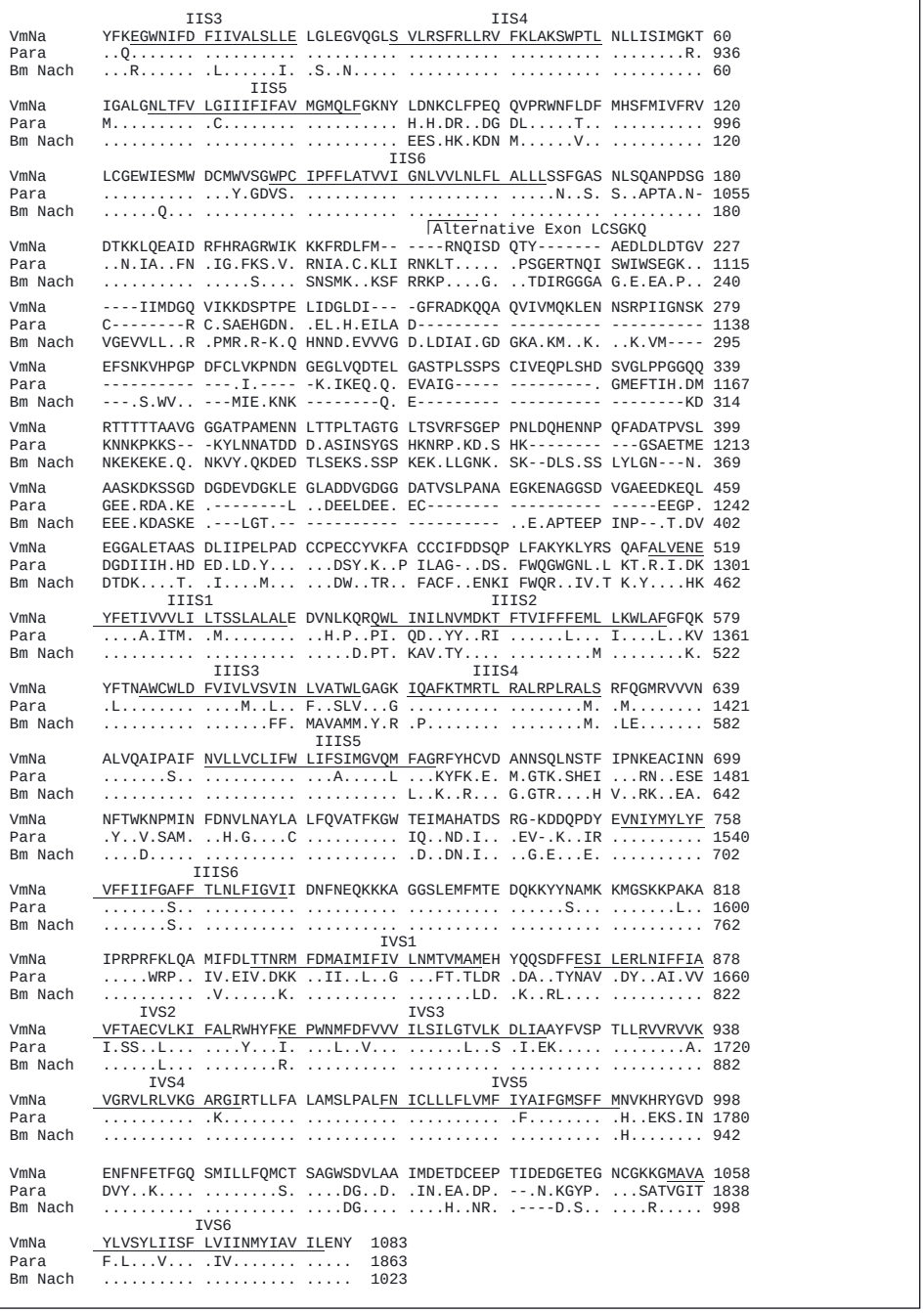

FIG. 2. Alignment of deduced amino acid sequences of the IIS3 to IVS6 region of *VmNa* (Michigan-S), the *para*-homologous protein of cattle tick (*Boophilus microplus*), and *Drosophila melanogaster* Para. Dots (.) refer to identical amino acid residues and dashes (-) refer to missing amino acid residues. Numbers on the right indicate the amino acid positions of each protein. Names above these sequences indicate domains and transmembrane segments. For example, IIS3 refers to transmembrane segment 3 of domain II and the amino acids residues of this segment are underlined. A short fragment encoding LCSGKQ was present in some clones and missing in the others, suggesting that this fragment likely represents an alternative exon, which is quite common for the *para* gene in *Drosophila* .

