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Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s.*

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Abstract

Pyrethroid-impregnated bednets are playing an increasing role for combating malaria, especially in stable malaria areas. More than 90% of the current annual malaria incidence (*c.* 500 million clinical cases with up to 2 million deaths) is in Africa where the major vector is *Anopheles gambiae s.s.* As pyrethroid resistance has been reported in this mosquito, reliable and simple techniques are urgently needed to characterize and monitor this resistance in the field. In insects, an important mechanism of pyrethroid resistance is due to a modification of the voltage-gated sodium channel protein recently shown to be associated with mutations of the *para*-type sodium channel gene. We demonstrate here that one of these mutations is present in certain strains of pyrethroid resistant *A. gambiae s.s.* and describe a PCR-based diagnostic test allowing its detection in the genome of single mosquitoes. Using this test, we found this mutation in six out of seven field samples from West Africa, its frequency being closely correlated with survival to pyrethroid exposure. This diagnostic test should bring major improvement for field monitoring of pyrethroid resistance, within the framework of malaria control programmes.

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Introduction

In the 1950s the worldwide Malaria Eradication Programme was almost entirely based on vector control using DDT and dieldrin insecticides. Since 1965, control of the major vector, *Anopheles gambiae s.s.*, has more or less been abandoned in Africa, partly because of insecticide resistance. This preventative measure was reintroduced only recently using pyrethroid-impregnated bednets (Choi *et al.*, 1995). As no insecticide class with similar efficiency is currently available, there is an urgent need for a reliable tool to detect and monitor pyrethroid resistance in malaria vector populations (Elissa *et al.*, 1993). Pyrethroids act on the nervous system by modifying the gating kinetics of voltage-sensitive sodium channels (Lund & Narahashi, 1983). One important type of resistance to pyrethroids is characterized by a marked reduction in the intrinsic sensitivity of the insect nervous system to these compounds. It confers resistance not only to pyrethroids but also to DDT which shares a similar mode of action. This mechanism was first identified in DDT-resistant houseflies, *Musca domestica*, and was termed knock-down resistance or *kdr* (Milani, 1954). It has recently been reported that a single mutation (leucine to phenylalanine) in the S6 transmembrane segment of domain II in the sodium channel sequence is associated with *kdr* to pyrethroids and DDT in *M. domestica* (Williamson *et al.*, 1996a) and the German cockroach, *Blattella germanica* (Miyazaki *et al.*, 1996). Moreover, a different mutation (leucine to histidine) at this same position has been found in pyrethroid-resistant populations of the tobacco budworm, *Heliothis virescens* (Park & Taylor, 1997). In *super-kdr* houseflies, this mutation is associated with a second substitution further upstream in the same domain which replaces a methionine with a threonine (Williamson *et al.*, 1996a). Since *kdr* is due to an alteration of the

insecticide affinity for its binding site on the sodium channel (Pauron *et al.*, 1989), it is possible that a limited number of modifications in the target site can promote nerve insensitivity against these compounds and therefore resistance.

In this study we have analysed the domain II region of the *para*-type sodium channel from pyrethroid susceptible and resistant strains of *A. gambiae* and identified in the resistant strains the same Leu to Phe point mutation as described for houseflies and cockroaches. This result has enabled us to develop a simple PCR-based diagnostic test for the rapid identification of this *kdr*-like allele, even when heterozygous, in natural mosquito populations.

Results and Discussion

Partial cDNA sequencing of the para*-type sodium channel in* Anopheles gambiae s.s.

Partial sodium channel sequencing was carried out on four *Anopheles gambiae* strains: Kisumu and Bobo are laboratory susceptible strains and Tola (from Côte d'Ivoire) and Kou Valley (from Burkina Faso) were obtained in 1993 and 1995 respectively from field resistant samples which were further selected with permethrin in the laboratory until all insects survived the current W.H.O. diagnostic permethrin concentration (0.25%). *Kdr* was implicated as the most likely mechanism because of the cross resistance to DDT and complete loss of the rapid knockdown effect by these compounds.

