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Running head: *Cx.pipiens* insecticide resistance in Morocco

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Abstract

BACKGROUND: Mosquitoes of the *Culex pipiens* complex are the vectors of several arboviruses, and are thus subjected to insecticide control worldwide. However, overuse of insecticides selects for resistance. While assessing the resistance status of the vectors is required for effective and sustainable disease control, resistance has so far only been sparsely studied in Morocco. In this study, we establish a first countrywide assessment of the levels of resistance to various insecticides and potential responsible mechanisms involved.

Cx. pipiens larvae have been collected from natural populations of five regions of Morocco, and their taxonomic status has been determined (molecular forms). The level of their susceptibility to insecticides was assessed by single-diagnostic-dose bioassays. Molecular identification of known resistance alleles was investigated to determine the frequency of target-site mutations.

RESULTS: This study confirms that Moroccan populations are an interbreeding mix of *pipiens* and *molestus* forms, with large gene flow for the resistance alleles. We also found that *Cx. pipiens* mosquitoes are resistant to all insecticide families, all over Morocco: resistance is high for insecticides used in mosquito control, but also present for other pesticides. Resistance alleles are similarly more frequent for mosquito control insecticides. However, their distribution is heterogeneous in the five regions, with significant genetic differentiation between populations, revealing the crucial role of local insecticide treatment practices.

CONCLUSION: This study provides reference countrywide data that highlight the need for further research to refine the distribution of resistance in Morocco and to understand the role of agriculture/urban residuals in its spread.

Key words: target-site resistance, metabolic resistance, arbovirus vectors, *molestus*, *pipiens*.

1 INTRODUCTION

Emerging and re-emerging vector-borne diseases are becoming increasingly important in animal and public health. According to the World Health Organization, they are responsible for more than 17% of infectious diseases and cause more than one million deaths each year [1].

Most vector-borne diseases affecting human health are zoonoses that require a cycle of amplification involving zoophilic mosquitoes and vertebrate hosts other than humans [2]. This is the case for many arboviruses (arthropod-borne viruses): the mosquito *Culex pipiens* is for example the vector of several arboviruses like West Nile (WNV), Rift Valley Fever and St Louis encephalitis viruses [3]. The reservoirs of WNV are birds, with epidemics occurring as a result of virus transmission to equidae and humans. Epizootics/epidemics have been regularly observed in Europe (1684 confirmed cases in December 2018, including 181 deaths, according to the European Centre for Disease Prevention and Control [4], and in Morocco, several outbreaks of WNV have been reported in 1996, 2003, 2010 [5–8] .

Mosquitoes of the *Cx. pipiens* complex proliferate in sites heavily polluted by organic matter produced by human activities (sewage treatment plants, sewers and wastewater collectors, etc.). In the southern Mediterranean regions in particular, the socio-economic changes of recent decades have favored the fast development of urban centers, sometimes however with inadequate sanitation facilities. Rural exodus to already-densely populated cities has created favorable conditions for *Cx. pipiens* proliferation. Several forms of the *Cx. pipiens* complex coexist in Morocco and hybridize [9]: the *pipiens* form is capable of diapause, anautogenic and eurygame, whereas the *molestus* form is autogenous and stenogamous [10–14], and does not diapause [15]. *Cx. pipiens* mosquitoes are now the predominant urban mosquito species, leading to an increased risk of arboviral epizootics and epidemics.

In the absence of effective treatment and vaccines against these arboviruses, mosquito population control is the main strategy for limiting pathogen transmission. In this context, the

