

The Knockdown Resistance (*kdr*) Mutation in Pyrethroid-Resistant German Cockroaches

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A point mutation in the *para*-homologous sodium channel gene has been shown to be associated with knockdown resistance (*kdr*) in several insect species including the German cockroach. In this study, we analyzed the genomic organization of the region where the *kdr* mutation resides and then performed polymerase chain reaction (PCR) and sequencing using genomic DNA as the template to detect *kdr* mutation in 24 pyrethroid-resistant German cockroach strains, most of which have been collected recently from the field. The *kdr* mutation, G to C at nt 2979 resulting in a leucine to phenylalanine amino acid substitution, was detected in 20 strains including 2 strains from overseas (China and Germany). Our results clearly indicate that the *kdr* mutation is widespread in German cockroach populations. However, the super-*kdr* mutation detected in super-*kdr* house flies was not found in any of the 4 strains that showed higher levels of knockdown resistance. Little correlation was observed between the presence of the *kdr* mutation and the level of knockdown resistance, suggesting the existence of multiple resistance mechanisms in many of these strains. ©1998 Academic Press

INTRODUCTION

The German cockroach is an important household insect pest. It is a major source of indoor allergens and responsible for an increased incidence of asthma (1). Pyrethroid insecticides are a large group of highly insecticidal compounds that are widely used in German cockroach control. Unfortunately, extensive use of these insecticides has led to the development of pyrethroid resistance resulting in control failures in German cockroach field populations (2–5).

Knockdown resistance (*kdr*)³ to pyrethroid insecticides, due to reduced neuronal sensitivity to these compounds, has been studied extensively in the German cockroach (6–12). One striking feature of *kdr* is its ability to confer

cross-resistance to the entire class of pyrethroids as well as DDT. VPIDLS was the first German cockroach strain described to exhibit *kdr*-type resistance (6). In electrophysiological experiments this strain exhibited reduced neuronal sensitivity to permethrin and DDT. Similar experiments also showed that another pyrethroid-resistant cockroach strain from Japan, Ocolony, possessed a *kdr*-type mechanism (7). Bioassays using synergists and pyrethroid metabolism studies have implicated the existence of a *kdr*-type mechanism in the Village Green strain (14).

Modification of voltage-gated sodium channels has long been suspected in the *kdr* mechanism based on cross-resistance of *kdr* insects to several sodium channel site 2 neurotoxins. Indeed, genetic studies have shown that *kdr* resistance in several insect species is closely linked to the *para*-homologous sodium channel gene (10, 15–17). Recently, several research groups have independently cloned and sequenced *para*-homologous genes from several

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³ Abbreviations used: *kdr*, knockdown resistance; PCR, polymerase chain reaction; RT, reverse transcriptase; PBO, piperonyl butoxide; DEF, S,S,S-tributyl phosphotriothioate; kb, kilobase; bp, base pair.

susceptible and *kdr*-resistant insects (12, 13, 18–20). Remarkably, an identical amino acid substitution, from leucine⁹⁹³ (G at nt 2979) to phenylalanine⁹⁹³ (C at nt 2979) of cockroach *para* sodium channel protein (13), in segment 6 of domain II (IIS6) was found in all *kdr*-type German cockroaches and house flies (12, 13, 19). A substitution from leucine to histidine at a position corresponding to leucine⁹⁹³ in the cockroach *Para* protein was also found in pyrethroid-resistant tobacco budworm strains (20). In addition, super-*kdr* house flies, which possess a higher level of resistance to pyrethroids than *kdr* house flies (21), carry an additional mutation that changes methionine⁹¹⁸ to threonine⁹¹⁸ (19).

An important question which remains to be answered is how widespread are the *kdr* and super-*kdr* mutations in insect populations in the field? In this study we first determined the cypermethrin resistance levels in 22 German cockroach strains collected recently from the United States and two strains from overseas (Germany and China). We then examined the occurrence of the *kdr* and super-*kdr* mutations in these strains. For this purpose, we conducted screening bioassays and analyzed the genomic organization of the IIS3-S6 region of the *para* gene where the *kdr* and super-*kdr* mutations are found. Genomic regions where *kdr* and super-*kdr* mutations reside were then amplified by the polymerase chain reaction (PCR) and sequenced to detect *kdr* or super-*kdr* mutations in these strains.