Total RNA extracted from the four strains was used to synthetize the first-strand cDNA. Primary PCR on the single-stranded cDNA was carried out with D1 and Dg2 primers (Table 1). As the fragment of interest was not always resolved as a unique single band because of primer degeneracy, we performed a secondary PCR using the nested primer Dipd1 with Dg2. A single specific fragment of 407 bp was then amplified and directly sequenced (Fig. 1). Comparison of the *Anopheles* sequence with the housefly and *Drosophila para*-type sodium channel sequences showed clearly that we had amplified a *para* homologous sodium channel fragment rather than that of *DSC1*, a second sodium channel gene described in *Drosophila* (Salkoff *et al.*, 1987). At the amino acid level, 95% and 97% identity were found between the *Anopheles* sequence and the *Drosophila* and housefly *para* sequences.

Table 1. Sequences of the primers used throughout this study.

D1	5′ AARYTNGCNAARTCTTGGCC 3′
D4	5′ ACRAARTCNARCCARCACCA 3′
Dg1	5′ TGGATHGARWSHATGTGGGAYTG 3′
Dg2	5′ GCDATYTTRTTNGTNGTNTCRTTTRTC 3′
Dipd11	5′ TGGCCSACRCTKAAYTTACTC 3′
Dip2	5′ TTGGACAAAAGCAARGCTAAG 3′
Dip3	5′ ATCATCTTCATCTTTGC 3′
Mos1	5′ ACYGTAGTGATAGGAAAT 3′
Agd1	5′ ATAGATTCCCCGACCATG 3′
Agd2	5′ AGACAAGGATGATGAACC 3′
Agd3	5′ AATTTGCATTACTTACGACA 3′
Agd4	5′ CTGTAGTGATAGGAAATTTA 3′

A comparison of the aligned nucleotide sequences obtained for the four *Anopheles* strains revealed only one nucleotide change (Fig. 1). This change is responsible for the substitution of a leucine residue (TTA) present in both susceptible strains (Bobo and Kisumu) by a phenylalanine (TTT) in both resistant strains (Kou Valley and Tola). This change coincides exactly with the reported Leu to Phe mutation in *kdr* houseflies (Williamson *et al.*, 1996a) and cockroaches (Miyazaki *et al.*, 1996). The observation of the same amino acid substitution in mosquitoes provides further evidence that this mutation is involved in resistance since the leucine residue at this position in susceptible insects is highly conserved during evolution in both vertebrates and invertebrates (Williamson *et al.*, 1996a). This striking convergence suggests that the mutated leucine probably plays a crucial role in the pyrethroid–sodium channel interaction and that its substitution by a different amino acid leading to *kdr* must be highly specific in order to keep the channel functional. Moreover, in *M. domestica*, *D. melanogaster* and several other insect species from different orders (Martinez-Torres *et al.*, 1997), this leucine is encoded by the codon CTT, and therefore to substitute a phenylalanine in *kdr* insects the first position of the triplet has changed to a T. Interestingly, in *A. gambiae* and the German cockroach the same conserved leucine is encoded by TTA and TTG respectively and the change into phenylalanine involves replacing the third position of the triplet. Therefore phenylalanine is probably one of a limited number of possible substitutions. The histidine found in *kdr Heliothis* replacing the same leucine (Park & Taylor, 1997) would be a second alternative, which appears at present to be less widespread. In this context, it is worth mentioning the reported convergence to the same amino acid substitution (Ala to Ser) in a GABA-gated chloride channel, *Rdl*, responsible for resistance to cyclodiene insecticides among a wide range of insect species (ffrench-Constant, 1994). The same *kdr*-associated mutation of Leu to Phe has so far been reported in pyrethroid resistant strains of five insect species from four different orders, Diptera (*M. domestica* and *A. gambiae*), Dictyoptera (*Blattella germanica*) and, very recently (Martinez-Torres *et al.*, 1997), Lepidoptera (*Plutella xylostella*) and Hemiptera (*Myzus persicae*). Further characterization of sodium

