Master thesis

Analysis of population structure and insecticide resistance in mosquitoes of the genus *Culex*, *Anopheles* and *Aedes* from different environments in Greece.

By

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Chapter 1

Analysis of population structure in mosquitoes of the genus *Culex*, *Anopheles* and *Aedes* from different environments in Greece.

Summary

The re-introduction of vector borne diseases in Greece poses a major public health problem and understanding vector population, composition and dynamics is fundamental to the development of effective disease control strategies. In this study we analyze the population structure in mosquitoes of the genus *Culex*, *Anopheles* and *Aedes* from different agricultural and urban environments in Greece. Our results highlight Thessaloniki, Evros and Attica regions as mosquito borne disease transmission hot spots and may prove useful in the guidance of control applications.

Introduction

From the early 20th century to date, Greece has had an intense mosquito borne disease history. In 1927, approximately 1 million people in Athens were infected with Dengue whereas in 1942 half the country's population was infected with malaria which was eventually eradicated in 1975 with the DDT spraying campaign. From then onwards mosquitoes were considered as a nuisance problem. This was the case until 2010 when the first West Nile Virus outbreak was recorded in Northern Greece in the region of Central Macedonia. In 2011 WNV spread through the country and by the end of 2012 the epidemic resulted in a total of 397 neuroinvasive cases and 57 deaths (1,2). To date neuroinvasive clinical cases and fatalities are still occurring but at a much lower frequency than the previous years (1).

Culex pipiens ss is considered a major vector of West Nile virus (WNV) in Europe (3,4) and is also involved in WNV transmission in Greece (5,6). This mosquito species comprises of two distinct forms, denoted *pipiens* and *molestus*, which are morphologically indistinguishable but exhibit important behavioral and physiological differences. Whereas *Cx. pipiens f. pipiens* diapauses, requires a blood meal to lay eggs (anautogeny), and is unable to mate in confined spaces, *Cx. pipiens f. molestus* does not diapause, is able to lay its first batch of eggs without a blood meal (autogeny), and mates in confined spaces (stenogamy) (7). Although conclusive evidence is still lacking, the two forms are thought to have different blood host preferences (*pipiens* biting mainly birds and *molestus* mainly mammals, especially humans) (8).

Hybridization between the two forms is hypothesized to make *Cx. pipiens* a superior bridge vector of WNV to humans (9) as hybrids may display a more opportunistic biting behavior (feeding both on birds / WNV reservoirs and humans) (10). Furthermore, according to Gunay et al (11), laboratory hybrid populations have an enhanced WNV vector competence relative to one or both parental stains.

Another important WNV vector belonging to the *Culex pipiens* complex is *Culex quinquefasciatus* whose morphological identification is difficult, time-consuming, limited to adult males and often impossible in the case of hybrids.

Added to WNV transmission, during 2011 malaria made a come-back in the country with a cluster of autochthonous cases in the Peloponnese and sporadic cases in Attika, Evros, Larisa , Viotia. Ecological settings favorable for breeding of potential malaria vector mosquitoes in combination with massive introduction of economic migrants from malaria endemic countries are considered the primary cause for malaria transmission in the country. *Anopheles sacharovi* which is the principal malaria vector in Turkey (12) is also the presumed vector for malaria transmission in Greece (1, 13).

It is evident that the re-introduction of vector borne diseases in countries such as Greece where they had been eradicated for many years poses a major public health problem. Certain areas, depending on the mosquito species and populations present in combination with a series of epidemiological events eg immigrations and climate changes may act as potential hot spots for mosquito borne disease outbreaks. Understanding vector population, composition and dynamics is fundamental to the development of pro-active effective disease control strategies.

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The main objectives of this study are:

1) To analyze the mosquito species composition and the population dynamics of the most prevalent species in 2 major mosquito breeding sites -agricultural ecosystems in Northern Greece (Evros and Thessaloniki). Such information on the population ecology is a pre-requirement for the development and application of appropriate control strategies.

2) By using modern diagnostic tools for species ID to assess differences in the molestus/ pipiens / hybrid form composition of *Cx. pipiens s.s.*, between Evros, Thessaloniki and urban settings in the Attika region. Biotype composition may be essential for WNV transmission and if so our study can prove useful in evaluating these foci as WNV transmission sites. Furthermore, biotype composition information is a pre-requirement for the development and application of appropriate control strategies targeting *Culex pipiens* mosquitoes.

1. Materials and Methods

1.1 Mosquito Surveillance

1.1.1 Study Area :

Two major agricultural sites were surveyed in 2014 from May till September, the "Thessaloniki Regional Unit" and the "Evros Regional Unit". Both foci have a history of mosquito borne disease transmission and have prolific mosquito breeding sites. They represent major agricultural ecosystems with relatively similar crops (cotton, corn, barley, rice-fields which is the predominant crop – approximately 20,000 hectares in Thessaloniki and 48,000 hectares in Thrace in the Turkish province right adjacent to the river Evros) and characteristics (river – based irrigation sources). In 2015 mosquitoes were collected from urban settings in the Attika region.

1.1.2 Mosquito Collections :

Collections were performed with CDC light traps baited with Carbon Dioxide (dry ice) in 16 sampling sites : Kastanies, Orestiada, Didimoticho, Soufli, Tychero, Feres, Apalo, Evros Delta within Evros region and M.Monastiri, Valtochori, Vrachia, Ag.Athanasios, Anatoliko, Malgara, Chalastra, Kalochori within Thessaloniki region. The traps were hung outdoors at ca. 1.5 m height and were located within the rice field zone following an evenly distributed pattern (Thessaloniki) or following the river pattern within a distance of 400m – 2 km from the rice fields (Evros) (figure 1). Traps were deployed biweekly from mid-May to mid-September in each study site from 18.00 – 8.00. Weather monitoring was also conducted throughout the surveillance period.

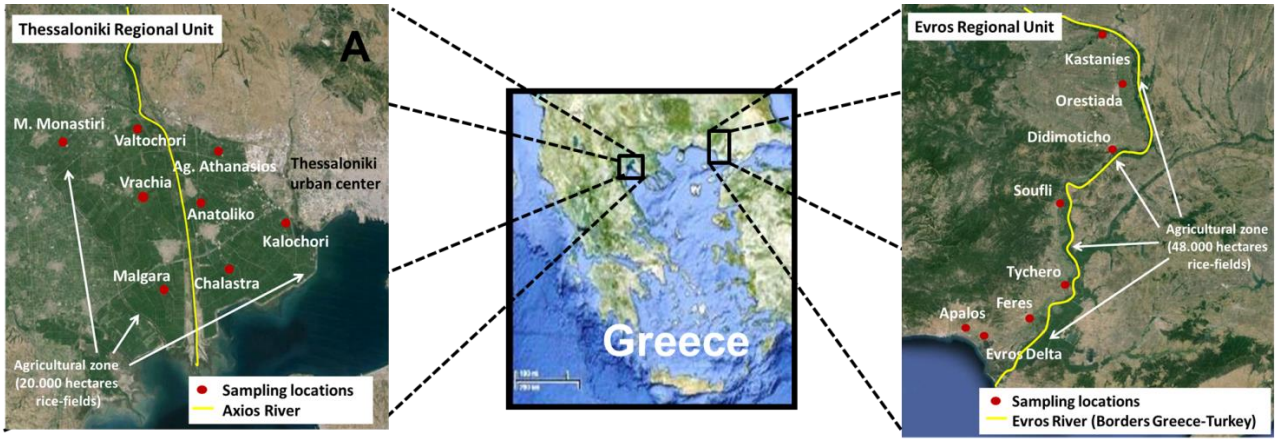


Figure 1: Study area in N.Greece and sampling locations of mosquito populations

In Attika larvae collections were performed in July and September from 3 urban sampling points : Nea Xalkidona (houses), Agios Stefanos (houses and stream). These collections and subsequent molecular analyses were conducted by Tsiamantas A.

1.1.3 Mosquito Samples, Sorting and Identification

Evros and Thessaloniki

Wild caught adult mosquitoes (of unknown age and of different physiological status) were killed with dry ice / carbon dioxide followed by a ten minute incubation period at -20 °C and subsequently females were identified morphologically down to species with the use of a stereoscope and a mosquito identification key (14). Catches of over 200 females per light trap were identified using a random sample of 200 mosquitoes, so that total species numbers could be extrapolated to counts for the entire sample. Individuals and batches of mosquitoes were stored in 2ml eppendorfs and 15ml tubes respectively containing (dry) silica gel.