MATERIALS AND METHODS

Strains

German cockroach strains used in this study are listed in Tables 1 and 2. Most strains were collected from the field and maintained in the laboratory without insecticide selection, except for Cypermethrin-Select and Village Green. Field collections were accomplished with a vacuum device (22). Cypermethrin-Select is the pyrethroid-resistant F₃ cypermethrin-selected progeny of a cross between a pyrethroid-resistant strain Munsyana (see Ref. 23 for details) and Johnson Wax, a susceptible strain. In addition to

those listed in Tables 1 and 2, CSMA (a susceptible strain) and Ectiban-R (a *kdr*-type strain), which were described previously (8), were used to isolate genomic DNA for analysis of *para* sodium channel genomic sequences.

Chemicals

S,S,S-tributyl phosphorotrithioate (DEF) (98% AI) was purchased from ChemService (Westchester, PA). Piperonyl butoxide (PBO) (90%) was purchased from Aldrich (Milwaukee, WI). Cypermethrin was a gift of Zeneca (Richmond, CA). All other chemicals were purchased from commercial suppliers.

Bioassays

To screen for potential *kdr* individuals in each strain, cockroaches were first treated with a mixture of the cytochrome P-450 monooxygenase inhibitor PBO (100 µg/cockroach) and the esterase inhibitor DEF (30 µg/cockroach) by topical application. One hour after the synergist treatment, cockroaches were subjected to a residue bioassay (10). Briefly, 300 µg of cypermethrin in 2 ml acetone was delivered to the walls of 1-pint jars by rotating the jars until the acetone evaporated. PBO- and DEF-pretreated cockroaches were subsequently placed into treated jars (25 individuals per jar, two jars per strain). The first five and the last five individuals and their corresponding times to knockdown were recorded. Knockdown was defined as the inability to exhibit coordinated walking after prodding with forceps. The first five and the last five knocked-down cockroaches were individually flash-frozen in liquid nitrogen for isolation of genomic DNA.

To determine the levels of resistance to the pyrethroid insecticide cypermethrin, a residue bioassay was used for six strains (Table 2) and a topical bioassay was conducted for the rest of the strains (Table 1). Residue bioassays were similar to that described above, except that 10 adult male cockroaches were placed in a 1-pint jar coated with 500 µg of cypermethrin. Topical bioassays were performed as described by Valles *et al.* (24). At least three replicates containing 10

TABLE 1
Toxicity of Topically Applied Cypermethrin to Adult Males of Various German Cockroach Strains

Strain	<i>n</i>	Slope ± SE	LD ₅₀ (95%CI) ^a	χ ²	RR ^b	Origin (year)
Orlando	120	5.69 ± 1.54	0.042 (0.038–0.047)	1.03	1	
Johnson Wax	150	5.90 ± 1.3	0.029 (0.024–0.034)	3.1	1	
Levy 405	150	2.32 ± 0.33	0.21 (0.17–0.27)	0.80	5 _{Orl}	Florida (1996)
Swine	120	3.03 ± 0.48	0.22 (0.17–0.26)	2.51	5 _{Orl}	Florida (1996)
Muncie'86	150	4.60 ± 0.70	0.15 (0.12–0.17)	1.3	5 _{JW}	Indiana (1986)
Sacramento	90	2.80 ± 0.61	0.36 (0.27–0.46)	1.31	9 _{Orl}	California (1996)
Shanghai ^c	—	—	—	—	14	China (1993)
Levy 616	150	1.22 ± 0.38	0.79 (0.20–1.24)	3.86	19 _{Orl}	Florida (1996)
Marietta ^d	150	3.8 ± 0.47	1.27 (1.06–1.45)	1.25	30 _{Orl}	Georgia (1992)
Union 507	150	1.99 ± 0.38	1.76 (1.34–2.28)	0.92	42 _{Orl}	Florida (1996)
Village Green ^e	150	3.99 ± 0.47	2.0 (1.78–2.77)	—	48 _{Orl}	Florida (1988)
Union 511	120	1.96 ± 0.49	2.63 (1.96–3.73)	1.01	63 _{Orl}	Florida (1996)
Woodland	150	1.47 ± 0.27	2.67 (1.90–4.00)	0.94	64 _{Orl}	Florida (1996)
Pinellis 214	150	2.24 ± 0.34	3.21 (2.53–4.27)	0.25	76 _{Orl}	Florida (1996)
Malo	150	1.40 ± 0.37	3.37 (2.36–6.96)	1.90	80 _{Orl}	Florida (1996)
Munस्याna	180	2.80 ± 0.70	2.88 (2.34–3.38)	4.20	99 _{JW}	Indiana (1994)
Fuerte	150	1.60 ± 0.30	7.74 (5.59–10.83)	4.60	184 _{Orl}	California (1996)
Parkview	150	2.90 ± 0.40	6.21 (5.36–7.31)	5.90	214 _{JW}	Indiana (1994)

