FIRST EVIDENCE OF *CULEX PIPIENS* RESISTANCE TO THE INSECT GROWTH REGULATOR DIFLUBENZURON

L. Grigoraki [(2)(3)], A. Puggioli [(1)], K. Mavridis [(2)], V. Douris [(2)], R. Bellini [(1)], J. Vontas [(2)(4)]

(1) Centro Agricoltura Ambiente "G. Nicoli", Crevalcore (BO), Italy
(2) Foundation for Research and Technology Heraklion-Crete, Greece
(3) University of Crete, Heraklion, Greece
(4) Agricultural University of Athens, Athens, Greece

INTRODUCTION

Culex pipiens is one of the most common mosquito in urban areas, it causes nuisance due to its biting behaviour, and it is the main vector of several human diseases, including West Nile Virus. Vector control by insecticides is the main tool to prevent these diseases and the insect growth regulator diflubenzuron is one of the most effective mosquito larvicides used in Europe. Its intense use can result in the selection of insecticide resistance, it is therefore of fundamental importance to monitor the susceptibility levels to this active principle in the target species in order to prevent and menage the insurgence of resistance phenomena.

MATERIALS AND METHODS

Diflubenzuron bioassays. Field populations were collected in 2015, 2016 and 2017 from Ravenna urban area, Italy, and tested for their susceptibility to diflubenzuron. A *Cx. pipiens* laboratory strain (Benaki), not exposed to insecticides for more than 20 years, was used as a reference susceptible strain. The bioassays were conducted on L_3-L_4 larvae, following WHO guidelines, testing doses of diflubenzuron ranging from 0.00015 to 0.468 ppm. The study was conducted in climatic chambers under controlled conditions (28 ±1 °C, 80% RH, 14:10 L:D).

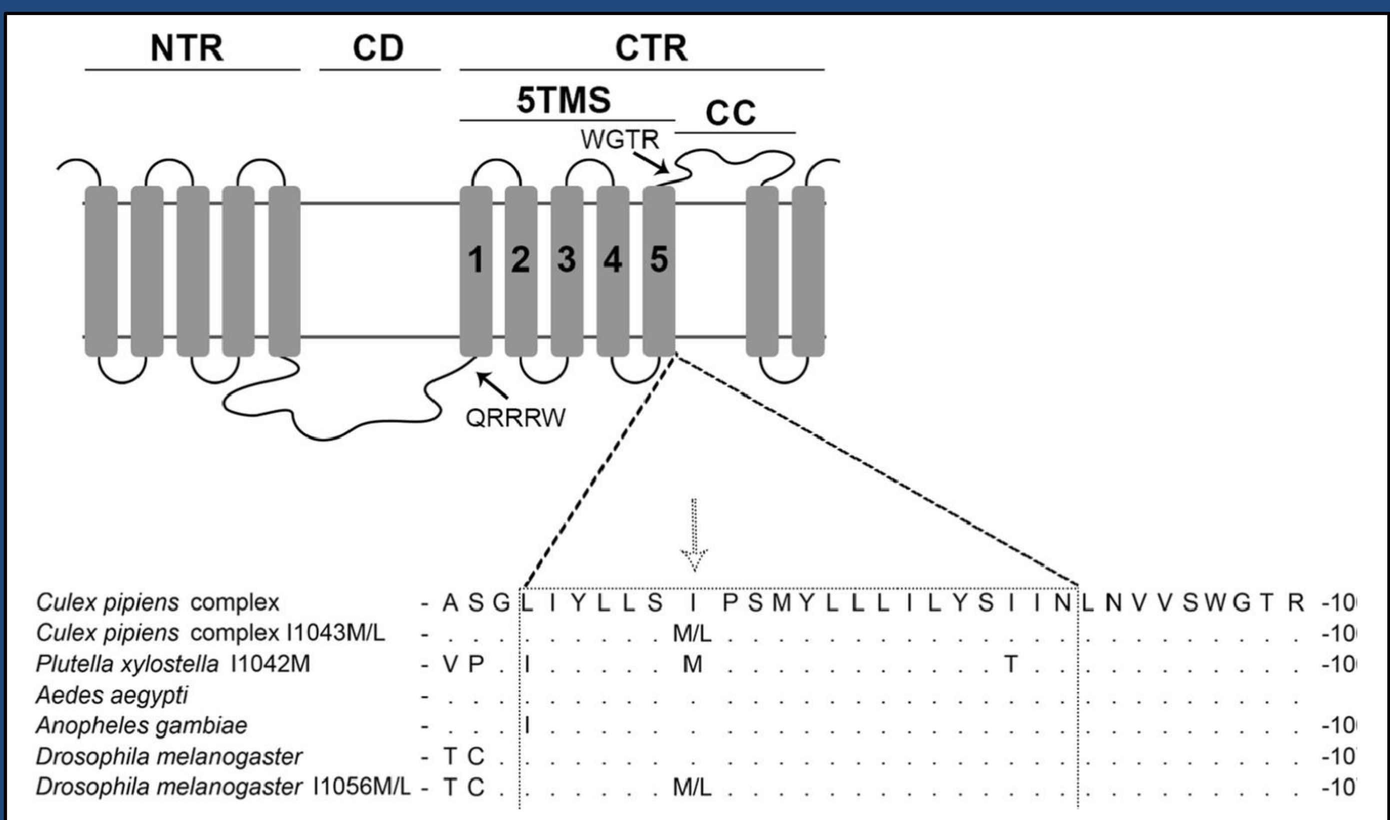
Treatment	DIFLUBENZURON DOSES (mg L^{-1})	
	Bioassay 2015	Bioassay 2016 - 2017
CONTROL	0	0
A	0.00015	0.00015
B	0.0003	0.00075
C	0.00075	0.00375
D	0.00225	0.0187
E	0.006	0.0937
F	0.015	0.468