(Promega, Madison, WI). Insert-positive clones were identified by restriction enzyme digestion. cDNA inserts were sequenced by primer walking at the W M Keck Laboratory at Yale University. Sequence data were analysed using DNASTAR (DNASTAR Inc.).

RESULTS

Two overlapping DNA fragments (M1 and M2) covering the majority of the *VmNa* coding region (IIS3 to IVS6) were isolated by RT-PCR from Michigan susceptible mites (Michigan-S) (fig. 1). The M1 clone (911 bp) was isolated using the poly(A)-containing first-strand cDNA as the template and degenerate primers A and B, which were designed based on the conserved amino acid sequences of sodium channels from *Drosophila melanogaster* (Loughney *et al.*, 1989), *Blattella germanica* (Dong, 1997a), and *Musca domestica* (Williamson *et al.*, 1996) (fig. 1). The M1 fragment was sequenced and the gene-specific primer F (fig. 1) was designed and used to prime the synthesis of a new round of cDNA. The M2 fragment (2.3-kb fragment) was then amplified by PCR using the new cDNA as the template and degenerate primer C and gene-specific primer H (fig. 1). DNA sequencing revealed that the M1 fragment is 911 bp long and encodes a polypeptide corresponding to the IIIS6 to IVS6 region (fig. 1). The M2 fragment is 2.3 kb long and encodes the IIS3-IIIS6 region. The alignment of the deduced amino acid sequence of the *VmNa* gene is shown with *D. melanogaster* Para (Loughney *et al.*, 1989) and the *para*-homologous protein of the Southern cattle tick (*Boophilus microplus*) (He *et al.*, 1999b) in figure 2. The deduced amino acid sequence of the IIS3 to IVS6 region of *VmNa* shares 71%, 65%, and 50% identity with the *para*-homologous protein of the *B. microplus*, *Drosophila* Para, and rat brain type II sodium channel α-subunit, respectively. The greatest similarities (60% to 98.2%) in amino acid sequence are in the six transmembrane segments of domain II, III, IV. The cytoplasmic linkers between these three domains are less conserved.

To determine whether tau-fluvalinate resistance in varroa is associated with specific mutations in the IIS3 to IVS6 region, we cloned and sequenced cDNA fragments encoding this region for both susceptible and resistant populations from Michigan and Florida. Comparison of the deduced amino acid sequences of this region obtained from the four mite populations using the Clustal method of DNASTAR revealed seven amino acid differences among the resistant and susceptible mites of the two locations (table 1). Comparison of the deduced amino acid sequences of various sodium channel proteins in the regions where our mutations reside is shown in figure 3.

DISCUSSION

The development of resistance to tau-fluvalinate in varroa is rendering the only US federally registered pesticide Apistan ineffective and has caused widespread concern among bee scientists and beekeepers. Understanding the resistance mechanism is the first step toward designing effective management strategies. Our study here clearly for the first time identified a major portion of a sodium channel gene (*VmNa*) from varroa. This conclusion is based on the high amino acid identity of the IIS3 to IVS6 region of *VmNa* to the same area of sodium channel proteins of other organisms. The transmembrane region of the sodium channel protein is highly conserved among organisms across different taxa and IIS3 to IVS6 covers most of the transmembrane region. In addition, most of the identified insect and arachnid *kdr*-type sodium channel mutations are located in the region of IIS3 to IVS6.

Among the seven mutations we found (table 1) three were associated with the origin of the varroa, not with the resistance. These are G^{247} (in Michigan-R and Michigan-S) to R^{247} (in Florida-R and Florida-S), E^{308} (in

TABLE 1. Amino acid differences in IIS3-IVS6 of the varroa sodium channel *VmNa* between susceptible and resistant populations.