^a Microgram per insect, 24 h mortality.

^b LD₅₀ resistant strain/LD₅₀ susceptible strain (Orl, Orlando; JW, Johnson Wax).

^c From Robinson (Personal communication).

^d Data taken from Valles and Yu, 1996.

^e Data taken from Atkinson *et al.*, 1991.

cockroaches per jar or per dose were conducted. Bioassay data were analyzed by probit analysis (25).

PCR and Sequencing

For analysis of genomic sequences containing the IIS3-6 region between leucine⁸³³ and alanine¹⁰²⁵ (13), genomic DNA was isolated from cockroaches following the method described by Dong and Scott (10). A sense primer, GL1F5 (5'GTTGATAGCAATGAACCCTAAGTA3'), and an antisense primer, PASA2 (5'GCAATCTTGTTGGTTTCATTGTC3'), were used in PCR to amplify a 4.5-kb fragment containing the IIS3-6 region. The locations of the two primers in the cockroach Para sequence are indicated in Fig. 1A. The 50-μl PCR mixture contained 0.5 μl genomic DNA (1 μg/μl), 0.5 μmol of each primer, 200 μM of each dNTP, 5 μl buffer A (Gibco/BRL), 5 μl buffer B (Gibco/BRL) and 1 U of eLONGASE Enzyme Mix (Gibco/BRL). Thirty amplification cycles of 30 s at 94°C, 30

s at 58°C, and 5 min at 68°C were performed. The PCR products were extracted with an equal volume of phenol:chloroform:isoamylalcohol (5:4:1) followed by agarose gel electrophoresis. The 4.5-kb fragment was then isolated from agarose gel using the Prep-A-Gene kit (Bio-Rad) and used for sequencing.

For detection of possible *kdr* and super-*kdr* mutations, genomic DNA prepared from individual cockroaches was used to amplify a 220-bp DNA fragment where the *kdr* mutation resides, and a 230-bp fragment where the super-*kdr* mutation resides. Primers IR1 and PASA1 (Fig. 1) were used for the amplification of the 220-bp fragment. Primers F5P2R3 and 2S5 (Fig. 1) were used to amplify the 230-bp fragment. The PCR products were extracted with an equal volume of phenol:chloroform:isoamylalcohol (5:4:1). Excess amount of primers were removed using Ultrafree-MC 30,000 NMWL filter units (Millipore) prior to sequencing. The PCR products of the first and last five knocked down

TABLE 2

Comparison of Time-Knockdown Response of Seven Resistant *B. germanica* Strains to Two Susceptible Strains

Strain, U.S. State or, Country	Slope \pm SE	χ^2	KT ₅₀ (95% CI) min	RR ^a
FT. Knox A, Kentucky	1.8 \pm 0.1	34.3	427 (368–515)	28
USDA, susceptible	5.3 \pm 0.3	21.9	15 (14–17)	
Ft. Knox B, Kentucky	1.2 \pm 0.07	30.4	536 (439–689)	35
USDA, susceptible	5.3 \pm 0.3	21.9	15 (14–17)	
APG, Maryland ^b			>1440	>96
USDA, susceptible	5.3 \pm 0.3	21.9	15 (14–17)	
Ft. Riley, Kansas	1.9 \pm 0.26	8.2	1234 (869–2229)	104
USDA, susceptible	7.3 \pm 0.6	9.1	12 (10–14)	
Ft. Leavenworth, Kansas	1.4 \pm 0.07	73.3	238 (201–293)	17
USDA, susceptible	5.5 \pm 0.4	19	14 (12–16)	
Hanau, Germany	1.2 \pm 0.07	37	210 (182–247)	18
USDA, susceptible	7.3 \pm 0.6	9.1	12 (10–14)	
Cypermethrin-Select	6.3 \pm 0.9	1.6	56 (51–61)	6
Johnson Wax, susceptible	10.6 \pm 1.4	0.4	8 (7.5–8.9)	