Identification of diflubenzuron resistance mutations. A sequence of 825 bp, part of the chitin synthase C-terminus, the putative binding site of diflubenzuron, spanning the 1043 position (*Cx. pipiens* numbering) was amplified from *Cx. pipiens* gDNA and examined for the presence of mutations.

RESULTS

Diflubenzuron bioassays. The Ravenna 2015 population showed considerable resistance to diflubenzuron (RR_{LC50}=32 fold), which increased to RR_{LC50}=128 fold in 2016 and to RR_{LC50}=206 in 2017 (Resistance Ratio calculated over the Benaki strain), exceeding the recommended WHO dosage of diflubenzuron in potable water containers (0.25 ppm). Log-dose probit-mortality data for diflubenzuron tested against third-fourth instar larvae of *Cx. pipiens* are shown in the table on the right.

Mosquito population	N	LC_{50} (95%CL) mg L^{-1}	RR_{LC50}	X^2 (df)
Benaki 2013 (control)	120	0.002 (0.001-0.003)	1	8.52 (10)
Ravenna 2015	600	0.065 (0.026-0.396)	32.5	16.838 (22)
Ravenna 2016	600	0.257 (0.146-0.619)	128.5	11.860 (10)
Ravenna 2017	400	0.413 (0.273-0.766)	206.5	5.7132 (10)

