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Brief Report

Sodium channel mutations (T929I and F1534S) found in pyrethroid-resistant strains of the cigarette beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae)

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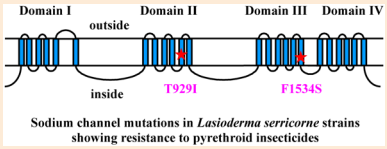
Supplementary material

RNA-seq data analysis of cigarette beetle (*Lasioderma serricorne*) strains having different sensitivities to pyrethroids identified sodium channel mutations in strains showing pyrethroid resistance: the T929I and F1534S mutations. These results suggest that reduced sensitivity of the sodium channel confers the pyrethroid resistance of *L. serricorne*. Results also showed that the F1534S mutation mostly occurred concurrently with the T929I mutation. The functional relation between both mutations for pyrethroid resistance is discussed.



Keywords: insecticide resistance, PCR-RFLP, stored products, target insensitivity, tobacco beetle.

Introduction

The cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae), which inhabits tropical to temperate zones worldwide, is recognized as the most serious pest affecting stored tobacco.[1)] *Lasioderma serricorne* infests tobacco leaves during curing, handling, and storage in tobacco manufacturers' warehouses and production facilities.[1)] Pesticide application to stored tobacco leaves and products in the warehouses is legally restricted. Consequently, phosphine fumigation has been adopted as the application method for *L. serricorne* disinfestation. However, continuous and extensive use of phosphine has produced insects showing phosphine resistance.[2)]

Application of contact insecticides by spraying them onto building surfaces (walls and floors) has been another key measure used to control *L. serricorne* in warehouses.[1)] Pyrethroids have been used as effective sprayed agents since the 1970s. In addition, pyrethroid-treated nets have recently been proposed as a means of mechanical and chemical control in *L. serricorne* disinfestation.[3)]

Pyrethroids act on voltage-sensitive sodium channels to keep them open, inducing hyperexcitation and/or conduction block in the nervous system, causing knockdown and/or death of the targeted insects.[4)] Pyrethroid resistance has been reported in diverse insects, including Coleoptera. The L1014F mutation in the sodium channel has been linked to nerve-insensitive pyrethroid resistance in *Sitophilus oryzae*,[5)] *Brassicogethes aeneus*,[6)] *Psylliodes chrysocephala*,[7)] and *Leptinotarsa decemlineata*.[8)] The involvement of the T929I mutation has been reported in *Sitophilus zeamais*[9)] and *L. decemlineata*.[8)] In *L. decemlineata*, some insects have the T929N mutation together with the L1014F mutation.[8)] The pyrethroid resistance of *L. serricorne* has also been reported in Germany.[10)] Nevertheless, no resistance mechanism for the pyrethroid resistance has been elucidated. This report describes, for the first time, study results demonstrating that *L. serricorne* strains showing resistance to pyrethroids have the T929I and F1534S mutations in the sodium channel.

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Materials and methods

1. Insecticides

Pyrethroids bifenthrin (Telstar 7.2% FL), cyfluthrin (Baythroid 5.0% EW), etofenprox (Trebon 20.0% EC), and silafluofen (Mr. Joker 19.0% EW) were purchased, respectively, from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan; Agro Kanesho Co., Ltd., Tokyo, Japan; Mitsui Chemicals Agro Inc., Tokyo, Japan; and Bayer Crop Science K.K., Tokyo, Japan. Permethrin (Adion 20.0% EC) and cypermethrin (Agrothrin 6.0% EC) were purchased from Sumitomo Chemical Co., Ltd., Osaka, Japan.

2. Insects and insecticidal assay

The seven *L. serricorne* strains used for this study are presented in Table 1. Insects were maintained on 10% yeast-added corn flour at 27°C and 60% relative humidity without insecticide selection.