^a Resistance ratio (RR) was calculated as ratio of KT₅₀ of test strain/KT₅₀ of susceptible strain.

^b There was no significant knockdown response in the APG strain.

individuals were combined for sequencing analysis. For the last five knocked-down individuals of the Marietta and Levy 405 strains, the PCR products of individual cockroaches were sequenced separately. DNA sequencing was performed in the W. M. Keck Laboratory at Yale University.

RESULTS

Bioassay Results

The bioassay results presented in Tables 1 and 2 indicate that all the strains are more resistant to cypermethrin compared with the susceptible strains (Johnson Wax or Orlando). Resistance ratios ranged from 5- to 214-fold. Slope values of dose-probit mortality curves were smaller for most resistant strains compared with those of susceptible strains (Tables 1 and 2), indicating heterogeneity of the resistant strains with regard to cypermethrin resistance. This result was expected as most strains have been collected recently from the field without further selection in the laboratory.

Sequence Analysis of Genomic DNA

Corresponding to the IIS3-IIS6 Region

The composite sequences of the *para* genes from the CSMA (*para*^{CSMA}) and Ectiban-R (*para*^{Ectiban-R}) strains were determined previously by

sequencing overlapping cDNA clones or reverse transcription (RT)-PCR products of *para* genes using messenger RNA of pooled cockroach individuals (13). To facilitate the detection of *kdr* or super-*kdr* mutations in individual cockroaches by PCR, we chose genomic DNA isolated from individual cockroaches as the template in PCR because isolation of messenger RNA from individual cockroaches and subsequent synthesis of cDNA proved to be technically difficult and time consuming. Therefore, we determined the *para* genomic sequence (both exon and intron) corresponding to the IIS3-IIS6 cDNA region. A 4.5-kb DNA fragment was amplified using the CSMA genomic DNA as the template and GLIF5 and PASA2 as primers (see Materials and Methods). The 4.5-kb DNA fragment was gel-purified and sequenced by primer walking. Alignment with the German cockroach *para*^{CSMA} cDNA sequence (13) confirmed that the 4.5-kb fragment corresponds to the IIS3-IIS6 cDNA region (Fig. 1A). The exon sequences in this region were interrupted by three introns of 0.8, 1.5, and 1.6-kb, respectively (Fig. 1A). The partial sequences of these three introns are presented in Fig. 1B. The same genomic organization of this region was found in *para*^{Ectiban-R}. The exon sequences were identical

to the corresponding cDNA sequences, including nt 2979 where the *kdr* mutation was found: G²⁹⁷⁹ in *para*^{CSMA} and C²⁹⁷⁹ in *para*^{Ectiban-R} (13). There is no evidence of any alternative exons or particular sequences that would indicate generation of G²⁹⁷⁹/C²⁹⁷⁹ by genomic or cDNA reorganization. On the contrary, the exons and sequenced portions of the introns in this region are identical in CSMA and Ectiban-R except for G²⁹⁷⁹/C²⁹⁷⁹, indicating that the *kdr* mutation in the Ectiban-R German cockroach strain most likely arose from a precise point mutation, rather than from genomic reorganization. Interestingly, the arrangement of exons and introns in this region is very similar to that of *Drosophila melanogaster* and *D. vilirix para* genes (26).