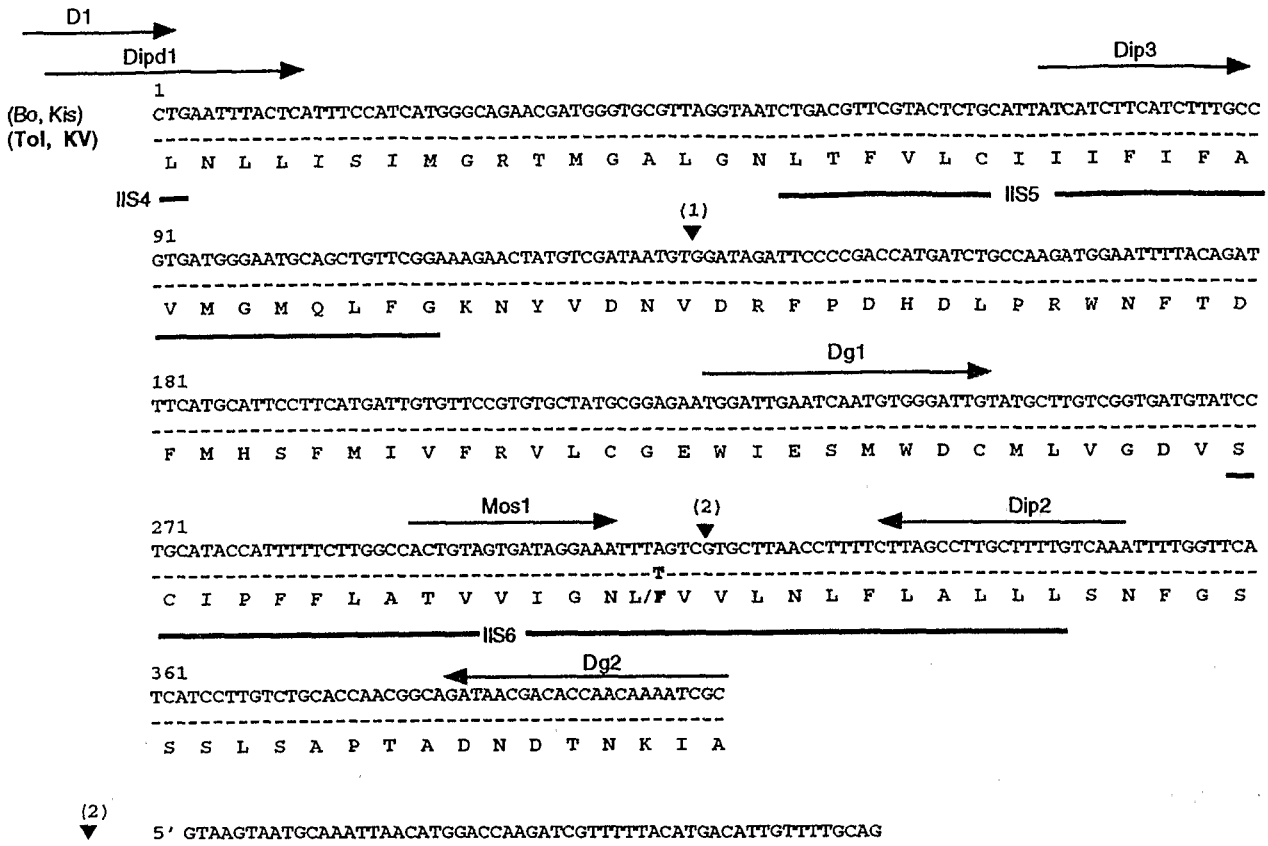

Figure 1. Sequence analysis of the IIS4–IIS6 region of the voltage-sensitive sodium channel in susceptible Bobo (Bo) and Kisumu (Kis) strains and in *kdr* Kou Valley (KV) and Tola (Tol) strains of *A. gambiae*. Identical positions are indicated by a dash (–). In the predicted amino acid sequence below, the single change (leucine/phenylalanine) is highlighted. The positions of the two introns present in this region are indicated by a ▼. The sequence of the second intron which was necessary for designing the '*kdr* specific' primer used in the diagnostic test is presented below. This sequence is identical in the four strains. The sequence has been deposited in the GenBank database (accession number Y13592).

channel genes in pyrethroid-resistant strains from other insect species will elucidate the extent of the convergence discussed here.