An insecticidal assay was conducted for the six pyrethroids described above. A group of 30 adult insects was dipped in one of seven to eight insecticide concentrations for 10 sec. After 24 hr, the numbers of dead or moribund insects and surviving insects were recorded. Using probit analysis, the LC_{50} value was estimated for each strain.[11)]

3. RNA extraction for RNA-seq and data analysis

Total RNAs were extracted from the whole bodies of three adult insects for each strain using an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). Then cDNA libraries prepared from the total RNAs were subjected to nucleotide sequencing using an Illumina NovaSeq 6000 (paired-end, 150 bp; Macrogen Japan Corp., Kyoto, Japan). The obtained raw reads of the respective strains were filtered using Trimmomatic ver. 0.36[12)] to remove adapter sequences and low-quality ends (quality score, <15). The resulting reads with high quality were *de novo* assembled into contigs (unigenes) using Trinity ver. 2.9.1.[13)] To determine the nucleotide sequence of the sodium channel in each unigene, a blastn search was performed against a manually prepared sodium channel nucleotide database derived from several insect species (GenBank/EMBL/DDBJ accession nos. KJ699123, MG813770, MG813771, M32078, KY123916, JX424546, and EU822499). Amino acid sequences of extracted transcript sequences encoding sodium channels were predicted using TransDecoder ver. 5.5.0 (https://github.com/TransDecoder/TransDecoder/releases). Based on the extracted transcript sequences, specific primers were designed for PCR amplification as described below.

4. RNA extraction and cloning of the sodium channel gene sequences

Total RNA was also extracted from the whole bodies of three adult insects for each strain using Sepasol RNA I Super G (Nacalai Tesque Inc., Kyoto, Japan). Then cDNA was constructed from 1 μg of total RNA using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan). The 309 bp DNA fragments with the T929I site were amplified from the seven strains using primers: 5′-tggccgacactaaacttgc-3′ and 5′-agcaacgccaagaagagattga-3′. Similarly, PCR amplification of

Table 1. Susceptibilities of *Lasioderma serricorne* strains to pyrethroids

Strain	Permethrin			Bifenthrin			Cypermethrin			Cyfluthrin		
	n	LC_{50} (mg/L) (95% CL)	RR	*n*	LC_{50} (mg/L) (95% CL)	RR	*n*	LC_{50} (mg/L) (95% CL)	RR	*n*	LC_{50} (mg/L) (95% CL)	RR
TSC	209	120.8 (107.4–136.8)	1.7	240	6.2 (5.6–6.9)	1.1	240	0.6 (0.5–0.8)	1.3	240	0.4 (0.4–0.4)	1.4
NGY	210	79.3 (67.5–91.0)	1.1	240	7.0 (6.1–7.9)	1.2	240	0.5 (0.4–0.5)	1	240	0.3 (0.3–0.3)	1
IWT	209	69.0 (58.6–78.8)	1	240	6.3 (5.4–7.2)	1.1	238	0.6 (0.4–0.6)	1.1	240	0.3 (0.3–0.3)	1
THR	209	97.2 (87.8–107.4)	1.4	240	5.9 (5.2–6.6)	1	241	0.7 (0.6–0.8)	1.1	240	0.4 (0.3–0.4)	1.3
SKG	90	>10000 (—)	>144.8	120	>10000 (—)	>1696.5	239	754.8 (629.7–911.6)	1584.2	240	759.8 (684.1–853.0)	2584.1
C87	90	>10000 (—)	>144.8	119	>10000 (—)	>1696.5	240	595.6 (547.9–645.6)	1250.0	240	655.7 (570.6–763.5)	2230.0
MLY	89	>10000 (—)	>144.8	120	>10000 (—)	>1696.5	238	539.3 (484.0–597.7)	1131.9	240	735.7 (634.3–879.9)	2502.0

Strain	Etofenprox			Silafluofen			Origin and collected year
	n	LC_{50} (mg/L) (95% CL)	RR	*n*	LC_{50} (mg/L) (95% CL)	RR	
TSC	211	123.0 (106.6–141.6)	1.3	239	197.2 (167.2–231.3)	1.1	Tokyo, Japan, 1991
NGY	210	105.6 (91.4–120.9)	1.1	240	177.6 (152.9–204.0)	1	Aichi, Japan, 1997
IWT	210	95.0 (82.1–108.4)	1	238	253.4 (229.2–278.7)	1.4	Shizuoka, Japan, 1999
THR	210	121.2 (106.9–137.0)	1.3	240	225.4 (198.4–254.3)	1.3	Tokyo, Japan, 1999
SKG	241	4953.4 (4470.7–5487.1)	52.2	119	>10000 (—)	>56.3	Fukushima, Japan, 2010
C87	239	3790.2 (3400.9–4156.6)	39.6	121	>10000 (—)	>56.3	Thika, Kenya, 2001
MLY	240	4451.7 (3943.2–4981.7)	46.9	119	>10000 (—)	>56.3	Shah Alam, Malaysia, 2012