Design of Primers for Single-Pass Sequencing Analysis of kdr and Super-kdr Mutations using Genomic DNA as Templates

The *kdr* mutation at nt 2979 is located 3 bp upstream of the 1.6-kb intron (Fig. 1A). To amplify a DNA fragment that contains the *kdr* mutation and that is suitable for single-pass sequencing analysis, an antisense primer, IR1, which corresponds to the sequence of the 5' end of the 1.6-kb intron was designed (Fig. 1B). Primers IR1 and PASA1 were used in PCR to amplify a 220-bp genomic DNA fragment. Another pair of primers, F5P2R3 and 2S5 (Fig. 1), were used to amplify a 230-bp genomic DNA fragment containing the nucleotide where the super-*kdr*-associated mutation was found in house flies. The amplified DNA fragments were sequenced using either PASA1 or F5P2R3 primers. Under the conditions used in this study, the success rate of sequencing PCR-amplified DNA fragments was 100%.

Survey of kdr and Super-kdr Mutations

In addition to the observation of the apparent heterogeneity in cypermethrin resistance in many of the strains, we also made an assumption that most strains possess multiple resistance mechanisms (i.e., metabolic detoxication, *kdr*, reduced cuticular penetration, etc.). Because we were interested solely in the detection of *kdr* in

each strain, PBO and DEF were topically applied prior to the residue bioassay to minimize the contribution of metabolic detoxication to knockdown resistance. If *kdr* (i.e., C²⁹⁷⁹) was present in a given population we expected that the last five knocked-down individuals would likely contain the *kdr* mutation(s) and the first five would not, provided the *kdr* mutation contributed to knockdown resistance in that strain. Genomic DNA was isolated from each of the 10 individuals and was used as the template in PCR to amplify the region carrying the *kdr* or super-*kdr* mutation (as described under Materials and Methods). Sequence results and knockdown times for each strain are presented in Table 3. The *kdr* mutation at nt 2979, i.e., C²⁹⁷⁹, was not detected in the last five knocked-down individuals in the Johnson Wax, Muncie'86, Fort Leavenworth, Swine, and Puerto Rico strains. In contrast, the *kdr* mutation (C²⁹⁷⁹) was detected in the last five knocked-down individuals of the remaining 20 strains, including those collected from China and Germany. Interestingly, only C²⁹⁷⁹ was detected in the last five knocked-down individuals of 12 strains (Table 3), indicating that the five individuals in these strains most likely were homozygous for the *kdr* mutation (RR). In contrast, both G²⁹⁷⁹ and C²⁹⁷⁹ were found in the last five knocked-down individuals of the remaining 8 strains including Marietta and Levy 405. Because the PCR products of the last five knocked-down individuals were pooled for sequencing analysis, we expected to detect both G²⁹⁷⁹ and C²⁹⁷⁹ if some of the five individuals only carry G²⁹⁷⁹ (SS) and others only carry C²⁹⁷⁹ (RR) or if they are heterozygous (RS), carrying both G²⁹⁷⁹ and C²⁹⁷⁹. To examine the genotype of the last five knocked-down individuals, the PCR products of these individuals from the Marietta and Levy 405 strains were individually sequenced. In Marietta, one individual was found to be homozygous for C²⁹⁷⁹ (RR), while the remaining four possessed G²⁹⁷⁹ (SS). In Levy 405, two individuals were homozygous for G²⁹⁷⁹ (SS), while three were heterozygous (RS) carrying both G²⁹⁷⁹ and C²⁹⁷⁹. These results confirmed that the detection of both G²⁹⁷⁹ and C²⁹⁷⁹