Designing a diagnostic test for kdr *alleles in* A. gambiae

Two introns are known to exist in the domain II region of the sodium channel gene at conserved positions in *Drosophila* and *M. domestica* (our unpublished results, see Fig. 1). One of these introns (i.e. intron 2 in Fig. 1) is located only 4 bp apart downstream of the *kdr* mutation and therefore knowledge of both its sequence and its level of conservation among the different strains was necessary in order to design specific oligonucleotides to be used on genomic DNA. We PCR-amplified through this region on genomic DNA from single mosquitoes using primers Dg1 and Dg2 (see experimental procedures). Using primers Mos1 and Dip2 directly on the amplified fragment, we read through the intron 2 sequence in the four *Anopheles* strains. We found that the position of this intron was conserved with respect to the houseflies and *Drosophila* sequences but no sequence homology was detected. Its size is also different: 55 bp in *Anopheles* and about 130 bp in the other Diptera (unpublished results). The sequence of intron 2 is entirely conserved in the four *A. gambiae* strains analyzed (Fig. 1).

These results enabled us to adopt a simple, single PCR test for identifying the resistance genotype using four primers (Williamson *et al.*, 1996b) as illustrated in Fig. 2A. Two 'common' primers asymmetrically flank the region of the mutation; another primer is specific for the susceptible genotype by having an A at the 3′ OH end of the sequence present in susceptible mosquitoes, and the last one is a *kdr* antisense specific primer ending at its 3′OH end with an A (complementary to the T specific for *kdr* strains) and which partially overlaps the intron sequence. Using genomic DNA extracted from single mosquitoes, unambiguous genotyping of susceptible and resistant individuals was possible as the method provides an internal control band (293 bp) and a band of different size in susceptible and *kdr* mosquitoes (137 and 195 bp, respectively). In the off-