Athens

Wild caught mosquito larvae were reared in the lab up to the adult stage and a representative subset was identified morphologically down to species and stored as previously described.

1.2 Molecular analyses of *Culex* mosquitoes : species and biotypes

Individual, adult, wild caught, female - morphologically identified *Culex pipiens* mosquitoes (from all three foci) were genotyped for the *Culex pipiens pipiens ss molestus* / *pipiens* biotype diagnostic marker (8).

A total of 100 *Culex pipiens* mosquitoes from Evros and Thessaloniki respectively and 30 mosquitoes from Athens were analyzed. The samples tested were representative of all mosquito collections conducted: all sampling sites - from May till September.

The presence of *Culex quinquefasciatus* and hybrids of the two species was also examined. All DNA extractions were performed on dead mosquitoes using the DNAzol method (Invitrogen).

1.2.1 DNA Extraction

Protocol

DNA extraction from individual females (use of DNAzol reagent)

Box 1

DNA Extraction	
1	Place individual mosquito in 1.5 ml sterilized eEppendorf
2	Add 50 µl DNAzol - with pestil grind mosquito
3	Add 150 µl DNAzol (Vfinal = 200 µl) - with pestil grind mosquito
4	Centrifuge at 10.000 rpm , 10 minutes , RT
5	Transfer supernatant into a new 1,5 ml eppendorf
6	Add 100 µl 100 % ethanol, mix. Incubate at RT for 1-3 minutes.
7	Centrifuge at 13.000 rpm ,20 min, RT
8	Discard supernatant
9	Add 1ml 75% ethanol
10	Centrifuge at 13.000 rpm , 5 min, RT
11	Discard supernatant
12	Airdry pellet at 30 °C for 30 min
13	Resuspend in 50 µl ddH20
14	Keep at 2°C for 30 minutes
15	Preserve at -20°C

Box 1 : Invitrogen DNA extraction protocol

1.2.2 *Cx. pipiens* Biotype diagnostic assay

This assay relies on polymorphisms (indels) in the 5' flanking region of the microsatellite locus CQ11 specific for *pipiens* and *molestus* alleles . A PCR reaction of three primers (reverse primers specific for molestus and pipiens forms respectively, forward primer common for both) produces a 200 bp PCR product - *pipiens* band, a 250 bp PCR product - *molestus* band and both amplicons for hybrids (8).

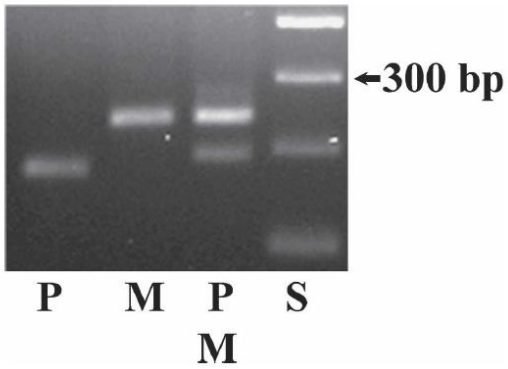


Figure 2: Reproduced from (8). Fragments amplified using pipiens reverse, molestus reverse and forward primers and run on a 2% agarose gel. P, *Cx. pipiens* f. pipiens , M, *Cx. pipiens* f. molestus, MP, f. molestus and f. pipiens hybrid.

Protocol

Box 2

Primers		**PCR Conditions**
pipiens reverse : 5' CATGTTGAGCTTCGGTGAA 3'		94°C / 5’
molestus reverse : 5' CCCTCCAGTAAGGTATCAAC 3'		94°C / 30’’
forward : 5' GATCCTAGCAAGCGAGAAC 3'		54°C / 30’’
		72°C / 40’’
Mastermix Solution		72°C / 5’
gDNA :	1,5 µl	10°C / f.e
Ampli-Taq Gold Buffer 10x :	2 µl	Steps 2-4 : 40 cycles
dNTPs :	0,4 µl	
MgCL2 (25Mm) :	1,6 µl	**Gel Electrophoresis**
Molestus Reverse primer :	1,5 µl	2% agarose gel
Pipiens Reverse primer :	1 µl	
Forward primer :	1,5 µl	
BSA 100x 10mg/ml :	0,3 µl	
Ampli-Taq Gold polymerase :	0,2 µl	
ddH20 :	10 µl	
Vfinal :	20 µl	

Box 2: *Cx. pipiens* Biotype diagnostic assay protocol

1.2.3 *Culex quinquefasciatus* molecular identification

This process was based on the combination of the ace- 2 assay (15) with the *Culex pipiens* biotype diagnostic assay (15).

The ace-2 assay relies on polymorphisms in the second intron of the acetylcholinesterase-2 (ace-2) locus for the identification of members of the *Cx. pipiens* complex. It generates different size products for *Culex pipiens ss* vs *Culex quinquefasciatus* but gives no information about the *Culex pipiens ss* biotype.

The assay was conducted on individuals that were homozygous or heterozygous for the *molestus* allele.

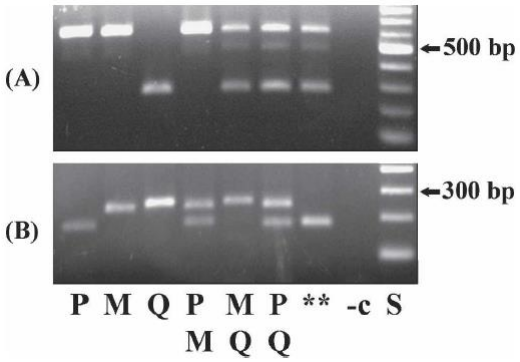


Figure 3: Reproduced from (8). Amplification products of (**A**) ACEpip, ACEquin, and B1246s primers and (**B**) pipiens reverse, molestus reverse and forward primers. P, *Cx. pipiens* f. pipiens; M, *Cx. pipiens* f. molestus; Q, *Cx. quinquefasciatus*;

Protocol

Box 3

Primers		**PCR conditions**
ACEquin : 5' CCTTCTTGAATGGCTGTGGCA 3'		94°C / 5'
ACEpip : 5 ' GGAAACAACGACGTATGTACT 3 '		94°C / 30''
B1246s : 5' TGGAGCCTCCTCTTCACGG 3'		55°C / 30''
		72°C / 1'
Mastermix Solution		72°C / 10'
gDNA :	1,5 µl	10°C / f.e
Kappa Taq Buffer A 10x :	2 µl	Steps 2-4 : 35 cycles
dNTPs :	0,5 µl	
MgCL2 (25Mm) :	1,6 µl	**Gel Electrophoresis**
ACE quinquef :	1µl	1% agarose gel
B1246S :	2 µl	
ACE pip :	1 µl	
BSA 100x 10mg/ml :	0,3 µl	
Kappa Taq polymerase :	0,2 µl	
ddH20 :	9,9 µl	
Vfinal :	20 µl	

Box 3: The ace-2 assay protocol for the identification of members of the *Cx. pipiens* complex *:Culex pipiens ss* and *Culex quinquefasciatus*

2. Results

2.1 Mosquito surveillance

A total of 100,000 and 140,000 mosquitoes were collected in CDC light traps in 2014 from Evros & Thessaloniki, respectively. Seven species were systematically recorded

in both regions with *Ae. caspius*, followed by *Cx. pipiens s.l.*, and *An. hyrcanus s.l.* being the most prevalent species. On the other hand, *Ae. geniculatus* and *Cu. longiareolata* were present only in Evros and *Ae. albopictus* was present only in Thessaloniki. (figure 4) .