use of insecticides plays a key role in the prevention and control of vector-borne diseases. In Morocco during the 1950s, treatment of larvae began with the use of DDT, an organochlorate insecticide. This was used until 1978 when an organophosphate insecticide (OP), temephos, was introduced [16]. However, mosquitoes control is threatened by insecticide resistance. Insecticide resistance is widespread worldwide, and multiple mechanisms have been selected due to the increasing pressure of insecticides used in public health but also in agriculture [17–20]. Two main groups of resistance mechanisms are recognized: metabolic resistance and resistance through modification of the insecticide target. In *Cx. pipiens* (as in most mosquito species), metabolic resistance results from strong increases in the expression of detoxifying enzymes that inactivate or sequester insecticidal molecules. These enzymes belong mostly to three major families: the glutathion S tranferases, cytochrome P450 oxidases, and the esterases. The latter, causing resistance to OP and carbamate (CX) insecticides, is the most studied in this species [21–23]. The main target modifications result in reduced susceptibility to the insecticide. In *Cx. pipiens*, they are due to mutations in the genes encoding i) the voltage-gated sodium channel (*vgsc* locus; L1014F *kdr* mutations), the target of the pyrethroids insecticides (PYR) target[24], ii) the acetylcholinesterase (AChE1; *ace-1* locus, G119S and F290V mutations), the target of OP and CX insecticides [25,26], and iii) the gamma aminobutyric acid receptor (GABA; *Rdl* locus, A302S mutation), the target of dieldrin (OC)[27]. Moreover, certain mechanisms allow resistance to several insecticide molecules, sometimes of different families: this cross-resistance severely limits the choice of alternative insecticides [28–30]. Finally, it has recently been shown that resistance can increase *Cx .pipiens'* vectorial competence for WNV, which may therefore lead to an increased risk of epidemics [31].

In order to anticipate and establish informed management of insecticide resistance as part of plans for the prevention and/or management of vector-borne disease outbreaks, knowing the mechanisms present in a given region and their frequency is required[32]. In contrast with Tunisia and Algeria, where various resistance alleles have been detected in *Cx. pipiens* mosquitoes, often at high frequencies [33,34], very little work has been done in Morocco on the mechanisms of insecticide resistance (this is also true for other vectors, as only *Anopheles labranchiae* has been studied so far[16]). Nevertheless, we have recently reported that wild

populations of *Cx. pipiens* in Mohammedia have developed resistance to OC, OP, CX and PYR insecticides [35].

The objective of the present study was thus to assess and map the resistance levels and the different resistance mechanisms present in natural populations of the *Cx. pipiens* species complex in Morocco. Only OPs and PYRs are used for mosquito control in Morocco, but mosquitoes can be exposed to other insecticide families, used in agriculture for example [20]. Therefore, resistance to the four families of insecticides usually used in vector control (PYR, OP, CX and OC) was evaluated for both *molestus* and *pipiens* forms in five regions of Morocco: Tangier (north), Larache (northwest), Mohammedia (center west), Marrakech (center) and Agadir (south west). Resistance mechanisms were characterized and the resistance alleles frequency analyzed in the different populations and taxons. In all of them, we found resistance to all insecticides, but with varying frequencies. This work constitutes a first reference to assess the dynamics of insecticide resistance in Morocco, with the ultimate goal of establishing a temporal and predictive projection of the risk of transmission, to assist the health authorities in their vector control strategy.

2 METHODS

2.1 Mosquito samples

Cx. pipiens was collected as larvae using the dipping sampling method during summer 2018 in 5 regions of Morocco (one sample per region), in various types of breeding sites and in different climatic regions representative of the country (Fig. 1): 1) sewer water in humid climate (Tanger, 35°46'44.3" N5°50'50.1"W), 2) wet meadows in sub-humid climate (Larache, 35°10'55.7" N6°07'56.8"W), 3) sewer water in humid climate (Mohammedia, 33°40'25.33"N7°26'42.5"W), 4) irrigation canals in arid climate (Marrakech, 31°38'12.7"N8°10'07.0"W), 5) sewage water mixing in Souss river in sub-arid climate (Agadir, 30°21'39.8"N9°29'22.6"W). The strain Slab was used as the susceptible reference strain [36]. Part of the larvae were analyzed directly using bioassay, the others were brought back to the lab and reared until adulthood for molecular analyses.

2.2 Bioassays

Mortality following insecticide exposure was determined by single-diagnostic-dose bioassays on fourth instar larvae collected in the field and brought to the lab. The insecticides, propoxur (CX), temephos (OP), permethrin (PYR), dieldrin (OC) and chlorpyrifos (OP), were purchased from Dr Ehrenstorfer GmbH, Germany. Each compound was dissolved in ethanol, with a final concentration of ethanol of 1% for all doses and all insecticides. For each insecticide, 2 cups were prepared for the Slab as susceptible strain, and 4 cups for the tested population. 2 cups of tested populations were prepared without insecticide as control. In each cup, 20-30 larvae were added to 100ml of water. The lowest dose of insecticide killing all the Slab larvae (100% mortality) was then added to each cup (the doses have been found through bioassays on Slab strain larvae with various does of insecticide) : 2 $mg.l^{-1}$ for propoxur, 0.0003 $mg.l^{-1}$ for temephos, 0.005 $mg.l^{-1}$ for permethrin, 0.01 $g.l^{-1}$ for dieldrin and 0.003 $mg.l^{-1}$ for chlorpyrifos. We then recorded the mortality after 24 hours.