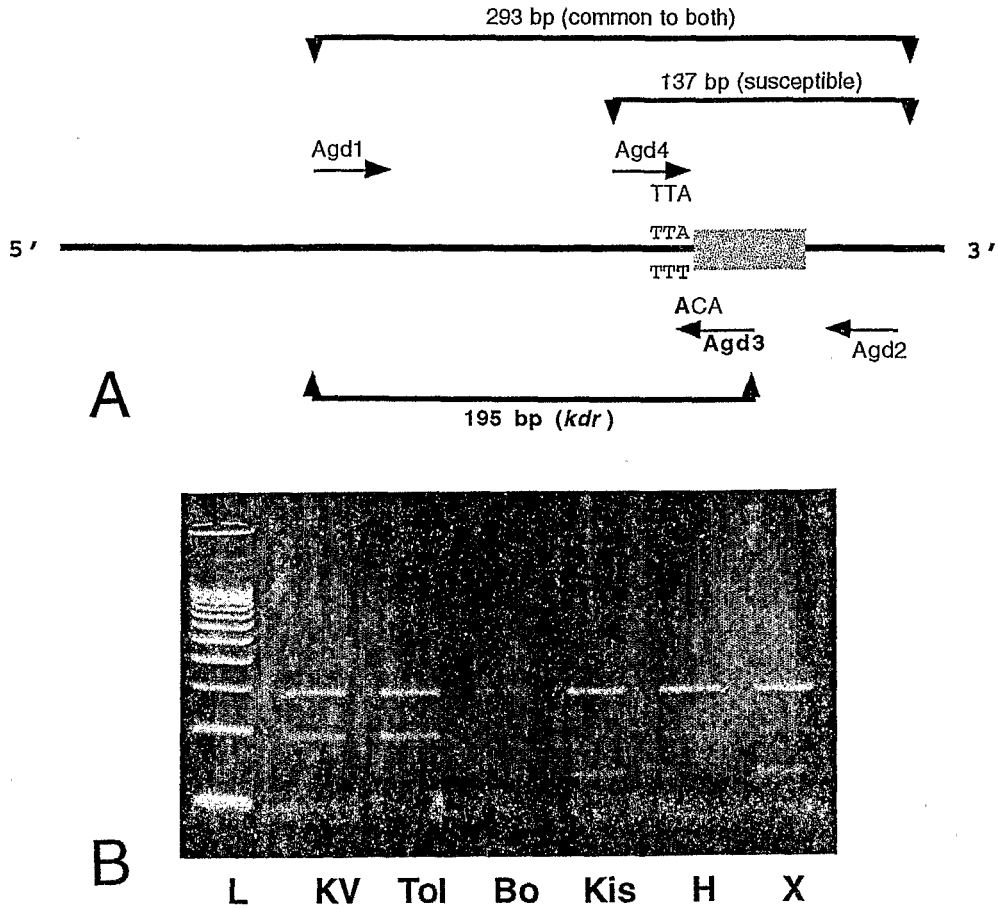

Figure 2. Identification of *kdr* by PCR-based diagnostic test. (A) Schematic representation of the experiment. The positions of the triplets encoding leucine (TTA) in susceptible mosquitoes and phenylalanine (TTT) in *kdr* individuals are indicated above and below the thick line representing the IIS4-IIS6 region of the sodium channel. The position of intron 2 is represented by a shaded box. (B) PCR products obtained using the test on four *Anopheles* strains after separation on a 1.5% agarose gel. Lane L: 100 bp ladder; lanes 2–7: products obtained using genomic DNA of single mosquitoes as templates; lanes KV and TOL: *kdr* insects (Kou Valley, Tola); lanes BO and KIS: susceptible insects (Bobo, Kisumu); lane H: equimolar mixture of genomic DNA from Kou Valley and Bobo insects; lane X: pattern obtained using the genomic DNA of the offspring of a Kou Valley × Bobo cross as a template.

spring of a susceptible × resistant cross, as well as in a mixture of equimolar amounts of genomic DNA from susceptible and resistant insects, three bands were systematically obtained, indicating that heterozygotes can also be distinguished (Fig. 2B).

Application of the diagnostic test to field populations

The validity of the PCR diagnostic test was checked by studying the sodium channel polymorphism in seven field samples collected in Côte d'Ivoire and Burkina Faso. The three genotypes (Leu/Leu, Leu/Phe, Phe/Phe) were clearly identified. The frequency of the resistant allele was strongly correlated ($P = 0.0005$) with the reduced mortality observed in bioassays with the W.H.O. discriminating concentration of permethrin (W.H.O., 1970) as shown in Fig. 3. This indicates that *kdr* is the major mechanism of pyrethroid resistance in these field populations. The efficacy of pyrethroids on resistant mosquitoes was shown to be dramatically reduced among mosquitoes of the two most resistant populations, Yao Koffikro and Korhogo: exposure for 3 min to nets treated with 25 mg/m^2 deltamethrin formulation using W.H.O. cones resulted in only 10% and 1.3% mortality, respectively versus 100% with a susceptible strain (unpublished data). It is not clear at this stage how the presence of *kdr* in the field will affect operational control. However, in view of the usual cross resistance conferred by this mechanism to other pyrethroids, immediate and long-term efficacy of impregnated bednets in the concerned areas should be closely investigated.