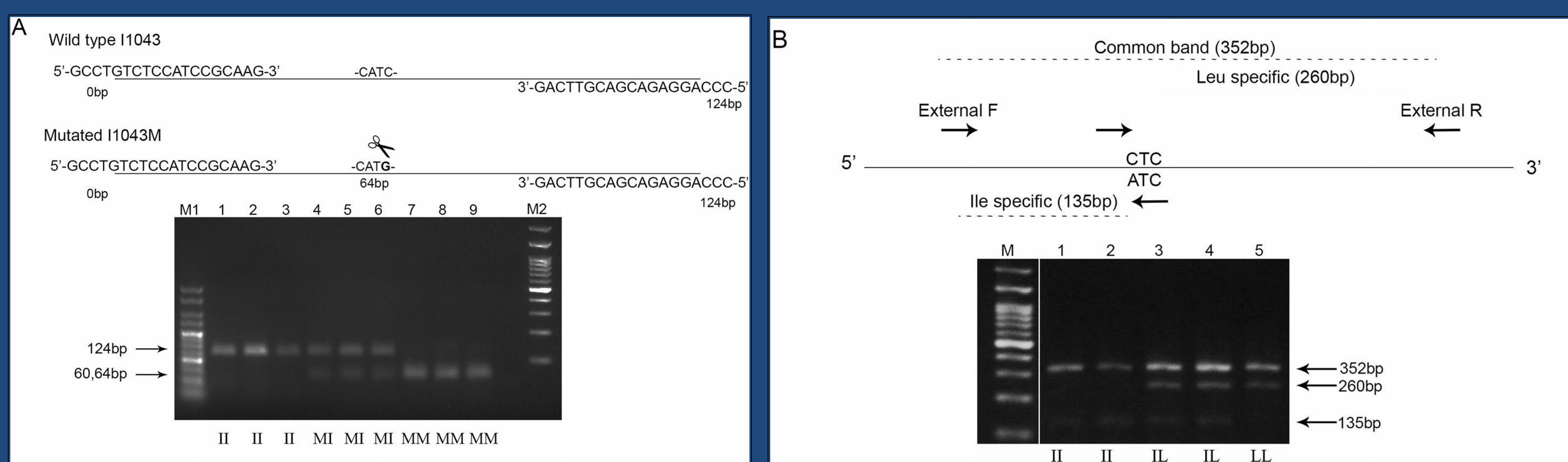


Identification of diflubenzuron resistance mutations. In the figure on the left, the schematic representation of the chitin synthase and the position of the diflubenzuron resistance mutations is depicted. The screening of *Cx. p*ipiens individuals for the presence of mutations at the I1043 site of the chitin synthase showed that the mosquitoes from the control strain (Benaki) were homozygous for the wild type Ile (I) amino acid, the presumed susceptible allelic form. For the Ravenna 2016 population, 9 out 34 tested individuals carried alleles with mutations at the 1043 site, either the I1043M (previously characterized in *Plutella xylostella*, Lepidoptera) or the I1043L. The frequency of mutated alleles increased significantly in the bioassay survivors of the Ravenna population (bioassay doses 0.0187–0.468 ppm) (Table below).

Population	Sample size (N)	Genotype at the chitin synthase position 1043					
		Homo (II)	Homo (MM)	Homo (LL)	Hetero (IM)	Hetero (IL)	Hetero (ML)
Benaki	14	14	-	-	-	-	-
Ravenna 2016	34	25	3	-	2	3	1
Ravenna 2016 (SURV)	30	-	17	13	-	-	-

Functional characterization of the I1043L mutation. Both mutations have been introduced in the *Drosophila melanogaster* chitin synthase gene using the genome editing method CRISPR/Cas9 and validated to confer significant levels of resistance, although at different levels. The I→M mutation results in a Resistance Ratio >2,900 fold and the I→L mutation >20 fold.

Diagnostic assays. Two PCR based diagnostics were developed for monitoring of the resistant mutations in field populations (Figures on the right).
Diagnostic PCR-RFLP for the mutation I1043M. Homozygous susceptible individuals show a single intact band of 124 bp, homozygous for the mutation individuals show a single band consisting of the 60 and 64 bp fragments and heterozygous individuals show both the 124 bp undigested band and the band consisting of the 60 bp and 64 bp fragments (Fig. A).
Allele specific PCR for the mutation I1043L. Four primers are used in a single PCR reaction: two common, external primers flanking the site of the mutation asymmetrically, a reverse primer specific for the wild type (susceptible) allele and a forward primer specific for the mutated allele (Fig. B).



CONCLUSIONS

- **High levels of resistance were identified in *Cx. pipiens* from Italy, with a Resistance Ratio of 128 fold in 2016 and 206 fold in 2017. The phenotype was associated with mutations at amino acid I1043 (I1043M and I1043L) of the chitin synthase gene, which showed significantly higher frequency in bioassay survivors.**

- **The selection of resistance to diflubenzuron seems to be associated with the extensive use of this larvicide in the Emilia-Romagna region over the past ten years to control *Ae. albopictus* in road drains, a larval habitat exploited by *Cx. pipiens* as well, and few decades ago in agriculture.The potential for resistance selection could be high, since within only one year of systematic diflubenzuron applications, resistance increased from 32- to 128-fold in Ravenna.**

- **The findings are of major concern for public health, as diflubenzuron is used in many places for the control of *Cx. pipiens* mosquitoes, transmitting West Nile and *Aedes* arbovirus vectors transmitting Dengue, Chikungunya and Zika. This is, particularly the case in regions such as Europe, where neurotoxic insecticides have been banned from use in mosquito breeding sites. Screening of *Cx. pipiens* and *Aedes* mosquito populations from several geographical regions for possible resistant CHS1 alleles must be conducted to guide appropriate resistance management strategies and ensure the sustainability of control interventions. The development of additional mosquito larvicides and the re-consideration of old active ingredients, such as temephos might need to be strongly supported. The use of alternative larvicides (i.e. Vectomax, methoprene, pyriproxyfen) in the areas where resistance has been detected is higly recommended.**

REFERENCES

World Health Organization. 2005. Guidelines for Laboratory Field Testing of Mosquito Larvicides. WHO/CDS/WHOPES/GCDPP/2005.13. WHO, Geneva.
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