2.3 Molecular analyses

For each population, ≈60 mosquito larvae were analyzed by PCR tests to characterize their taxonomy and genotypes for 5 resistance mutations. The DNA fragments were separated on 1.5% agarose gel electrophoreses and visualized by ethidium bromide staining under ultraviolet light.

2.3.1 Molecular identification of Cx. pipiens forms

DNA was extracted individually using the DNAzol method according to the manufacturer's protocol. Multiplex PCR tests were used to identify the *Cx. pipiens* complex, as described by Bahnck & Fonseca[37]. The CQ11 locus was used to distinguish the two forms of *Cx. pipiens* (*pipiens* and *molestus*) and their hybrids, using the primers pipCQ11R and molCQ11R, with the following PCR conditions: 30 seconds at 94°C, 30 seconds at 54°C and 40 seconds at 72°C. The different sizes of the amplified DNA fragment, 200 bp for *pipiens* and 250 bp for *molestus*, allows distinguishing the two forms and their hybrids in a single PCR reaction.

2.3.2 Detection of the L1014F mutation in the vgsc locus

The PASA diagnostic test of Martinez Torrez et al [38] was used to genotype the samples for the *vgsc* L1014F mutation, except that the two separate PCRs were performed in parallel on each mosquito, PCR1 for the susceptible S alleles (L1014), using the primers Cgd1, Cgd2 and Cgd3, and PCR2 for the resistance R allele (F1014), Cgd1, Cgd2 and Cgd4. PCR conditions were 1 min at 94°C, 2 min at 48°C and 2 min at 72°C for 40 cycles. PCR1 amplifies a common fragment for all individuals, and a specific fragment if the mosquito carries a S allele. PCR2 amplifies a common fragment for all individuals, and a specific fragment if the mosquito carries a R allele. The two PCR together thus allows distinguishing homozygotes and heterozygotes.

2.3.3 Detection of the G119S and F290V mutations in the ace-1 locus

The PCR-RFLP diagnostic test of Weill et al [39] was used to genotype the individuals for the *ace-1* G119S mutation. Briefly, a 374 bp fragment was amplified from exon 3 using the primers CpEx3dir and CpEx3rev, with the following PCR conditions: 30 seconds at 95°C, 30 seconds at 52°C and 1 minute at 72°C, for 30 cycles. The PCR product was then digested by the restriction enzyme AluI, according to the manufacturer's instructions (Jena Bioscience, Jena, Germany). The S allele is not digested, whereas the R allele is cleaved into two fragments, which allows distinguishing homozygotes and heterozygotes.

The PASA diagnostic test of Alout et al [26] was used to genotype the individuals for the *ace-1* F290V mutation. Briefly, 543 bp control fragment was amplified using the primers CxEx5dir and CxKrev2, 148 bp fragment specific of phenylalanine was amplified using the primers Valdir/CxKrev2 and 435 bp fragment specific of valine was amplified using the primers, with the following PCR conditions : were 30 seconds at 94°C, 30 seconds at 51°C and 40 second at 72°C, for 30 cycles.

2.3.4 Detection of A302S mutation in the Rdl locus

The PCR-RFLP diagnostic test of Tantely et al [27] was used to genotype the individuals for the *Rdl* A302S mutation. Briefly, a 232 bp fragment was amplified by PCR with mqGABAdir and mqGABArev, with the following PCR conditions: 30 seconds at 94°C for, 30 seconds at 52°C and 1 minute at 72°C, for 30 cycles. The PCR product was then digested by the

restriction enzyme BstAPI according to the manufacturer's instructions (Jena Bioscience, Jena, Germany) instructions. The R allele is not digested, whereas the S allele is cleaved into two fragments, which allows distinguishing homozygotes and heterozygotes.