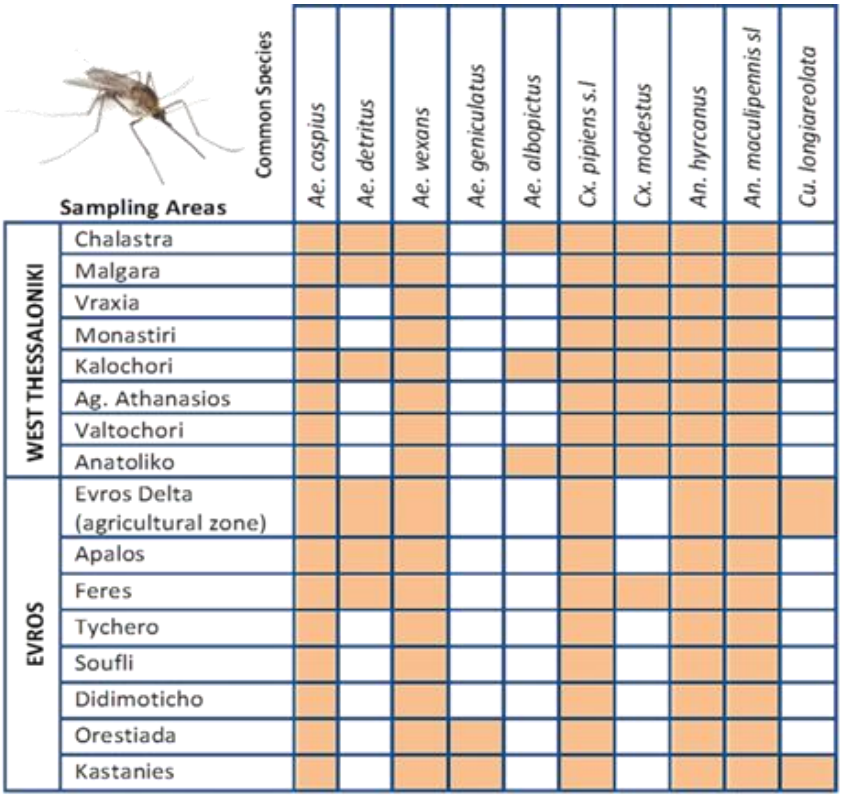


figure 4 : Composition of the most prevalent mosquito species collected from two major agricultural regions in Thessaloniki and Evros.

Figure 5 presents the population dynamics of the most prevalent mosquito species collected from the two major agricultural regions in Thessaloniki and Evros in correlation with temperature and precipitation In Evros (A) : *Ae.caspius* was by far the most dominant species from May till Sep and *Culex* , *Anopheles* populations peaked in the 2nd week of September. In Thessaloniki (B): *Ae. caspius* relevant activity was the highest until mid-July when *Culex* populations started to increase significantly. *Cx. pipiens s.l.* and *An. hyrcanus s.l.* were the most dominant species in August and September respectively.

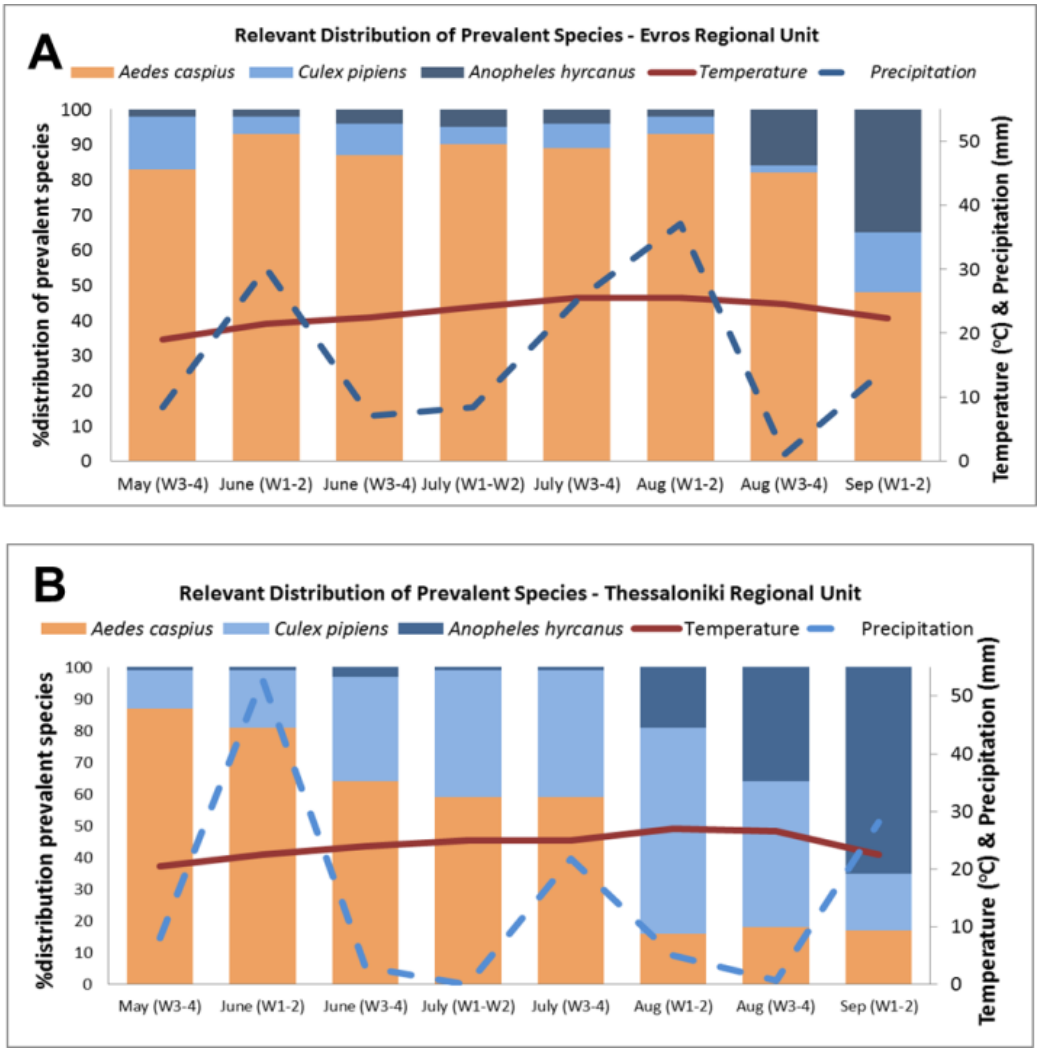


Figure 5: Population dynamics of the most prevalent mosquito species collected from two major agricultural regions in Thessaloniki and Evros.

2.2 Molecular analyses of *Culex* mosquitoes : species and biotypes

2.2.1 *Culex pipiens ss* biotype analysis

A total of 100 adult individual female *Culex pipiens* mosquitoes from Evros and Thessaloniki, respectively, were molecularly identified for *Culex pipiens ss* biotype.

Different biotype composition between the 3 foci was observed. The dominant biotype in Attica is *Cx. pipiens molestus*, followed by hybrids and last by *Cx. pipiens pipiens*. The dominant biotype in Thessaloniki is *Cx. pipien pipiens* followed by hybrids and *Cx. pipiens molest*us. In Evros dominant biotype is *Cx. pipiens pipien*s,

followed by *Cx. pipiens molestus* and *hybrids*. Substantial higher representation of *Cx. pipiens pipiens* in Evros, but hybrids in Thessaloniki and Attica (figure 6).

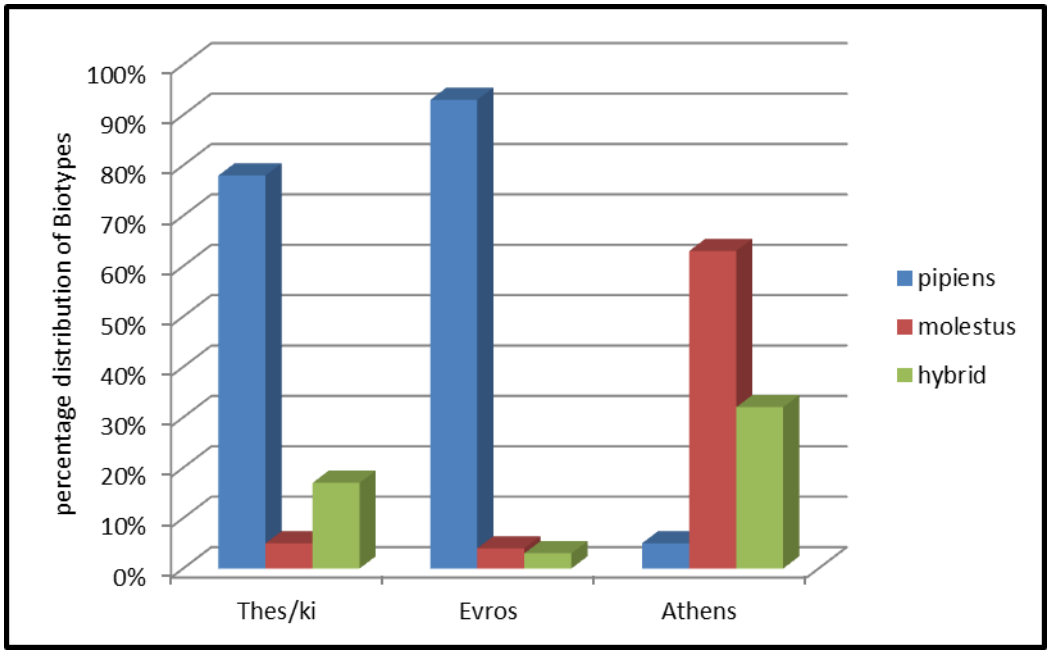


Figure 6 : *Cx. pipiens* biotype representation of mosquitoes collected in Thes/ki , Evros and Athens

2.2.2 *Cx. quinqeufasciatus* identification

Few specimens have been tested and initial results indicate the absence of *Cx. quinquefasciatus* from these populations. However, presence of hybrid mosquitoes of *Cx. quinquefasciatus* and any of the two *Cx. pipiens* forms cannot be ruled out. More specimens must be analyzed and cases where the ACE-assay and CQ11-assay do not agree should be investigated further.