Position[a]	Michigan-S aa (nt)[b]	Michigan-R aa (nt)	Florida-S aa (nt)	Florida-R aa (nt)	Location
247	G (g)	G (g)	R (a)	R (a)	IIS6-IIIS1 linker region
308	E (g)	E (g)	K (a)	K (a)	IIS6-IIIS1 linker region
519	E (g)	E (g)	K (a)	K (a)	IIIS1
758	F (t)	F (t)	F (t)	L (c)	IIIS6
826	L (t)	P (c)	L (t)	P (c)	IIIS6-IVS1 linker region
982	I (a)	I (a)	I (a)	V (g)	IVS5
1055	M (g)	I (a)	M (g)	I (a)	IVS6

[a]The amino acid residue position according to fig. 2
[b]aa: one-letter code of amino acid; nt: nucleotide

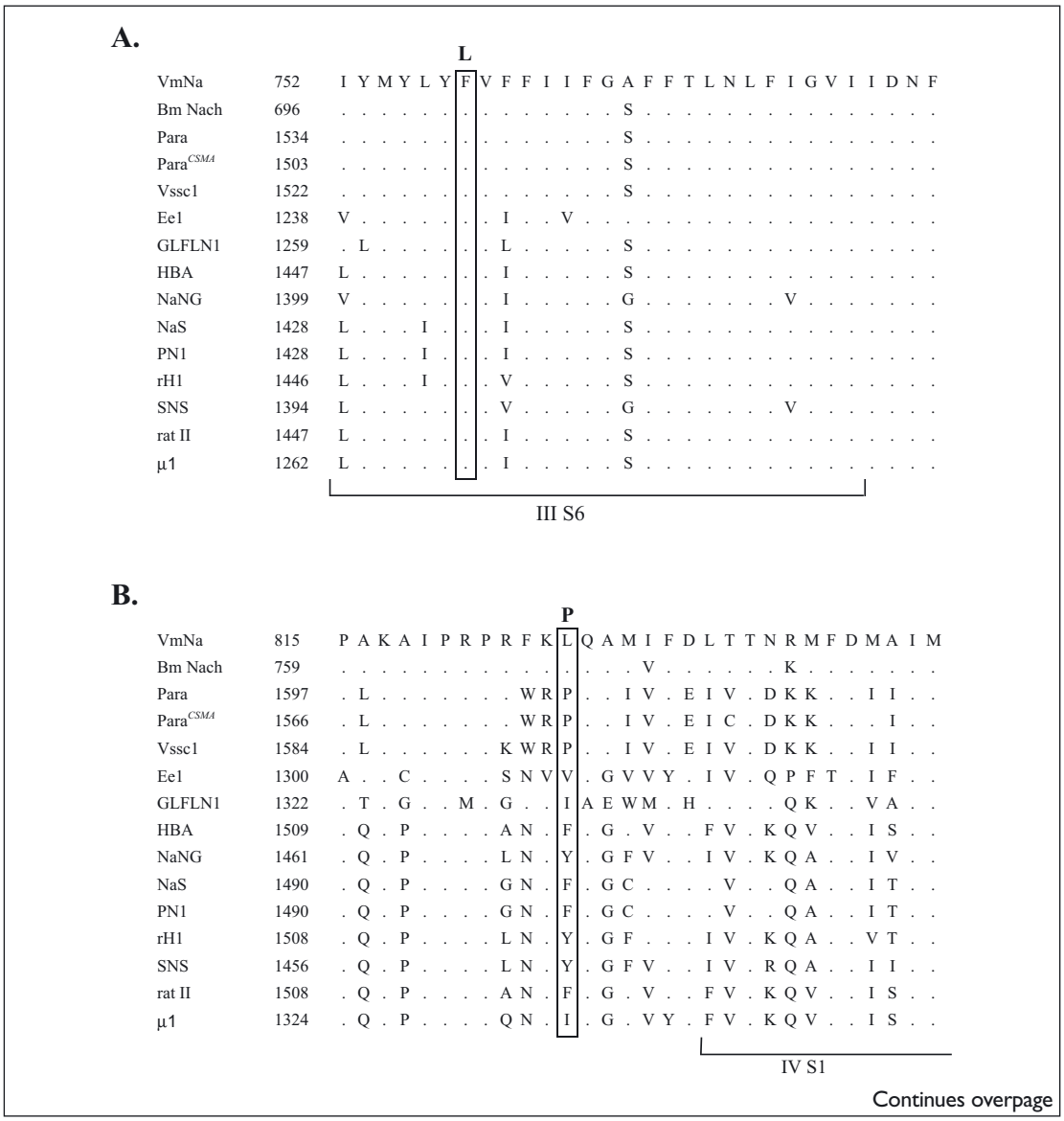

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FIG. 3. Alignment of amino acid sequences of regions where mutations reside among sodium channel α-subunits from various species and tissues. Sequence alignment was carried out with the amino acid sequences of *VmNa* and other sodium channel α-subunits using the Clustal method (DNASTAR). L ^{826}, F ^{758}, I ^{982}, and M ^{1055} in the susceptible populations and in the corresponding amino acid residues in other sodium channel sequences are boxed. Indicated above each box is the amino acid substitution found in the resistant populations. Bm Nach is the Southern cattle tick sodium channel sequence (He *et al.*, 1999b). The next three sequences are insect *para*-protein sequences (with accession numbers and references): cockroach Para^{CSMA} (U73583, Dong, 1997), *Drosophila* Para (M24285, Loughney *et al.*, 1989) and house fly Vssc1 (U38813, Ingles *et al.*, 1996). Ee1 is from *Electrophorus electricus* (M22252, Noda & Numa, 1987); GFLN1 is from the giant axon of the squid *Loligo opalescens* (L19979, Rosenthal & Gilly, 1993); HBA is from human brain (M94055, Ahmed *et al.*, 1992); NaNG is from dog nodose ganglion neurons (U60590, Chen *et al.*, 1997); NaS is from rabbit Schwann cell (U35238, Belcher *et al.*, 1995); PN1 is from rat peripheral neurons (U79568, Toledo-Aral *et al.*, 1997); rH1 is from rat cardiac muscle (M27902, Rogart *et al.*, 1989); SNS is from rat sensory neurons (X92184, Akopian *et al.*, 1996); Rat II is from rat brain (X61149, Auld *et al.*, 1988) and μ1 is from rat skeletal muscle (M26643, Trimmer *et al.*, 1989).