2.3.5 Detection of the Ester^2 allele at the Ester locus

The PCR-RFLP diagnostic test Berticat et al [40] was used to genotype the individuals for *Ester^2* (A2-B2). The EsterA allele fragments were amplified using the primers EstAdir and EstArev, whereas the EsterB allele fragments were amplified using the primers EstBdir and EstBrev, with following PCR conditions: 30 seconds at 95°C for, 30 seconds at 52°C and 1 minute at 72°C, for 30 cycles for the EsterA and 30 seconds at 95°C for, 30 seconds at 52°C and 1 minute at 72°C, for 30 cycles for the EsterB. The PCR product was then digested by the restriction enzyme HaeIII for EsterA and HinfI for EsterB according to the manufacturer's instructions (Jena Bioscience, Jena, Germany) instructions.

2.4 Statistical analyses

All computations were performed using the R free software (v.3.3.1, http://www.r-project.org, The R core Team).

Frequency data for the different mutations were analyzed, using the *genepop* R package: the Hardy-Weinberg equilibrium was verified for each sample, and the genotypic differentiation of the different samples from Morocco was evaluated using F_{st} [41,42].

For each resistance mutation independently, the number of individuals for each genotype (*NG*, *i.e.* RR, RS and SS numbers) were also compared between the different forms of *Cx. pipiens* (FORM) and in the different localities (LOC). The significance of the observed differences was assessed with the following multinomial log-linear model (MLLM), using the *nnet* R package: *NG* = FORM + LOC + FORM: LOC, where FORM is a 3-level factor (*pipiens*, *molestus* or hybrid), LOC is a 5-level factor (one for each locality), and FORM:LOC represents the interaction between the two factors. The models were simplified as follows (Crawley 2007): significance of the different terms was tested starting from the higher-order terms using likelihood ratio tests (LRT). Non-significant terms ($p > 0.05$) were removed. Factor levels that were not significantly different (LRT) were grouped.

3 RESULTS AND DISCUSSION

3.1 Moroccan *Culex pipiens* are resistant to most common insecticides

To assess the diversity and prevalence of insecticide resistances in *Cx. pipiens* from Morocco, we first used single-diagnostic-dose bioassays on field-collected fourth instar larvae from five regions (Fig. 1). The proportion of individuals surviving the minimal dose killing all Slab larvae (*i.e.* the reference susceptible strain) is shown in Figure 2 for various insecticides from different families. Resistance to temephos (OP), chlorpyrifos (OP) and permethrin (PYR), was found very prevalent in all five populations, with mortality rates ranging from 4.34% to 26.5% for both OP, and from 4.3% to 19.33% for permethrin. By contrast, mortality was generally high with propoxur (CX) (range= 63% in Tanger to 93 % in Larache), and moderate to high for dieldrin (OC) (range=50% in Larache to 96% in Marrakech). Resistance to all main families of insecticides is thus present in mosquito natural populations from Morocco. It confirms previous studies and suggests that many types of insecticides are used in Morocco. Only OPs and PYRs are allowed for public health, which could explain the high level of resistance observed for these families. However, the presence of resistance to CXs and OCs also suggest that mosquitoes are exposed to other sources of insecticides, for example from agriculture or urban residuals, as found for several mosquitoes [43–46].

3.2 Moroccan *Cx. pipiens* populations are a mix of two molecular forms with many hybrids

Two distinct forms of the *Cx. pipiens* complex, *pipiens* and *molestus*, are morphologically identical but have significant behavioral and physiological differences. In the northern regions of Europe, Russia and the USA, these forms occupy different habitats, respectively surface and underground, whereas in Morocco a study found that both forms can co-occur in breeding sites [9]. To analyze the taxonomic diversity of *Cx .pipiens* in the different sampled localities, we genotyped 60 mosquitoes per population using the CQ11 PCR diagnostic test [37]. We found that most individuals appear as hybrids (from 31.7% in Tanger to 61.7% in Agadir), and that the *pipiens* form (18.3 to 63.3%) was more common than the *molestus* form (1.7 to 16.7%) in these populations (Tab. 1).

Table 1: Diversity and distribution of the *C. pipiens* forms in Morocco. The number of individuals from each form or hybrid and their frequencies in the sampled populations are indicated.