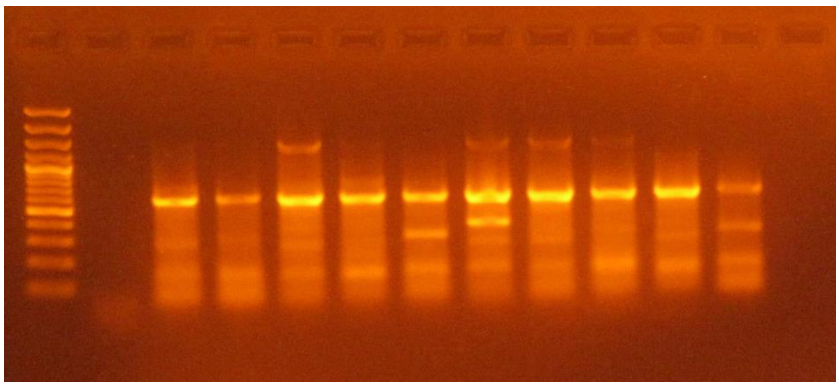


Figure 7 : ACE-assay. Amplification products of ACEpip, ACEquin, and B1246s primers run on a 1% agarose gel .Lane 1: 100bp marker , Lane 2: negative control, Lane 3-6 / 9-11 : *Cx.pipiens ss* diagnostic band, Lanes 7,8,12 : Heterozygote mosquitoes (?)

3. Discussion

3.1 Mosquito Surveillance

Mosquito surveillance results from Evros and Thessaloniki revealed similar species composition in the two foci with *Ae. caspius*, followed by *Cx. pipiens s.l.*, and *An. hyrcanus s.l.* being the most prevalent species (figure 2). *Aedes caspius* is a major nuisance mosquito whereas *Cx pipiens ss* is involved in WNV transmission in Greece (5, 6).

Anopheles hyrcanus (highly exophilic and anthropophilic (14), on the other hand, is considered as a potential malaria vector (16, 17, 18) and its high abundance point to Thessaloniki and especially Evros (due to the presence of immigrant detention camps) as potential malaria transmission hot spots. The prevalence of these species in Northern Greece is in accordance with previous studies (5, 19, 20, 21, 22).

The presence of *Aedes albopictus* (vector of Yellow fever virus, Dengue and Chikungunya fever) in Thessaloniki may be correlated with the fact that Thessanoliki has the main harbor facility of Northern Greece, since it has been shown that this species has moved from continent to continent and within countries via trade (23).

As shown in figure 3 the population dynamics of *Aedes caspius*, *Cx. pipiens* and *An. hyrcanus* differ. *Ae caspius* dominance in both foci in the early summer is due to the fact that this species overwinters in the egg stage and populations explode with each rainfall in a pulsed egg hatching manner (24). (2014 year was an unusually rainy summer).). On the contrary, *Cx. pipiens* and *An. hyrcanus* populations develop gradually and peak in August and September partly due to the increase in temperature. Differences in the population dynamics between Evros and Thessaloniki are possibly correlated with trap positioning. Unlike Thessaloniki, in Evros the traps are positioned at a distance (400m – 2 km) from the rice fields and both *Culex* and *Anopheles* mosquitoes don't have the same distance flying ability as *Aedes* mosquitoes (14). Thus, the mosquito catches in Evros may be biased towards *Aedes caspius*.

3.2 Species molecular analyses

The genetic analysis conducted in this study revealed important differences in *Cx. pipiens ss* composition between the three regions studied. The dominance of *molestus* biotype in Attica urban foci and *pipiens* biotype in both agricultural foci is in accordance with their mating: stenogamy vs eurygamy and feeding: mammophilic vs ornithophilic behaviors respectively and these results are in agreement with a previous study in Greece (25). However, Gomes et al study in 2010 did not display the high hybrid representation (30%) we found in the Attica populations. This may indicate an increase in hybridization over the last years even though *pipiens* frequency is very low (5%). Thessaloniki seems to be exactly the reverse situation of Urban Attica, with

a high hybrid representation (17%) and a low *molestus* representation (5%). High hybrid frequency compared to low *pipiens* and *molestus* representation in Athens and Thessaloniki respectively may indicate a selective advantage of hybrid mosquitoes in these environments and the higher frequency of hybrids in Thessaloniki compared to Evros (3%) may explain the occurrence of the major WNV epidemic in The/ki region when no WNV transmission has been recorded in Evros despite the close proximity of the 2 regions. In any case, the high hybrid frequencies observed both in Athens and Thessaloniki indicate these foci as ongoing hot spots for WNV transmission.

The presence of *Cx. quiquefasciatus* or hybrids of *Cx. quinquefasciatus* and *Cx. pipiens ss* is still an open question. Gunay et al (11) found in 2014 for the first time the widespread presence of this species in West Turkey and according to a recent study (26) hybrids of *Cx. Pipiens* and *Cx. quinquefasciatus* were found in Kos island. In our case further population analyses are required including positive – *Cx. quiquefasciatus* mosquitoes to help interpret the diagnostic assay gel bands generated.

Conclusion

The mosquito population structure analysis we conducted provides information that can prove useful in the guidance of control applications : when (species dynamics), where and how (species composition and distribution) to intervene and highlights the need for surveillance and pro - active mosquito control in the mosquito borne disease transmission hot spot regions of Thessaloniki, Evros and Attica.

Chapter 2 : Analysis of insecticide resistance in mosquitoes of the genus *Culex*, *Anopheles* and *Aedes* from different environments in Greece.

Summary

The re-introduction of vector borne diseases in Greece poses a major public health problem and use of insecticides (larvicides and adulticides) is the primary method for control of pathogen transmitting mosquitoes as well as nuisance species. Target species and knowledge about their insecticide resistance is an important pre-requirement for effective control strategies. In this study we analyze the insecticide resistance status of mosquito populations of the genus *Culex*, *Anopheles* and *Aedes* from different agricultural and urban environments in Greece. Our results indicate pyrethroid resistance in *Cx. pipiens* and *Anopheles hyrcanus* populations and susceptibility to organophosphate and carbamate insecticides.

Introduction

Mosquito borne disease epidemic history in Greece and the wide spread distribution of important mosquito vectors in the country make mosquito control a necessity. Current control strategies rely mainly on insecticidal applications. There are four main classes of insecticides used for mosquito control: Organochlorines (DDT), Pyrethroids, Carbamates and Organophosphates.

An ever-lasting problem of chemical mosquito control is the development of insecticide resistance. The extensive use, limited range of active ingredients, off target application, inadequate coverage rates and untimely implementation of insecticides targeting mosquitoes or applied for agricultural purposes act like a selective force, which results in the formation of mosquito populations exhibiting, either physiological or behavioral (27,28) resistance. The physiological resistance is based on molecular mechanisms, which mainly include increased activity or abundance of detoxification enzymes (metabolic resistance) and changes at the insecticide target molecules (altered target site resistance) disabling the insecticide binding. Target-site insensitivity includes insecticide target-site mutations in structural genes of the central nervous system, such as the synaptic acetylcholinesterase G119S and F290V mutations (Ace-1 mutations) and the voltage-dependent sodium channel L1014 mutations conferring resistance to organophosphates / carbamates and pyrethroids respectively (29-32)
Mutations in the sodium channel result in the characteristic “knock-down resistance” or *kdr* phenotype. The behavioral resistance is associated with the feeding and resting

preferences of mosquito species that can change as a result of insecticidal applications.