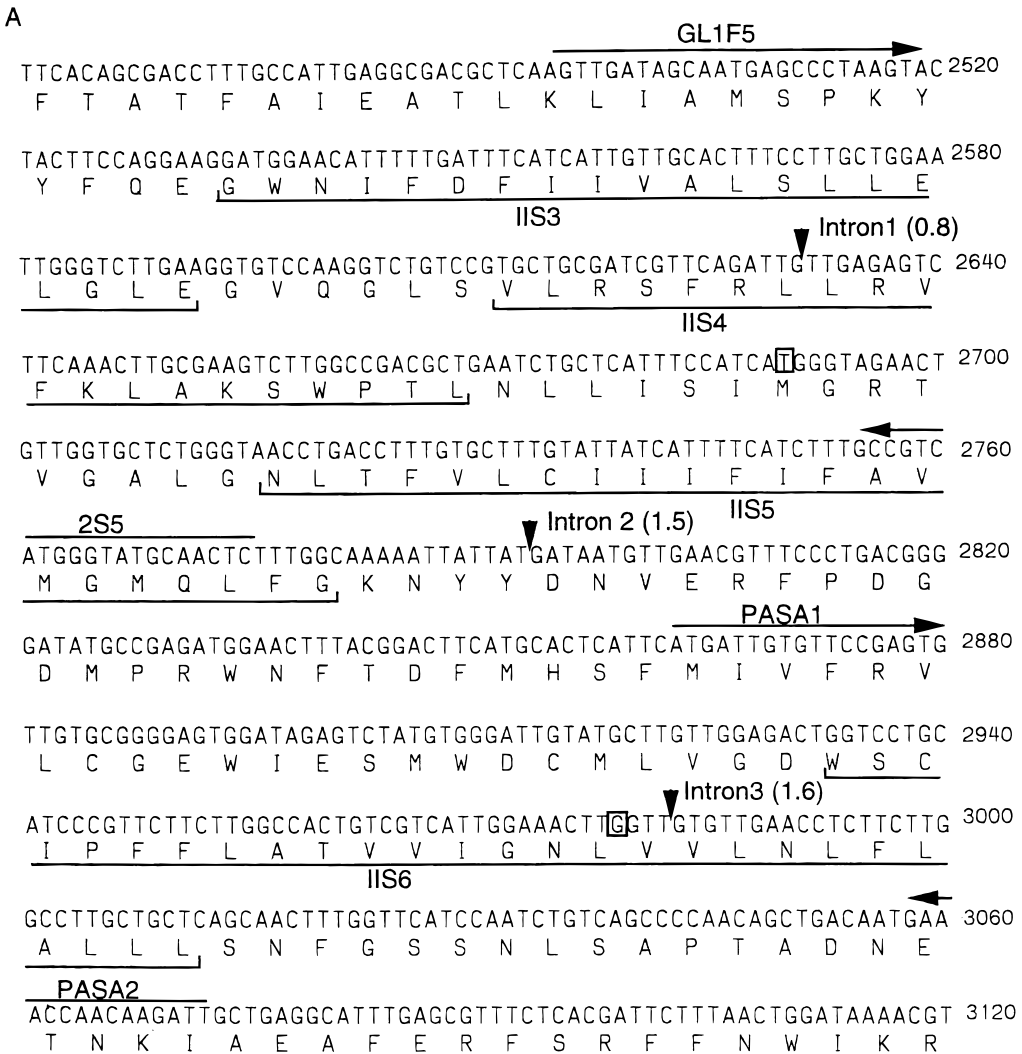


FIG. 1. Genomic organization of the IIS3-6 region of the German cockroach *para* gene. (A) Intron positions in the IIS3-6 region. Nucleotide and amino acid sequences of the IIS3-6 region are presented. The nucleotide sequence is numbered on the right. The IIS3-6 regions are indicated. Locations of three introns are marked by arrowheads. The intron sizes are given in kb. The locations of PCR primers, GL1F5, 2S5, PASA 1, and PASA 2, are indicated by arrows above the nucleotide sequences. The nucleotide T (in box) in the linker region between IIS4 and IIS5 is substituted by a C in super-*kdr* house fly strains. The G (in box) in IIS6 is substituted by a C in *kdr* house fly and German cockroach strains. (B) Portions of intron sequences. The locations of two primers (F5P2R3 and IR1) are marked with arrows. Consensus splice sites flanking exons are underlined.

in the last five individuals of 8 strains could result from the presence of RS individuals or from a mixture of SS and RR individuals. These results also demonstrated that our method of combining PCR products of five cockroaches for DNA sequencing is sensitive enough to

detect the C^{2979} mutation when only one of the five individuals possesses C^{2979} .