Resistance ratio (RR): LC_{50} of each strain/ LC_{50} of the most susceptible strain. CL: confidence limit. *n*: The number of insects examined.

the 325 bp DNA fragments with the F1534S site was conducted using 5′-cgatcacgtcggaaaggctt-3′ and 5′-ttcgaagaggatcgcttgtg-3′. The PCR conditions were 1 cycle of 3 min at 94°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C, and a final extension of 72°C for 7 min. Quick Taq HS DyeMix (Toyobo Co., Ltd.) was used for PCR amplification. The amplified DNA fragments were cloned into a pMD20 Vector (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions.

5. Genomic DNA extraction and PCR amplification of sodium channel gene sequences

Genomic DNA was extracted individually using a sample-preparation reagent (PrepMan Ultra; Thermo Fisher Scientific K.K., Tokyo, Japan). Briefly, a single insect was introduced into 20 μL of the reagent and incubated at 95°C for 10 min, followed by standing at room temperature for 2 min. After centrifugation at 15,000×g for 2 min, the supernatant was recovered as a DNA sample. The DNA sample (0.5 μL) was used for PCR amplification of the sodium channel gene fragments.

The 606 bp DNA fragments with the T929I site were amplified using the primers 5′-agaagccagcatgaagctgg-3′ and 5′-ctcaagagcaacgccaagaa-3′. Subsequently, a nested PCR was conducted using primers 5′-actacttccaggaaggctgg-3′ and 5′-cgatgactactgtcgctaga-3′ to amplify the 465 bp DNA fragments. Similarly, for amplification of the 383 bp DNA fragments with the F1534S site, a first PCR using 5′-cgatcacgtcggaaaggctt-3′ and 5′-caccaaacgaagaactctgc-3′ was followed by a second PCR using 5′-cgatcacgtcggaaaggctt-3′ and 5′-ttcgaagaggatcgcttgtg-3′. The PCR conditions were as described above. A Tks Gflex DNA polymerase (Takara Bio Inc.) was used for PCR amplification. When a sufficient amount of DNA with high purity is extracted, the first PCR might be avoided.

6. Genotyping for the T929I and F1534S sites

The DNA fragments with the T929I site (465 bp) amplified from genomic DNA were digested with a restriction enzyme, *Hpy*AV. Then 5 μL of PCR products out of 10 μL was incubated at 37°C for 3 hr with 0.4 units of enzyme and 0.5 μL of the manufacturer-supplied buffer. Reliability of the method was confirmed by nucleotide sequencing using some amplified DNA fragments (data not shown). The F1534S sites on the amplified DNA fragments were sequenced directly using the primer 5′-tttccatcttggtcgcgg-3′.