In stable malaria areas of Africa the strategy for prevention currently recommended by W.H.O. relies almost entirely on the use of pyrethroid-impregnated bednets at community level (W.H.O., 1993). As more and more bednet programmes are launched, it is

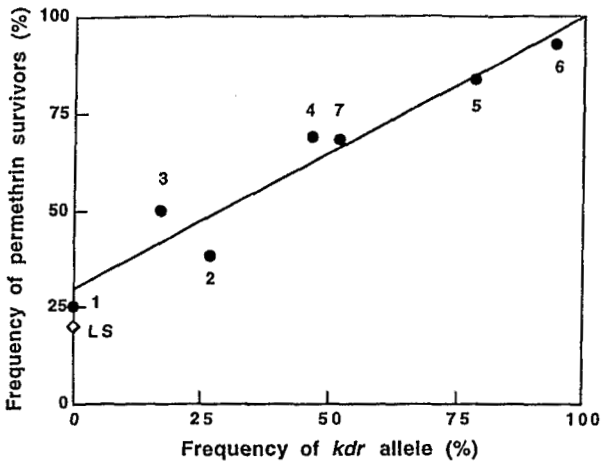

Figure 3. Relationship between the frequencies of surviving adults and of the *kdr* allele, as determined by the PCR test in seven field samples of *A. gambiae s.s.* from West Africa ($y = 0.70x + 29.3$; $r_{yx} = 0.96$; $P = 0.0005$ for H_0: $r_{yx} = 0$). The frequency of the *kdr* allele was determined for thirty individuals and the frequency of permethrin survivors was measured from 100 insects. Numbers refer to localities as follows: Côte d'Ivoire: 1, Abidjan; 2, Odienne; 3, Daloa; 4, Kafine; 5, Yaokoffikro; 6, Korhogo; Burkina Faso: 7, Kou Valley (before selection); LS: laboratory susceptible strain (Kisumu).

urgent to evaluate the prevalence of *kdr* alleles and to monitor pyrethroid resistance in the target mosquito populations. One important aspect of a molecular diagnostic such as the PCR test described here over conventional bioassays for monitoring is that, *kdr* being recessive, it can also discriminate between heterozygotes and susceptible homozygotes. Hence the molecular diagnostic is far more powerful at picking up very low frequencies of resistance genes which are then mainly present in heterozygous form, and resistance management could be implemented at a very early stage. Other important vectors of malaria and other diseases in the world have also developed pyrethroid resistance and *kdr* has been implicated in some cases (Omer *et al.*, 1980). Because the sodium channel region involved in *kdr* resistance is apparently highly conserved, the diagnostic test proposed here might be usable with few or no modifications for monitoring pyrethroid *kdr* as part of the planning and implementation of malaria vector control operations in Africa and elsewhere.

Experimental procedures

Mosquito populations and bioassays

Samples of *Anopheles gambiae s.s.* were collected in six localities from Côte d'Ivoire separated by at least several hundred kilometres (Abidjan, urban area; Odienne, peri-urban; Daloa, urban; Kafine, rural; Yaokoffikro, rural; Korhogo, rural) and one from Burkina Faso (Kou Valley, rural). Mature larvae were transported to the laboratory and reared to adults under standard conditions. Tests were carried out with 2–4-day-old non-bloodfed females emerged from wild larvae or on generation of laboratory rearing using W.H.O. insecticide susceptibility tests. Five replicates of twenty females were exposed during 1 h to papers impregnated with permethrin (cis/trans ratio: 25/75) at a concentration of 0.25%. Mortality was checked 24 h post exposure. Mosquitoes exposed to untreated papers served as controls. Resistance status of wild populations was compared to a susceptible reference strain of *A. gambiae s.s.*, Kisumu (Kenya).

Two resistant field populations were submitted to permethrin selection in the laboratory for fifteen and twenty-five generations, respectively, for Kou Valley (Burkina Faso) and Tola (Côte d'Ivoire) by exposing adults of each generation for 1 h to permethrin-impregnated papers at concentrations providing 80–90% mortality.