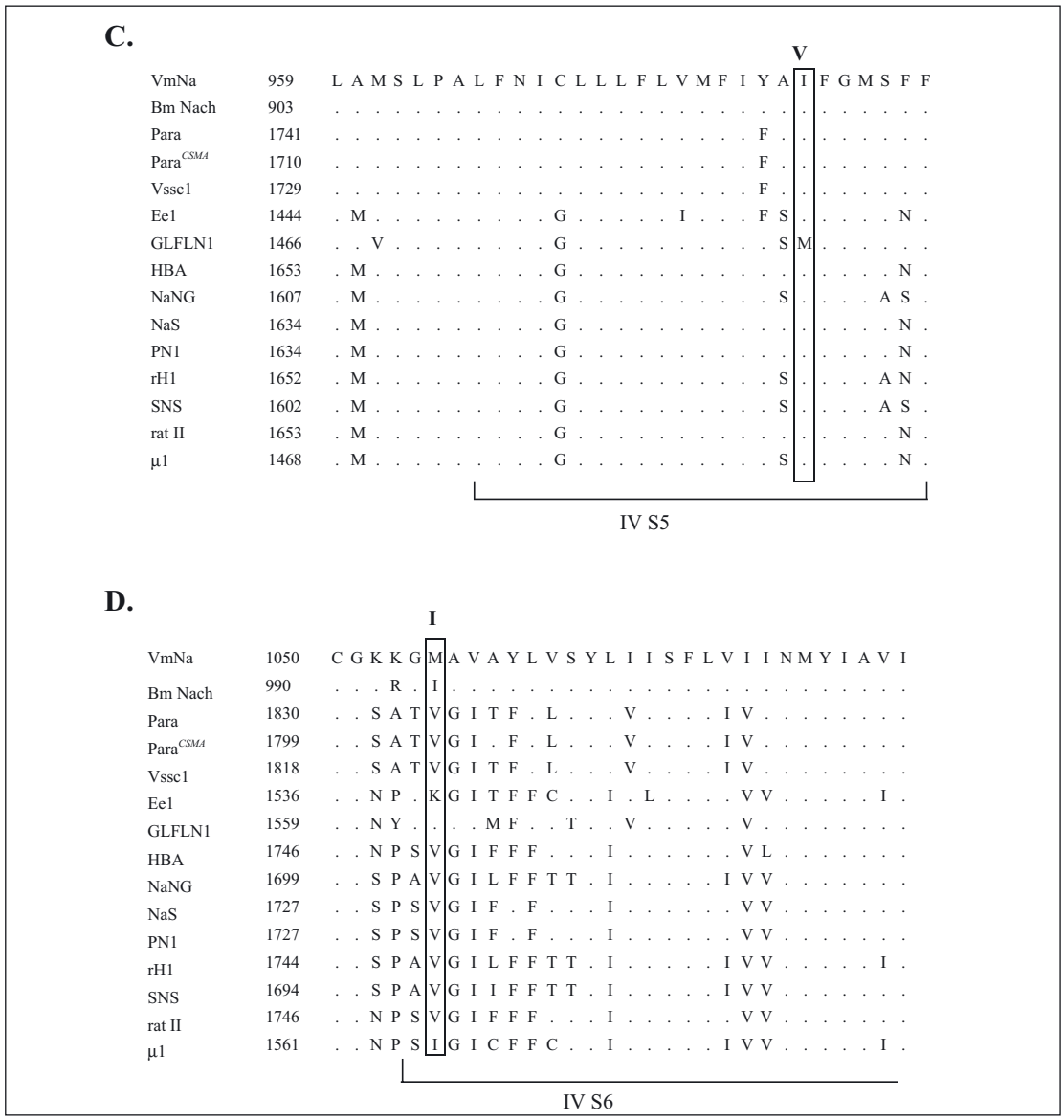

FIG. 3. Continued

Michigan-R and Michigan-S) to K^{308} (in Florida-R and Florida-S) in the linker region between IIS6 and IIIS1, and E^{519} (in Michigan-R and Michigan-S) to K^{519} (in Florida-R and Florida-S) in IIIS1. Four amino acid changes were correlated with tau-fluvalinate resistance and were independent of mite origins. L^{826} to P^{826} in the IIIS6–IVS1 linker region and M^{1055} to I^{1055} in IVS6, were found in both Michigan-R and Florida-R. The remaining two amino acid changes, F^{758} to L^{758} in IIIS6 and I^{982} to V^{982} in IVS5, were found only in the Florida-R population. Interestingly, comparison of the deduced amino acid sequences of representative vertebrate and invertebrate sodium channel proteins in the regions where the four mutations reside (fig. 3) reveals that F^{758} is conserved in all sodium channel proteins, and I^{982} is also conserved except for GLFLN1, a squid sodium channel protein, which has an M at the corresponding position, suggesting possible functional importance of these residues. However, the amino acid residues corresponding to L^{826} or M^{1055} are less conserved among sodium channel proteins. Whether they are involved in tau-fluvalinate resistance needs further investigation.

The identified insect and arachnid sodium channel mutations that are located in IIS3 to IVS6 include the L to F mutation in IIS6 (Taylor, 1993; Williamson *et al.*,1996; Miyazaki *et al.*, 1996; Park *et al.*, 1997; Dong, 1997; Guerrero *et al.*, 1997; Martinez-Torres *et al.*, 1997; 1998; 1999a; 1999b; Lee *et al.*, 1999b), the M to T mutation in the linker connecting S4 and S5 of

domain II (Williamson *et al.*, 1996; Lee *et al.*, 1999a), the D to V and E to G mutations in the linker connecting domains III and IV (Head *et al.*, 1998) and the F to I mutation in IIIS6 (He *et al.*, 1999a). However, none of those mutations was detected in resistant varroa populations. Our findings thus support the emerging notion that distinct sodium channel mutations have been selected in response to intensive pyrethroid use in different insect and arachnid species.

Further sequence analysis of the remaining *VmNa* coding region is required to identify whether additional mutations are involved in mite tau-fluvalinate resistance. The complete sequence information will be useful for making full-length *VmNa* clones, which can be expressed in *Xenopus* oocytes to determine whether any of the four resistance-associated mutations is indeed involved in tau-fluvalinate resistance in varroa. With the mutations confirmed, we should be able to monitor the frequency of these mutations in the field populations using molecular detection methods, such as allele-specific PCR, which is critical for the development of resistance management strategies.

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