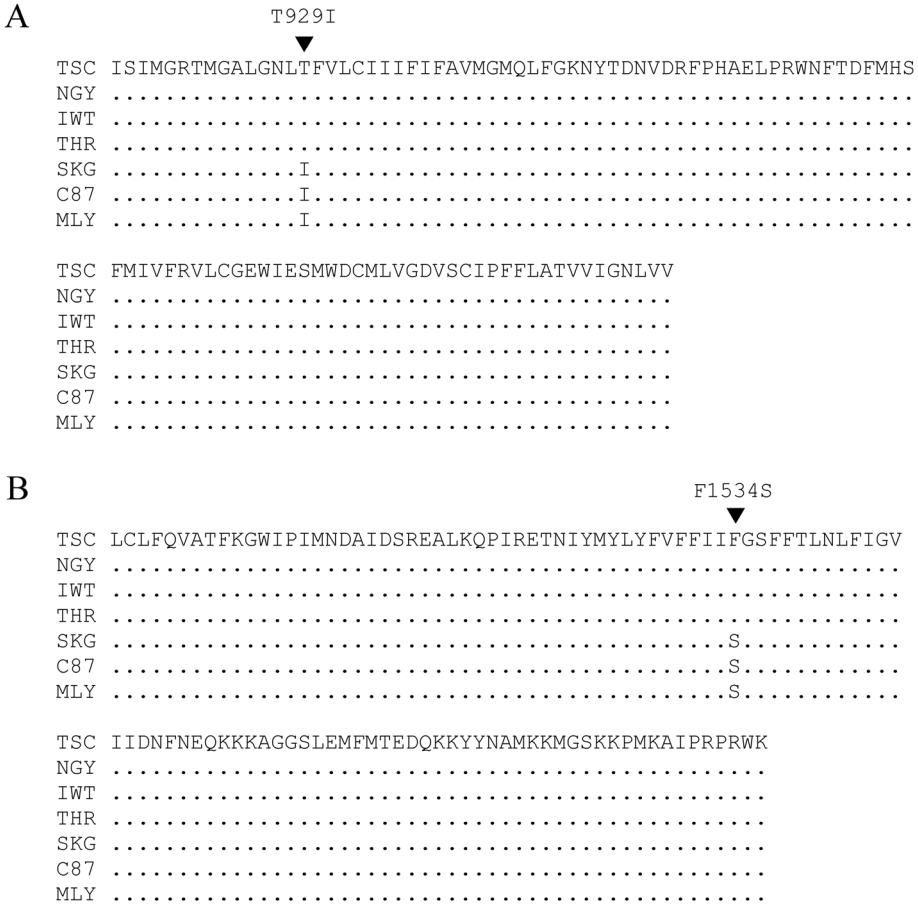

Fig. 1. Deduced amino acid sequences of the domain IIS4–IIS6 (A) and IIIS6 (B) regions in the sodium channels of seven *L. serricorne* strains. The sequences encoded in the DNA fragments amplified from cDNAs are shown. Identical amino acids are represented by dots. The positions of the T929I and F1534S mutations are represented by triangles.

7. Plasmid preparation and nucleotide sequencing

The plasmid DNA used for nucleotide sequencing was purified using a Plasmid Mini Extraction Kit (Bioneer Corp., Daejeon, South Korea). The nucleotide sequence was determined using a dye terminator cycle sequencing kit (Thermo Fisher Scientific K.K., Tokyo, Japan) and a DNA sequencer (3500 Genetic Analyzer; Applied Biosystems, Waltham, MA, USA). Nucleotide and deduced amino acid sequences were analyzed using software (Genetyx ver. 13; Genetyx Corp., Tokyo, Japan).

Results and discussion

Based on LC_{50} values for six pyrethroids, the seven *L. serricorne* strains were divided into two groups: pyrethroid resistant (SKG, C87, and MLY) and pyrethroid susceptible (TSC, NGY, IWT, and THR) (Table 1). The three resistant strains were collected in Japan, Malaysia, and Kenya. The pyrethroid resistance of *L. serricorne* was also reported in Germany.[10] These results suggest the worldwide prevalence of pyrethroid-resistant insects in *L. serricorne*.

The numbers of RNA-seq reads (raw reads and filtered reads) of each strain are presented in Supplemental Table S1. Sequence data have been deposited in the DDBJ Sequence Read Archive (DDBJ Sequence Read Archive accession nos. DRR299545–DRR299551). The predicted amino acid sequence obtained from the NGY strain (1,801 amino acids) was found to have the highest identity (87.1%) with that of *B. aeneus* (UniProtKB accession no. AJM87404). Comparison of the predicted amino acid sequences revealed mutations in the resistant SKG (T929I and F1534S mutations), C87 (T929I mutation), and MLY (F1534S mutation) strains (numbering according to *Musca domestica*) (data not shown). The transcript sequences obtained from the MLY and C87 strains did not include the T929I and F1534S sites, respectively, possibly because of the shortage of RNA-seq reads corresponding to the sodium channel (data not shown).

DNA fragments amplified from the cDNA of the seven strains were cloned and sequenced. The predicted amino acid sequences of the amplified DNA fragments confirmed the presence of the T929I and F1534S mutations for the SKG strain, the T929I mutation for the C87 strain, and the F1534S mutation for the MLY strain and indicated the presence of the T929I mutation for the MLY strain and the F1534S mutation for the C87 strain (Fig. 1).