Partial sequencing of the sodium channel gene

The IIS4–IIS6 coding region of the sodium channel gene was amplified by RT-PCR using 1–5 μg of total RNA as template for cDNA synthesis. RNA was extracted from single mosquitoes by the guanidinium–hydrochloride/phenol–chloroform method (Logemann *et al.*, 1987). First-strand cDNA synthesis was performed according to the supplier's protocol (SuperscriptII, Gibco-BRL) using as primers a mixture of 200 ng of oligo-dT (Gibco BRL), plus 200 ng of an antisense sodium channel primer (D4) corresponding to a conserved sodium channel sequence downstream the region of interest (Table 1). Two rounds of PCR were carried out to selectively amplify this region. The primary PCR reaction was carried out on the cDNA using the degenerate sodium primers D1 and Dg2 that flank the region of interest. One unit of Taq polymerase (Eurogentec) in buffer supplied by the manufacturer (75 mM Tris-HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v)Tween 20, 1.5 mM $MgCl_2$), 250 ng of each primer and 0.2 mM dNTP were used in a 50 μl total volume PCR reaction consisting of 35 cycles at 94°C for 1 min, 50°C for 2 min, 72°C for 2 min and a final extension step at 72°C for 10 min. A secondary PCR was then carried out on the primary product using at the 5′ end a nested Diptera specific primer (Dipd1) based on the *Drosophila melanogaster* (Loughney *et al.*, 1989) *Musca domestica* (Williamson *et al.*, 1996a), and *Culex pipiens* (our unpublished data) *para*-type sodium channel sequences and keeping at the 3′ end the same primer used in the primary PCR (Dg2). PCR conditions were the same as for the primary PCR except that the annealing temperature was reduced to 48°C and the total reaction volume increased to 100 μl. The 407 bp amplified fragment was then recovered by ethanol precipitation and used as template for direct sequencing with the automated ABI PRISM Dye Terminator Cycle Sequencing Kit (ABI). Sequencing primers (Dip3, Dip2 and Mos1) were based on conserved sequences present in *Drosophila*, housefly and *C. pipiens*. The positions of all the primers used both for PCR and sequencing are indicated in Fig. 1, and their sequences are listed in Table 1.

Intron sequence determination

Genomic DNA was extracted from single mosquitoes according to Martinez-Torres *et al.* (1992) with some modifications. Single individuals were homogenized in 100 μl 10 mM Tris-HCl, 60 mM NaCl, 5% sucrose, 10 mM EDTA, pH 7.8. Then, 125 μl of 1.25% SDS, 300 mM Tris-HCl, 5% sucrose, 10 mM EDTA, pH 7.8

were added. The mixture was incubated at 65°C for 30 min and 40 μl of 5 M potassium acetate pH 4.8 were added afterwards. After incubation at −20°C for 10 min the mixture was centrifuged at 13,000 *g* and the supernatant was collected. Two volumes of cold absolute ethanol were added and the mixture kept at −20°C for 15 min prior to centrifugation at maximum speed to precipitate nucleic acids. The genomic region containing the intron of interest (intron 2 in Fig. 1) was PCR amplified on 10–50 ng of genomic DNA using primers Dg1 and Dg2 (see Table 1 and Fig. 1). One unit of Taq polymerase (Eurogentec) and 200 ng of each primer were used in a 100 μl total PCR volume. Amplification was performed for 35 cycles at 94°C for 1 min, 54°C for 2 min and 72°C for 2 min with a final extension step at 72°C for 10 min. After ammonium precipitation, automated sequencing was performed through the intron sequence using primers Mos1 and Dip2.

DNA diagnostic test for kdr alleles in single mosquitoes

10–50 ng of genomic DNA prepared as above were combined in a 50 μl total volume with the four primers Agd1, Agd2, Agd3 and Agd4. The PCR reaction conditions were 1 min at 94°C, 2 min at 48°C and 2 min at 72°C for forty cycles with a final extension step at 72°C for 10 min. Amplified fragments were analysed by electrophoresis on a 1.5% agarose gel and were visualized by ethidium bromide staining under UV light.