	Tanger	**Larache**	**Mohammedia**	**Marrakech**	**Agadir**
Forms	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
pipiens	38 (63.33)	25 (41.67)	27 (45.76)	18 (30)	11 (20.34)
molestus	1 (1.67)	4 (6.67)	7 (11.86)	10 (16.67)	9 (15.25)
hybrids	19 (31.67)	31 (51.67)	24 (40.68)	32 (53.33)	37 (62.71)
Total	*58*	*60*	*59*	*60*	*59*

These results thus confirm that the two forms coexist in the same breeding sites in Morocco. Note however that while the CQ11 PCR diagnostic test is sufficient to demonstrate hybridization at the population level, it provides only a crude approximation for the frequency of this hybridization, as it can be less accurate at the individual level. Further studies based on more discriminating tests (e.g. microsatellites,) are thus required for better estimations of the gene flow between the two forms.

3.3 Common target-site, but also metabolic resistance mutations, are present in Morocco, but their frequencies vary

We then measured the frequency of the most common resistance mutations, *i.e.* mutations at three locus encoding target proteins (*vgsc*, *ace-1* and *Rdl*) and a gene encoding detoxifying esterases (*Ester*).

i) The A302S *Rdl* mutation allows resistance to dieldrin, an OC insecticide. For logistical reasons, it was tested only in Mohammedia, and found at a frequency of 0.19, with similar frequencies between the molecular forms (LRT, χ^2= 1.5, *df*= 4, *p*= 0.83). While dieldrin has

been prohibited for a while, the persistence of *Rdl* resistance alleles is common in *Cx. pipiens* populations and could be due to other pesticides, used against termites for example.

ii) The L1014F *vgsc* mutation (*kdr*), found worldwide in *Cx. pipiens* (see review in [22]) and in other mosquitoes [47], allows resistance to PYR insecticides. As suggested by previous studies, the L1014F mutation is present in Morocco [35,48]: we found it in all populations tested, its frequency ranging from 0.08 in Mohammedia to 0.38 in Larache (Tab. 2). Two populations showed a significant departure from panmixia: a deficit of heterozygotes in Mohammedia, and excess in Agadir (Tab. 2).

Table 2: Resistance mutations frequencies in Morocco. For each population, the number N of mosquitoes analyzed is indicated (NB: the numbers can vary from one mutation to another, because some individuals could not amplify for all mutations tested, probably for technical reasons, e.g. conservation, resulting in poor quality for the extracted DNA), *f(R)* indicates the frequency of the resistance allele, F_{is} measures an excess ($F_{is} < 0$) or a deficit ($F_{is} > 0$) of heterozygotes compared to the panmixia expectation (Hardy-Weinberg equilibrium), and *P* indicates the probability that these deviations are statistically significant (bold when significant).

Samples	***ace-1* G119S**				***ace-1* F290V**				***vgsc* L1014F**			
	N	***f(R)***	F_{is}	***P***	**N**	***f(R)***	F_{is}	***P***	**N**	***f(R)***	F_{is}	***P***
Tanger	**60**	0.195	-0.018	1	**60**	0.033	-0.026	1	**57**	0.34	-0.2	0.15
Larache	**59**	0.242	-0.311	**0.01**	**60**	0.192	-0.229	0.10	**57**	0.38	-0.25	0.09
Mohammedia	**60**	0.083	-0.083	1	**58**	0.112	-0.118	1	**57**	0.08	0.4043	**0.03**
Marrakech	**61**	0.172	-0.2	0.19	**59**	0.051	-0.045	1	**59**	0.11	0.0576	0.52
Agadir	**59**	0.161	-0.058	1	**58**	0.009	-	-	**59**	0.31	-0.432	$\mathbf{5.10^{-4}}$

Samples	***Rdl* A302S**				***Ester*[2]**			
	N	***f(R)***	F_{is}	***P***	**N**	***f(R)***	F_{is}	***P***
Tanger		-	-		**58**	0.328	-0.009	1
Larache		-	-		**54**	0.583	0.3605	**0.01**
Mohammedia	**56**	0.188	0.2466	0.08	**59**	0.737	0.3512	**0.01**
Marrakech		-	-		**59**	0.636	0.2397	0.09
Agadir		-	-		**56**	0.482	-0.207	0.18