Knowledge of the resistance status of populations in areas of interest and the mechanisms responsible for resistance are essential for effective mosquito surveillance and control programs' planning and implementation.

In Greece (Northern prefectures) organized mosquito control applications have only taken place in the recent years with both larviciding and adulticiding interventions conducted in the rice-fields with aerial applications of Insect Growth Regulators - IGRs, diflubenzuron and Aerial ULV applications with pyrethroid insecticides - unsynergized-deltamethrin respectively. Furthermore, pyrethroid, carbamate and organophosphate insecticides have been used in the past and are still applied for agricultural purposes.

The main objectives of this study are :

1) To analyze the insecticide resistance status of the three most prevalent mosquito species in Thessaloniki and Evros : *Aedes caspius* , *Culex pipiens* and *Anopheles hyrcanus* to the pyrethroid deltamethrin, with the use of CDC Bottle Bioassays.

2) By using modern diagnostic tools for the insecticide resistance markers / target site mutations : L1014F, G119S and F290V to further analyze pyrethroid resistance and incipient organophosphate / carbamate resistance respectively.

4. Materials and Methods

The Study Area and Mosquito Collections for this study are described in chapter 1, section: 1.1.1 and 1.1.2

4.1 CDC Bottle Bioassays:

Bioassay experiments were conducted for the analysis of *Ae. caspius*, *Cx. pipiens s.l.*, and *An. hyrcanus s.l.* (most prevalent species in Thessaloniki and Evros) insecticide resistance status to deltamethrin.

4.1.1 Mosquito samples in bioassays

Bioassays were conducted on wild caught, adult, live, female mosquitoes collected from 2 locations in Evros (Feres agricultural region & river delta region, collection

dates : 31/7 till 20/8) and Thessaloniki (Chalastra & Vrachia agricultural region, collection dates 31/7 till 13/9).

A standard *Cx.pipiens molestus* laboratory strain that had not been exposed to insecticides for more than 20 years (Benaki Phytopathological Institute, Athens, Greece) was also included in the bioassays.

4.1.2 Bioassay set-up and Insecticides

CDC Bottle Bioassays were executed following the standard CDC procedures (33). Wild caught female mosquitoes were placed in cages with 10% sucrose solution for 24 hours prior to experimentation to allow for acclimatization to lab conditions. The mosquitoes were anaesthetized with a cold shock (40 seconds on ice) and subsequently identified morphologically. They were allocated to separate cages based on morphological species ID and were given 1 h to recover from the chilling effects of the ice prior to the treatment. The insecticide stock solutions were prepared based on the CDC guidelines and tests were conducted using batches of 20-25 mosquitoes per bottle. For every experimental set there was one control / untreated bottle and a total of at least 100 mosquitoes divided among 4 replicate bottles (treated) exposed to deltamethrin (when not possible to collect this number on a single occasion, results of multiple bioassays over a few days were pooled to achieve the recommended sample size).

Diagnostic times and doses for mosquitoes tested :

- *Aedes caspius* : 10 μg diagnostic dose Deltamethrin / bottle , Diagnostic time 30 minutes (33).
- *Cx. pipiens s.l.* : 20 μg diagnostic dose Deltamethrin / bottle , Diagnostic time 23 minutes
- *Anopheles hyrcanus s.l.* : 12,5 μg diagnostic dose Deltamethrin / bottle , Diagnostic time 30 minutes (33).

After exposure to the insecticide for 2 hours (recording of dead and alive mosquitoes was conducted every 15 minutes), the mosquitoes were transferred into insecticide-clean/ uncontaminated tubes and maintained on 10% sucrose solution for 24 hours. Then, the final numbers of dead and surviving mosquitoes were recorded.

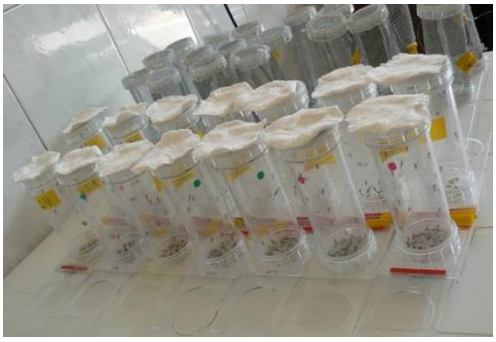



Figure 8 : CDC bioassay bottles and secondary chambers used in our experiments.

4.1.3 Data Analysis

The percent mortality during the 2 hour exposure to insecticide and at 24 h post-treatment, was used to determine insecticide susceptibility status. Mortality 98-100% at the recommended diagnostic time indicates susceptibility, 80-97% mortality suggests the possibility of resistance that needs to be further confirmed and mortality at less than 80% denotes resistance (33).

The data obtained from the CDC bottle bioassays was compared with the diagnostic times given in the CDC guidelines for *Aedes caspius* and *Anopheles hyrcanus* whereas *Cx. pipiens* were compared with a susceptible baseline population (Benaki *Cx. pipiens molestus* laboratory strain).

4.2 Insecticide resistance molecular analyses

Culex pipiens mosquitoes were analyzed for the presence and frequency of the target-site resistance mutations : L1014F/C (pyrethroid resistance) , F290V and G119S (organophosphate / carbamate resistance) using PCR-based diagnostic assays. Genotypes were confirmed with sequencing. *Anopheles hyrcanus s.l* mosquitoes were also genotyped in search of KDR mutations conferring pyrethroid resistance. All DNA extractions were performed using the DNAzol method (Invitrogen).

4.2.1 Culex pipiens molecular analyses

4.2.1.1 Mosquito samples : A representative subset of 200 hundred *Culex pipiens* mosquitoes from Evros and Thessaloniki (from all collection stations - throughout the surveillance period) and 40 *Culex pipiens* mosquitoes from Attica region that were sorted, identified and stored as described in section.. were analyzed individually for the pre-mentioned mutations.

4.2.1.2 DNA Extraction : see section 1.2.1

4.2.1.3 KDR mutation L1014F (TTA to TTT) diagnostic assay

For each mosquito sample 2 PCR reactions are conducted containing primers for the susceptible and resistant allele respectively (34).

- Cgd1, 2 primers amplify a sequence (PCR control band) which is common in all individuals
- Cgd3, 2 amplify for a sequence found only in susceptible alleles
- Cgd4 ,2 amplify for a sequence found only in resistant alleles

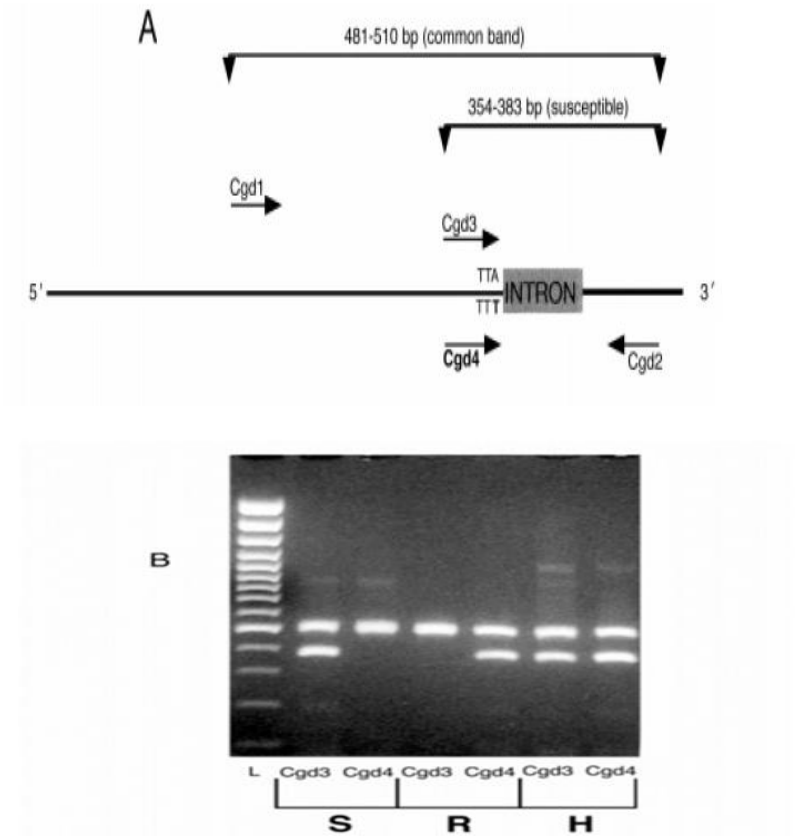


Figure 9: Reproduced from (34). A) KDR diagnostic assay diagram, B) Amplification products of Cgd1,2,3,4 primers run on a 1,5% agarose gel .Lane 1: 100bp marker , Lane 2-3: susceptible (SS) individual, Lane 4-5: resistant (RR), Lane 6-7: heterozygote (RS)