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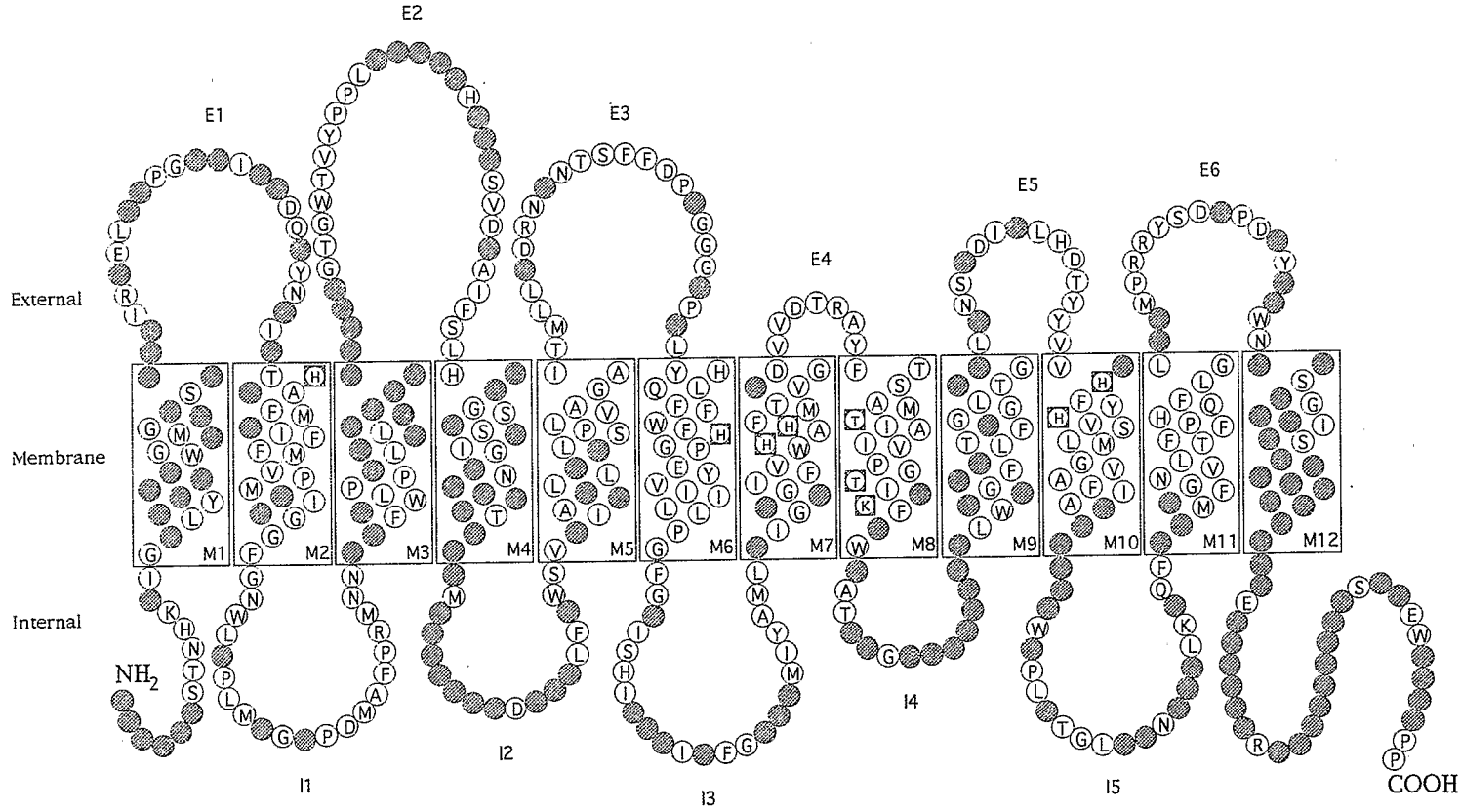

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