The importance of the T929I mutation, located at domain IIS5, in the channel properties and sensitivities to pyrethroids has been elucidated by electrophysiological studies using *Xenopus* oocytes.[14–17] The T929I mutation was found together with the L1014F mutation in pyrethroid-resistant strains of *Plutella xylostella*[18] and *Pediculus humanus capitis*.[19] Similarly, the T929C and T929N mutations located at the same position were linked with the L1014F mutation in *Frankliniella occidentalis*[20] and *L. decemlineata*.[8] In some insects, such as *Thrips tabaci*,[21] *Thrips palmi*,[22] and *S. zeamais*,[9] the T929I mutation was found as a single mutation. In this study, the L1014F mutation was not found in any of the seven strains. Consequently, the T929I mutation is not linked with the L1014F mutation in *L. serricorne*.

The association of the F1534S mutation, located at domain IIIS6, with pyrethroid resistance has been reported in *Aedes albopictus*[23] and *Tetranychus urticae*.[24] In *Ae. albopictus*, the F1534S mutation was found as a single mutation. However, the F1534S mutation was found together with the F1538I mutation in *T. urticae*. To ascertain the conjunctive relation between the T929I and F1534S mutations in *L. serricorne*, the DNA fragments with the T929I site or those with the F1534S site were amplified from individually extracted genomic DNA for the seven strains. The T929I site of the amplified fragments was examined by digesting it with *Hpy*AV. *Hpy*AV digestion produced a single 465 bp fragment (no restriction site) for resistant homozygotes, fragments of 218 bp and 247 bp for susceptible homozygotes, and all fragments for heterozygotes (Supplemental Fig. S1). It should be noted that fragments of 218 bp and 247 bp might be observed as a single band on an agarose gel, as shown in Supplemental Fig. S1B. The amplified DNA fragments with the F1534S site were sequenced directly. The results revealed that the T929I mutation is mostly found together with the F1534S mutation, but not always (Table 2). Insects homozygous for I929 and F1534 were found in the SKG and C87 strains. However, no insect homozygous for T929 and S1534 was found in any resistant strain. Insects homozygous for the I929 mutation and heterozygous for the F1534S site were found in the C87 and MLY strains. The cause of detection of the T929I mutation in the susceptible

Table 2. Genotyping of the T929I and F1534S sites in the sodium channel genes of *L. serricorne* strains

Strain	*n*	T929I			F1534S		
		SS (T929/T929)	SR (T929/I929)	RR (I929/I929)	SS (F1534/F1534)	SR (F1534/S1534)	RR (S1534/S1534)
TSC	10	10			10		
NGY	16	16			16		
IWT	11	11			11		
THR	10	9	1		10		
SKG	21			21	3		18
C87	23	2	1	20	3	1	19
MLY	23			23		1	22

SS: Susceptible homozygote, SR: heterozygote, RR: resistant homozygote. *n*: The number of insects examined.

THR strain remains unclear.

Reportedly, the F1534S mutation substantially reduced sodium channel sensitivity to type I pyrethroids, permethrin and bifenthrin, in *Ae. albopictus* but not to type II pyrethroids, deltamethrin and cypermethrin.[25] In the present study, the LC_{50} values against type I pyrethroids, permethrin and bifenthrin, were found to be greater than those against type II pyrethroids, cypermethrin and cyfluthrin (Table 1). The T929I mutation is known to reduce the sensitivity to both types of pyrethroids.[15] These observations suggest that the F1534S mutation plays an important role in enhancing resistance to type I pyrethroids, while the T929I mutation confers a basal level of resistance to pyrethroids in *L. serricorne*. If this is the case, detection of the T929I mutation using the PCR-RFLP method, as in this study, might be useful as a primary tool for detecting resistant insects. Further analyses of their functional relations remain tasks for future investigations.

For this study, we identified the T929I and F1534S mutations associated with the target-site-insensitive pyrethroid resistance of *L. serricorne*. Reportedly, cytochrome P450 (CYP450)-mediated detoxification is involved in the pyrethroid resistance of diverse insect pests.[26] To examine the involvement of CYP450 in the pyrethroid resistance of *L. serricorne*, synergism tests using piperonyl butoxide, an inhibitor of CYP450, and RNA-seq data analyses using more biological replicates must be conducted in future studies.

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Electronic supplementary materials

The online version of this article contains supplementary materials (Supplemental Table S1 and Supplemental Fig. S1), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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