Protocol

Box 4

Primers			
Cgd1 : 5’ GTGGAACTTCACCGACTTC 3’			
Cgd2 : 5’ GCAAGGCTAAGAAAAGGTTAAG 3’			
Cgd3 : 5’ CCACCGTAGTGATAGGAAATTTA 3’			
Cgd4 : 5’ CCACCGTAGTGATAGGAAATTTT 3’			
Mastermix Solutions			
Mastermix 3		**Mastermix 3**	
gDNA :	1 µl	gDNA :	1 µl
Kappa Taq Buffer A 10x :	2,5 µl	Kappa Taq Buffer A 10x :	2,5 µl
dNTPs :	0,5 µl	dNTPs :	0,5 µl
MgCL2 (25Mm) :	0,75 µl	MgCL2 (25Mm) :	0,75 µl
CgD1 :	1µl	CgD1 :	1µl
CgD2 :	2 µl	CgD2 :	2 µl
CgD3 :	1 µl	CgD4 :	2 µl
Kappa Taq polymerase :	0,2 µl	Kappa Taq polymerase :	0,2 µl
ddH20 :	16 µl	ddH20 :	15 µl
Vfinal :	25 µl	Vfinal :	25 µl
PCR conditions		**Gel Electrophoresis**	
95°C / 5’		1,5% agarose gel	
94°C / 30’’			
48°C / 30’’			
72°C / 1’			
72°C / 10’			
10°C / f.e			
Steps 2-4 : 40 cycles			

Box 4 : KDR (L1014F) diagnostic assay protocol

To clarify whether the ‘’resistant’’ bands generated were due to the presence of L1014F or L1014C mutation a subset of RR individuals was sequenced.

4.2.1.4 Ace-1 mutation G119S (GGC to AGC) diagnostic assay

The presence of the G119S mutation creates an AluI restriction site in the ace-1 gene and the resistance mutation is detected with a PCR-RFLP diagnostic test (35).

Primers CxEx3dir and CxEx3rev produce a fragment close to 550bp which is undigested by AluI for susceptible homozygous mosquitoes (SS), and cut into two

fragments (approximately 370 bp and 180 bp) for homozygous resistant (RR) mosquitoes. Heterozygous individuals (RS) display a combined pattern (36).

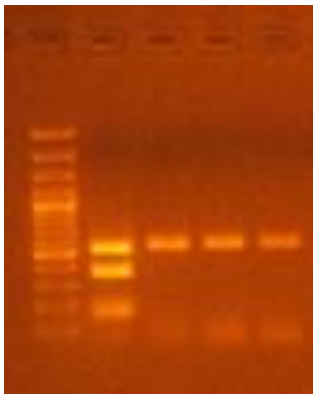


Figure 10: Amplification products of CxEx3dir and CxEx3rev primers after AluI digestion, run on a 1,5% agarose gel .Lane 1: 100bp marker , Lane 2:Heterozygote (RS) individual, Lane 3-5: susceptible (SS)

Protocol

Box 5

Primers	**PCR conditions**
CxEx3dir : 5' CGACTCGGACCCACTGGT 3'	95°C / 5'
CxEx3rev : 5' GTTCTGATCAAACAGCCCCGC 3'	95°C / 30''
	54°C / 30''
	72°C / 1'
Mastermix Solution	72°C / 10'
gDNA : 1 µl	10°C / f.e
Kappa Taq Buffer A 10x : 2,5 µl	Steps 2-4 : 35 cycles
dNTPs : 0,5 µl	
CxEx3dir : 1 µl	**AluI digestion**
CxEx3rev : 1 µl	PCR product : 10 µl
Kappa Taq polymerase : 0,2 µl	10x AluI buffer : 1,5 µl
ddH20 : 18,8 µl	BSA (10mg/ml) : 0,5 µl
Vfinal : 25 µl	AluI (10u/µl) : 0,6 µl
	ddH20 : 2,4 µl
	Vfinal : 15 µl
	for 3 hours at 37 °C
	digestion products on 1,5 % agarose gel

Box 5 : G199S diagnostic assay protocol

4.2.1.5 Ace-1 mutation F290V (GTT to TTT) diagnostic assay

A PCR reaction of four primers for the coding exon 5 of the ace-1 gene discriminates individuals with a valine at position 290 (resistance) from those having the wild-type phenylalanine (37).

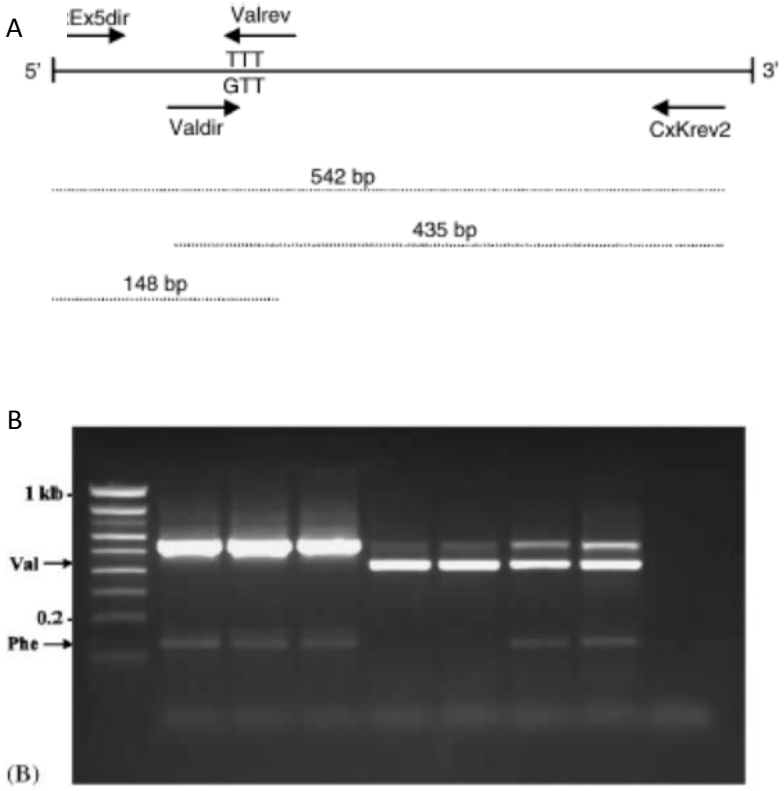


Figure 10 : Reproduced from (37). A)F290V diagnostic assay diagram :148 bp band / specific to phenylalanine (susceptible allele), 542 bp band / internal control band, 435 bp band / specific to valine (resistant allele). B) Amplification products of CxEx5dir, CxKrev2 , Valdir, Valrev primers run on a 1,5% agarose gel .Lane 1: 100bp marker , Lane 2-34: susceptible (SS) individual, Lane 5-6: resistant (RR), Lane 7-8: heterozygote (RS)

Protocol

Box 6

Primers	**PCR conditions**
CxEx5dir : 5' GTCTGGCCGAGGCCGTCA 3'	95°C / 5'
Valdir : 5' ACGCTGGGGATCTGCGAGG 3'	94°C / 30''
Valrev : 5' TCCACAACCGGAACGAACGGAAA 3'	51°C / 30''
CxKrev2 : 5' TGCTTCTGTGCGTGTACAGG 3'	72°C / 40''
	72°C / 5'
Mastermix Solution	10°C / f.e
gDNA : 1,5 µl	Steps 2-4 : 30 cycles
Kappa Taq Buffer A 10x : 2,5 µl	
dNTPs : 0,5 µl	**Gel Electrophoresis**
CxEx5dir : 0,75 µl	1,5% agarose gel
Valdir : 1,5 µl	
Valrev : 0,75 µl	
CxKrev2 : 0,75 µl	
Kappa Taq polymerase : 0,25 µl	
ddH20 : 16,5 µl	
Vfinal : 25 µl	

Box 6 : F290V diagnostic assay protocol

4.2.2 *Anopheles hyrcanus* sequencing for detection of KDR mutations

A total of 38 adult female field caught *Anopheles hyrcanus* mosquitoes were sequenced.