We also surveyed the first five knocked-down individuals of nine strains for the *kdr* mutation. The first five knocked-down individuals of Marjietta, Union 507, Union 511, and Pinellis 214

B

Intron 1

gtaagttcagttaggctaaaggtaataatgaacaacttcaaaatgttttagttatgtttagaatgcttct
 tatgtgggtaacttgagatagaaacccatantccagtggtctangttatggtacaaaatcaaaactt-

 ----- tggaattttcaatatgtataaacctttctcattgagttggtgaattcc
 cattatttttgtfactcctttattatatgaaattaaattatgattgatctttcag

F5P2R3

Intron 2

ggtgagtgatgctcttcacattctttgtacttacactaatattatctgaatccaatatatttccttaattgc
 atattaaattcagacctatgttctataaaatgtttgtaaataaacacacaaacaaaattcacaca
 caaaatgatgacagaggagttcaaatgaaataaaatagtagttatacctaataaaacagatttaa

 -----tttggtgaaaggctctgtattttgtgagtaaaaaaaaaatgtttccgttggca

Intron 3

gtaagt agaaataattgaaccatctgaattgctcttttttaaaattttatgtatttgagtgaacca
 gaaatctatcaaaaatcttcacagggtggcagggatattttctgaatgttt-----

 ----- gcaaggatgatgccaacaaatttctagatactgtataactgtttgt ttcag

IR1

FIG. 1—Continued

were found to carry only G^{2979} , whereas both C^{2979} and G^{2979} were detected among the first five knocked-down individuals of Malo. In contrast, only C^{2979} was detected in all first five knocked-down individuals of the Cypermethrin-Select, Shanghai, and Hanau strains.

Higher levels of knockdown resistance were detected in Malo, Fuerte, Parkview, and Marietta compared to other strains (Table 3). To examine whether the super-*kdr* mutation is present in the *para* gene of these four strains, we amplified and sequenced the 230-bp PCR fragments where the super-*kdr* mutation was detected in super-*kdr* strains of house fly using genomic DNA from the last five individuals of these four strains. The super-*kdr* mutation was not detected

in any of the German cockroach strains surveyed.

DISCUSSION

In this study, we have examined the occurrence of the *kdr* mutation among field-collected German cockroach strains. Our results clearly show that the *kdr* mutation is widespread in German cockroach populations in the United States. The *kdr* mutation was also detected in pyrethroid-resistant strains collected from China and Germany. Only 4 of the 24 strains surveyed do not appear to carry the *kdr* mutation. Interestingly, the super-*kdr* mutation was not detected in the 4 German cockroach strains that exhibit high levels of resistance.

TABLE 3
Detection of the *kdr* Mutation in Pyrethroid-Resistant German Cockroach Strains

Strain	Origin (yr)	Time to knockdown ^a (min)		<i>kdr</i> mutation ^b	
		First five	Last five	First five	Last five
Johnson Wax		2–5	14	No	No
Muncie'86	Indiana (1986)	10	30–40	— ^d	No
Ft. Leavenworth	Kansas (1996)	12	16–18	—	No
Puerto Rico	Puerto Rico (?) ^c	5–15	20–30	—	No
Swine	Florida (1996)	15–20	60–85	No	No
APG	Maryland (1996)	20	45–50	—	Yes*
Ft. Riley	Kansas (1996)	20	35–40	—	Yes*
Marietta	Georgia (1992)	17–30	175–190	No	Yes*
Union 511	Florida (1996)	22–24	175–180	No	Yes*
Village Green	Florida (1988)	15–21	85–95	—	Yes*
Levy 616	Florida (1996)	16–20	110–125	—	Yes*
Levy 405	Florida (1996)	24–33	95–100	—	Yes*
Woodland	Florida (1996)	14–30	119	—	Yes*
Malo	Florida (1996)	50–81	275–300	Yes*	Yes
Munsyana	Indiana (1994)	20	180	—	Yes
Parkview	Indiana (1994)	<20	180	—	Yes
Cypermethrin Select	Indiana (1995)	10	20	Yes	Yes
Ft. Knox A	Kentucky (1995)	<10	30–40	—	Yes
Ft. Knox B	Kentucky (1995)	<10	30–40	—	Yes
Union 507	Florida (1996)	17–30	100–120	—	Yes
Sacramento	California (1996)	17–24	40–55	—	Yes
Pinellis 214	Florida (1996)	8–37	120	No	Yes
Fuerte	California (1996)	14–48	280	—	Yes
Hanau	Germany (1996)	22	30	Yes	Yes
Shanghai	China (1993)	5	25	Yes	Yes

^a The time at which the first five (first 10%) or last five (last 10%) individuals of 50 cockroaches were knocked down in the residue bioassay described under Materials and Methods.