No clear geographical pattern emerges, but there is significant geographic structuration at this locus (Fig. 3A). This is confirmed by statistical analyses using multinomial log-linear model (MLLM): we found no significant interaction of the *Culex* form and the locality on the genotypic frequencies distribution (LRT, χ^2= 11.6, *df*= 20, *p*=0.93), no effect of the form (LRT, χ^2= 3, *df*= 6, *p*= 0.80), but the locality effect was significant (LRT, χ^2= 78.5, *df*= 8, $p=9.9x10^{-14}$). PYR treatments thus appear pervasive in Morocco, although probably not homogenous in their intensity. Moreover, the high level of PYR resistance compared to the L1014F *vgsc* mutation frequencies (particularly in Mohammedia or Marrakesh, Figs. 2 and 3) suggests that other mechanisms, most probably overproduction of detoxifying enzymes from the cytochrome P450 oxidase family [22], also contribute to this resistance in Morrocco (but they were not tested here).

iii) At the *ace-1* locus, two mutations allow resistance to OP and CX insecticides. The F290V substitution is found only in *Cx. pipiens*, around the Mediterranean Sea, usually at low frequencies [26,34,49]. By contrast, the G119S substitution is found worldwide, close to fixation in some populations, and has been selected independently in many pest species [25,39,50]. While the G119S mutation had already been described in Morroco [35,51], this is the first report for F290V in this country. We found both mutations in all regions, generally at relatively low frequencies, from <0.01 in Agadir to 0.19 in Larache for F290V, and from 0.08 in Mohammedia to 0.24 in Larache for G119S (Tab. 2). For both mutations, all populations displayed tendencies towards an excess of heterozygotes (F_{IS} <0), although only one was significant, for G119S in Larache (Tab. 2). Such heterozygote excess has already been described in other populations of *Cx. pipiens*, and may suggest the presence of duplicated alleles associating a susceptible and a resistant copy of *ace-1*[23,34,49,52,53]. These alleles are quite frequent in *Cx. pipiens*: they are selected because they display intermediate resistance and cost, which is adaptive in an environment with heterogeneous insecticide treatments [54–56]. However, further studies are required to confirm the presence and assess the identity of this or these alleles.

While the F290V mutation appeared more frequent on the central coast, with strong geographic structuration (Fig. 3B), no clear geographical pattern emerges for G119S, with only very limited geographic structuration (Fig. 3C). MLLM revealed significant locality effect in both cases (LRT, F290V: χ^2= 40.8, *df*= 8, *p*=2.2x10^{-6}; G119S: χ^2= 19.7, *df*= 8, *p*=1.09x10^{-2}), but again no effect of the form (LRT, F290V: χ^2= 4.7, *df*= 6, *p*= 0.58; G119S: χ^2= 0.99, *df*= 6, *p*= 0.98) and no interaction (LRT, F290V: χ^2= 15.6, *df*= 18, *p*=0.62; G119S: χ^2= 12.3, *df*= 20, *p*=0.9).

iv) Several alleles of the *Ester* locus also allow resistance to OP insecticides: among them *Ester*^{2} is found all over the World, [21,57] and appears to be the only *Ester* allele found in Morocco. It was found in all populations at relatively high frequencies, from 0.33 in Tanger to 0.74 in Mohammedia. Two populations showed significant departures from panmixia, deficits in heterozygotes in Larache and Mohammedia that are probably due to Wahlund effects (Tab. 3). No clear geographical pattern emerges, but we found significant geographic structuration for this locus (Fig. 3D; MLLM, LRT; significant locality effect: χ^2= 51.2, *df*= 8, *p*=2.4x10^{-14}; no form effect: χ^2= 3,6, *df*= 6, *p*= 0.73; no interaction: χ^2= 24.4, *df*= 18, *p*=0.14). The high prevalence of OP resistance in Moroccan *Culex* populations probably reflects the continuous use since 1978 of temephos and chlorpyrifos to control mosquito larvae in this country [35]. It also explains why the *ace-1* mutations (G119S and F290V) remain at lower frequencies than *Ester*^{2}, as the latter confers generally a higher resistance to OPs [21,57].

Table 3: Linkage disequilibrium. For each population (Pop), the p-value of the linkage disequilibrium between two loci (locus 1 and locus 2) is indicated; p-values > à 0.05 are italicized, but none remained significant after multiple testing correction (Bonferoni). The overall disequilibrium (over all populations, Fisher method) is bolded. These statistics were computed using genepop.