Primers used :

- *An. hyrcanus* F : 5' TGGATTGAATCAATGTGGGATTC 3'
- *An. hyrcanus* R : 5'AAGGATGAAGAACCGAAATTGGAC 3'

5. Results

5.1 CDC Bottle Bioassays:

As shown in figure 11, *Ae. caspius* susceptibility to deltamethrin was recorded in both regions with a mortality over 90% at the LT90 diagnostic time point. *An. hyrcanus s.l* mortality at LT90 was 40 % and 60% for Thessaloniki and Evros respectively indicating resistance to deltamethrin. For *Cx. pipiens* mortalities recorded at the LT50

diagnostic timepoint were 22% and 4% for Thessaloniki and Evros respectively, results that also indicate *Cx. pipiens* resistance to deltamethrin.

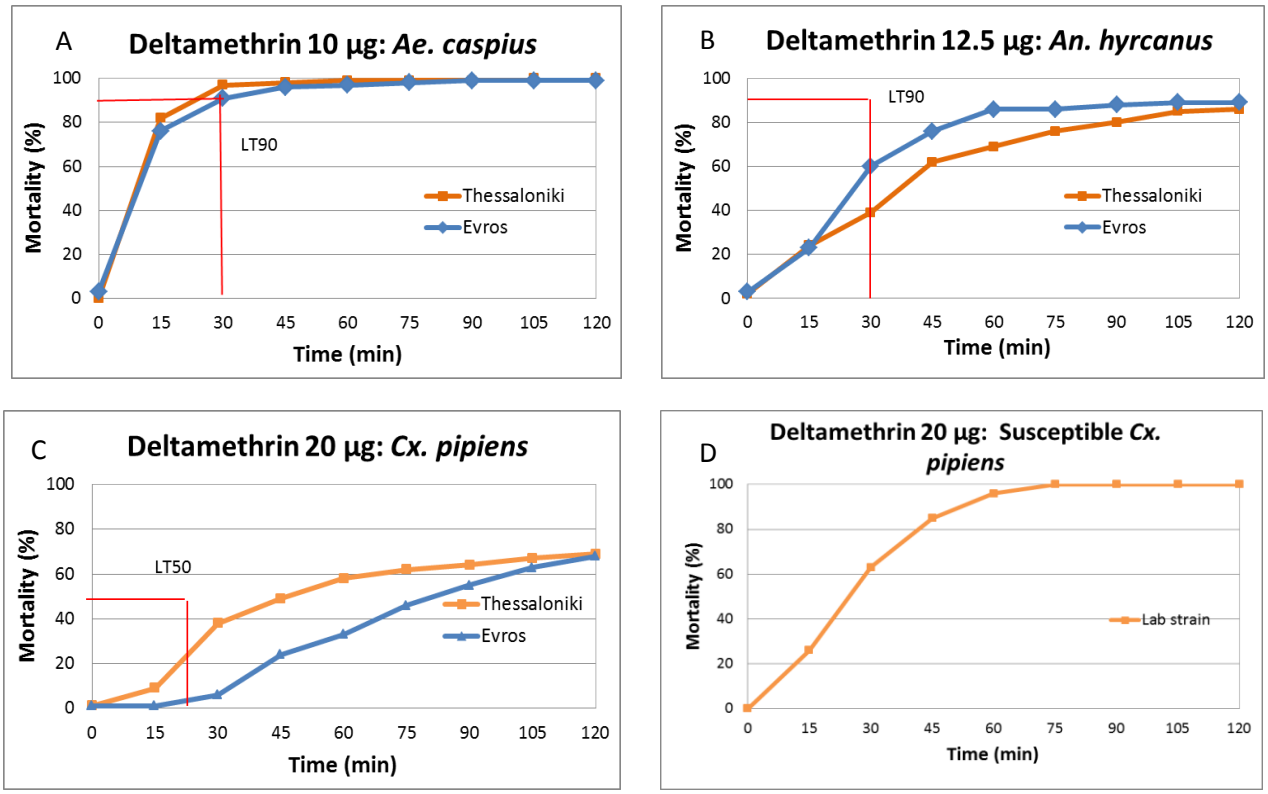


figure 11: CDC bottle deltamethrin time response bioassays in *Ae. Caspius* (A), *An. hyrcanus* (B), *Cx. pipiens* (C) populations from Evros and Thessaloniki and *Cx. pipiens* susceptible laboratory strain (D). In red : diagnostic LT90s and LT50S

5.2 Insecticide resistance molecular analyses

5.2.1 *Culex pipiens* molecular analyses

Culex pipiens insecticide resistance was further analyzed by molecular means due to the phenotypic resistance displayed in the bioassays and the public health importance of this species as it is the major vector of WNV in our country. Analyses of the Attica populations and susceptible laboratory strain were conducted by Tsiamantas A. and Kampouraki A.

5.2.1.1 KDR mutations

Higher frequency of (RR) KDR mosquitoes was observed in Evros and Athens compared to Thessaloniki and close to zero frequency of (SS) mosquitoes in all foci. These results are generated from the diagnostic assay that detects the KDR mutation L1014F. However this assay does not distinguish between this mutation and L1014C.

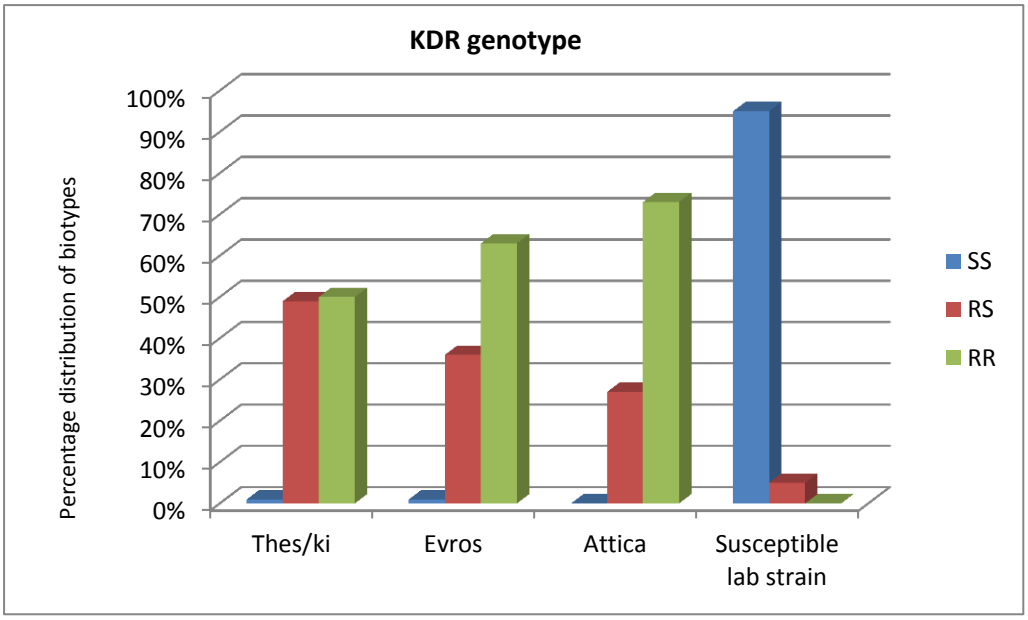


figure 12 : Frequency and distribution of KDR mutations L1014F, L1014C in Thes/ki, Evros, Attica, Susceptible lab strain (RR=mutation present in both alleles).

A subset of 30 (RR) mosquitoes from all foci were sequenced in order to clarify the mutation present.