^b C²⁹⁷⁹.

^c (?) Date of collection unknown.

^d Analysis not performed.

* Both C and G were detected at nt 2979.

In a previous study (13), a good correlation was observed between the presence of the *kdr* mutation and a higher level of knockdown resistance to deltamethrin in the O-colony German cockroach strain that appears to possess only the *kdr* mechanism (7). In the present study, however, no direct relationship between the presence of the *kdr* mutation and the high levels of knockdown resistance to cypermethrin was noted (Table 3). The level of knockdown resistance varied drastically among cypermethrin-resistant strains that carry the *kdr* mutation. For example, Cypermethrin-Select, Shanghai, and Hanau strains all carry the *kdr* mutation, but tested individuals of these strains were knocked

down within 30 min. In contrast, the knockdown time for the last five individuals (all carrying the *kdr* mutation) of the Malo strain was more than 4 h. Interestingly, the time to knockdown for the last five individuals of the Swine strain that lacks the *kdr* mutation was almost 1 h. These results strongly suggest that in these field-collected strains the *kdr* mutation alone may not confer high levels of knockdown resistance and high levels of the knockdown resistance observed could be the result of a synergistic effect of *kdr* and other resistance mechanisms. It is possible that additional mutations in the *para* gene or modification of other pyrethroid target sites may contribute to a high level of

knockdown resistance. Alternatively, enhanced metabolic detoxication or reduced cuticular penetration may also be involved in knockdown resistance.

Although PBO and DEF were used in our bioassays to suppress monooxygenase-mediated and hydrolytic detoxication of pyrethroids, these synergists may not suppress all forms of metabolic detoxication, as suggested previously by Scott (27) and Valles and Yu (5). Pretreatment with PBO and DEF actually increased the cypermethrin resistance ratio by twofold in the Marietta strain (5). A high level of knockdown resistance, in the presence of PBO and DEF, was also observed for the Marietta strain in the present study. The *kdr* mutation was detected only in one of the last five knocked-down individuals in the Marietta strain. Therefore the *kdr* mutation is not a main factor for the knockdown resistance in this strain. Thus, the role of other mechanisms, such as enhanced metabolic detoxication, additional mutations in the *para* gene, or reduced cuticular penetration, alone or in combination, may confer a high level of knockdown resistance to pyrethroids in the Marietta strain.

It is remarkable that most pyrethroid-resistant German cockroach strains which were independently collected from various locations in the United States and other countries carry the same *kdr* mutation at nt 2979. A common feature of *kdr* resistance is cross-resistance to DDT. The widespread occurrence of the *kdr* mutation could be due to the intensive use of DDT before the introduction of pyrethroid insecticides. For example, in China from the 1950s to 1970s, a large scale and intensified use of insecticides, especially DDT in urban areas, was undertaken during the nationwide "Eradication of Four Pests Movement" to eradicate mosquitoes, house flies, rodents, and cockroaches. Similarly, DDT was used on a wide scale in the 1950s and 1960s in the United States and Great Britain (28). Apparently, no fitness disadvantage is associated with the *kdr* mutation. Although the level of resistance to deltamethrin in the German cockroach *kdr* strain Ectiban-R has slowly declined over the past 5 years, the strain is still homogenous

for the *kdr* mutation (Dong, unpublished). The basis for the decline of pyrethroid resistance in Ectiban-R is not known but it is likely due to some other mechanism(s).

A single nucleotide change within the γ -aminobutyric acid (GABA) receptor gene *Rdl* was found to confer cyclodiene resistance in *D. melanogaster* (29). Subsequently developed PCR-based monitoring methods were shown to be more accurate than insecticide bioassays in detecting cyclodiene-resistant individuals and estimating the resistance frequency in field populations (30). Furthermore, such methods require a smaller sample population than conventional bioassays (30). It is evident from this study that bioassays alone are not reliable for the detection of *kdr* individuals in field populations, even when inhibitors of metabolic detoxication are used. In contrast, PCR/sequence analysis as described in this paper, when combined with bioassays, can accurately and effectively detect *kdr* individuals in a given field population.

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