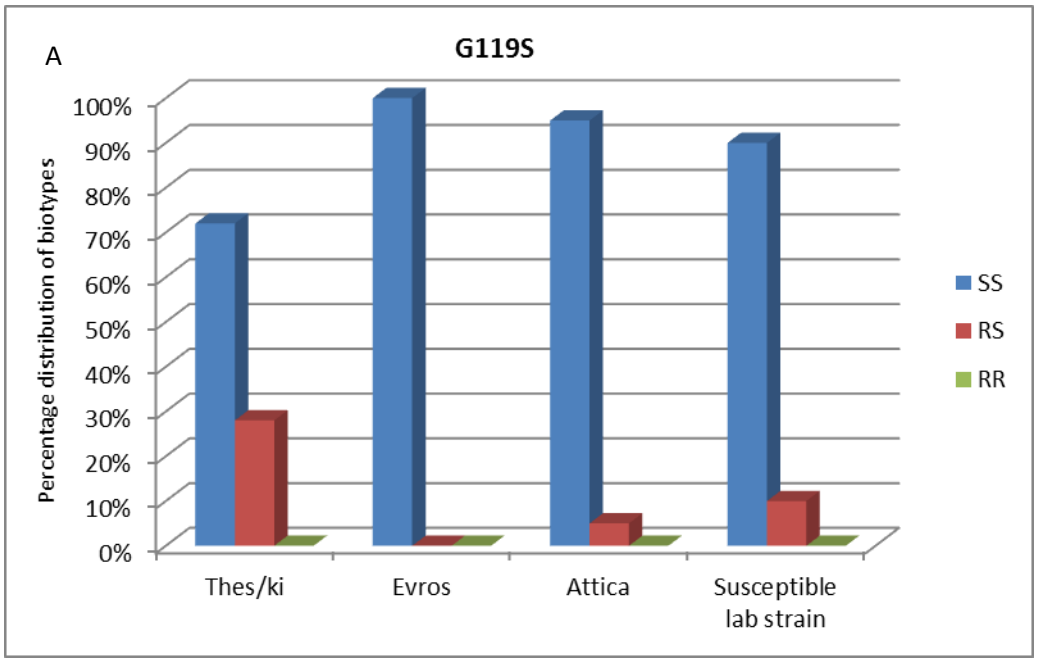
KDR genotype	Thes/ki	Evros	Attica
L1014F	1	0	2
L1014C	17	8	1
L1014F/C	0	1	0
total # of individuals	18	9	3

Table1 : KDR sequencing results

Higher frequency of the KDR mutation L1014C (vs) L1014F was recorded in both Evros and Thes/ki.

5.2.1.2 Ace-1 mutations : G119S and F290V

Similar frequencies of G119S and F290V mutations were observed within mosquito populations of Thes/ki, Evros and Athens respectively with the exception of a higher (RS) G119S frequency observed in Thes/ki.



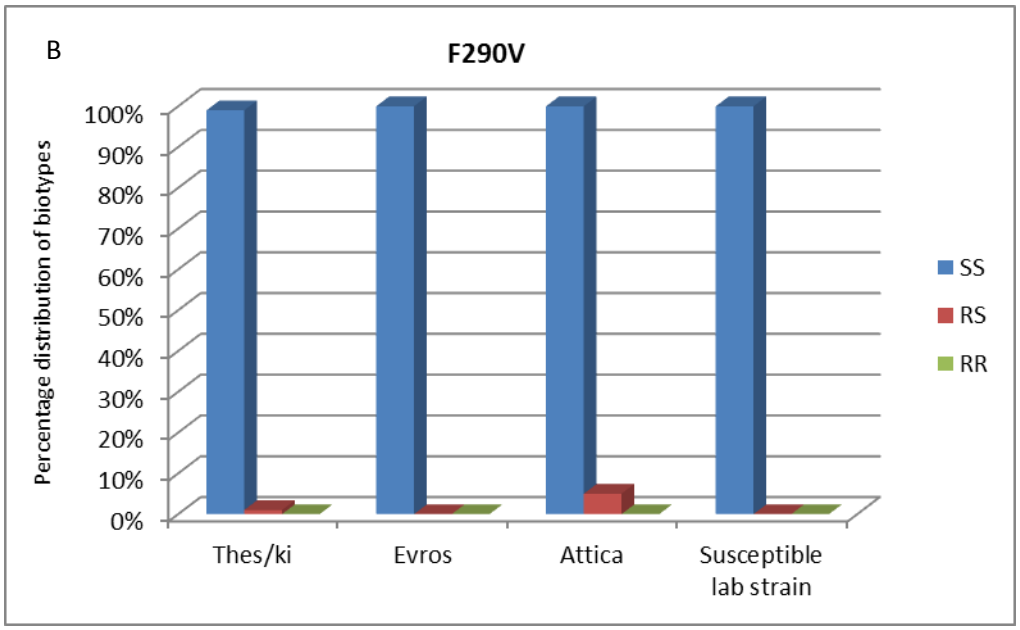


figure 12 : Frequency and distribution of ACE-1 mutations : G119S (A) and F290V (B) in Thes/ki, Evros, Attica, Susceptible lab strain (RR=mutation present in both alleles).

5.2.2 *Anopheles hyrcanus* molecular analysis

All mosquitoes (n=38) sequenced for the presence of KDR mosquitoes displayed an (SS) genotype. Even mosquitoes which had survived the bioassays had no mutations (n=12).

6. Discussion

6.1 Pyrethroid resistance

By comparing the LT90s of susceptible (diagnostic LTs) and our wild caught mosquitoes the bioassay results indicate *Anopheles hyrcanus* and *Culex pipiens* resistance to deltamethrin. However in the case of *An. hyrcanus* the 2 hour exposure to the insecticide led to a 90% mortality in both Evros and Thessaloniki whereas *Cx. pipiens* mortality at the same timepoint was 70% which raises even more our concerns on the resistance of this species. The higher resistance recorded in both species from Evros compared to Thessaloniki may be correlated with agricultural insecticide applications and their spill-over effect on mosquitoes. In Greece vector control chemical applications are minimal compared to the total volumes of insecticides used in agricultural practices and added to this, control practices are not applied in Evros. On the other hand, in Thrace – Turkish province (48,000 hectares of rice fields) little or no regulation on agricultural pesticides is applied hence possibly acting as a selective pressure for insecticide resistance development in mosquitoes.

The KDR analysis conducted on *Cx. pipiens* displayed higher frequency of (RR) KDR mosquitoes in Evros compared to Thessaloniki and this is in accordance with the bioassay results. The high (RR) and (RS) and close to zero frequency of (SS) mosquitoes in all foci is alarming.

The diagnostic assay we used (34) was designed for the detection of the mutation L1014F, however the primer Cgd4 (generates the R band) can also hybridize to genomes with a Cysteine codon at position 1014. Subsequently mosquitoes genotyped as (RR) may have the L1014F, L1014C or both mutations. We sequenced a subset of these mosquitoes and the mutation L1014C was detected in Evros, Thessaloniki and Attica. This is the first time this mutation is identified in *Culex* mosquitoes in Greece. More so the L1014C mutation seems to be the main KDR mutation found in mosquitoes from Evros and Thessaloniki and it is present in all three biotypes : pipiens , molestus and hybrids (combined findings from biotype and resistance analyses). There have been few reports of the L1014C mutation in field populations of mosquitoes from different parts of the world (38-41) and so far, this mutation has been reported to be associated with permethrin and deltamethrin resistance in *An.*

sinensis (39,42). A study in the Aegean region of Turkey (41) showed that homozygous 1014C/C was the most commonly occurring pyrethroid-response genotype found, with a decreasing frequency in the absence of insecticide selection pressure. This study in combination with our results indicates the natural existence, wide spread distribution and high frequencies of this mutation in the Eastern Mediterranean basin.

As for *Anopheles hyrcanus*, despite their phenotypic resistance to deltamethrin no KDR mutations were detected. Possibly, metabolic detoxification is the cause of this resistance observed rather than KDR target site mutations. In Thrace - Turkey, both *An. hyrcanus s.l.* and *An. sacharovi* had become highly resistant to insecticides after many years of developing in larval sites contaminated by crop spraying (43-45). *Anopheles maculipennis* strains tested showed elevated levels of NSEs (non specific esterases) and GSTs(glutathione S transferases) (46).

6.2 Organophosphate / Carbamate resistance

Both of the known AChE1 insensitivity mutations, G119S and F290V, were found in the populations tested but at a very low frequency. These observed frequencies are consistent with the findings of a previous study in Greece (36) except for the elevated frequency of (RS) G119S mosquitoes in Thessaloniki. The data obtained from the field samples revealed that the frequencies of resistant homozygous individuals for both insensitivity mutations are extremely low (<0.2%). In general the low frequency of the AChE1 insensitivity mutations observed may result from the weak selection pressures applied in these regions throughout the collection season together with the fitness cost of the mutations.

Conclusion :

Based on our results *Culex pipiens* mosquitoes in Evros, Thessaloniki and Attica and *Anopheles hyrcanus* populations in Northern Greece seem to be resistant to deltamethrin which is one of the adulticiding products used for mosquito control in Greece. The current insecticide resistance status in combination with the lack of alternative adulticides is a particularly worrying situation and alternative control strategies with insecticides with different modes of action should be examined. For efficient control guidance follow up studies and further investigation into the resistance mechanisms of these mosquito populations with emphasis on metabolic resistance is required.

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