Pop	Locus1	Locus2	P-Value
Mohammedia	*kdr*	*ace-1(F290V)*	0.85
	kdr	*ace-1(G119S)*	1.00
	kdr	*Rdl*	0.91
	kdr	*Ester*	0.44
	ace-1(F290V)	*ace-1(G119S)*	1.00
	ace-1(F290V)	*Rdl*	1.00
	ace-1(F290V)	*Ester*	0.08
	ace-1(G119S)	*Rdl*	0.39
	ace-1(G119S)	*Ester*	0.43
	Rdl	*Ester*	0.30
Agadir	*kdr*	*ace-1(F290V)*	1.00
	kdr	*ace-1(G119S)*	0.14
	kdr	*Ester*	0.59
	ace-1(F290V)	*ace-1(G119S)*	1.00

	ace-1(F290V)	*Ester*	0.18
	ace-1(G119S)	*Ester*	0.38
Marrakech	*kdr*	*ace-1(F290V)*	*0.01*
	kdr	*ace-1(G119S)*	0.20
	kdr	*Ester*	0.34
	ace-1(F290V)	*ace-1(G119S)*	0.16
	ace-1(F290V)	*Ester*	0.30
	ace-1(G119S)	*Ester*	*0.02*
Tanger	*kdr*	*ace-1(F290V)*	0.48
	kdr	*ace-1(G119S)*	*0.03*
	kdr	*Ester*	0.10
	ace-1(F290V)	*ace-1(G119S)*	1.00
	ace-1(F290V)	*Ester*	1.00
	ace-1(G119S)	*Ester*	0.78
Larache	*kdr*	*ace-1(F290V)*	0.35
	kdr	*ace-1(G119S)*	0.78
	kdr	*Ester*	0.07
	ace-1(F290V)	*ace-1(G119S)*	1.00
	ace-1(F290V)	*Ester*	0.72
	ace-1(G119S)	*Ester*	0.09
All populations	***kdr***	***ace-1(F290V)90V)***	**0.26**
	kdr	***ace-1(G119S)119S)***	**0.15**
	kdr	***Ester***	**0.14**
	ace-1(F290V)90V)	***ace-1(G119S)119S)***	**0.96**
	ace-1(F290V)90V)	***Ester***	**0.33**
	ace-1(G119S)119S)	***Ester***	**0.08**

4 CONCLUSION

Overall, we thus found that resistance is pervasive in Moroccan populations of *Cx. pipiens*. We found no effect of the taxonomic form, which further suggests high gene flow between *pipiens* and *molestus* in Morocco, at least for the resistance alleles. While resistance levels and resistance allele frequencies against insecticides used for mosquito control in Morocco, OP and PYR, are high in all the populations, we also found that *Cx. pipiens* populations are moderately resistant to more insecticide families. This suggests that they are exposed to many other pesticides than those used for mosquito control. The origin of these pesticides, whether

from agriculture or urban residuals, should be investigated further to understand and measure their impact on resistance, which also requires a detailed knowledge of the histories of local treatments (a quite challenging endeavor). Our study is of course only a first assessment of the resistance distribution in Morocco, and more samples from many localities will be required to refine it, but general observations can already be made.

Resistance patterns are relatively similar between populations (Fig. 2), but we nevertheless found significant heterogeneity between the various localities. This suggests that the local selection intensity varies, and thus highlights the role of the local insecticide treatment practices or local pesticide exposure in the spread of resistance. Moreover, we did not find any linkage disequilibrium between the different mutations in any populations (Tab. 3): this suggests that the mosquitoes are also exposed to various selective pressures at the local scale. Part of this absence of linkage disequilibrium could also be due to the nature of the resistance mechanisms selected locally: similar PYR resistance levels are found in Larache and Mohammedia (Fig. 2), while L1014F frequencies are very different (Fig. 3), which strongly suggests the presence of other mechanisms (P450 oxidases).

More worryingly, the fact that OP and PYR resistances are already quite prevalent all over Morocco could impede mosquito control, while alternative insecticides are very limited. Beyond the nuisance and its impact on tourism, control failures could in this country lead to increased arbovirus transmission, as the WNV is actively circulating in Morocco [58], which increases the risk of epizootics and epidemics. This risk is even further increased by the prevalence of resistance, as a study recently showed that resistance alleles tend to increase *Cx. pipiens* competence for arboviruses [31]. This work must thus be furthered and refined to help the health authorities defined sustainable and effective vector control strategies.

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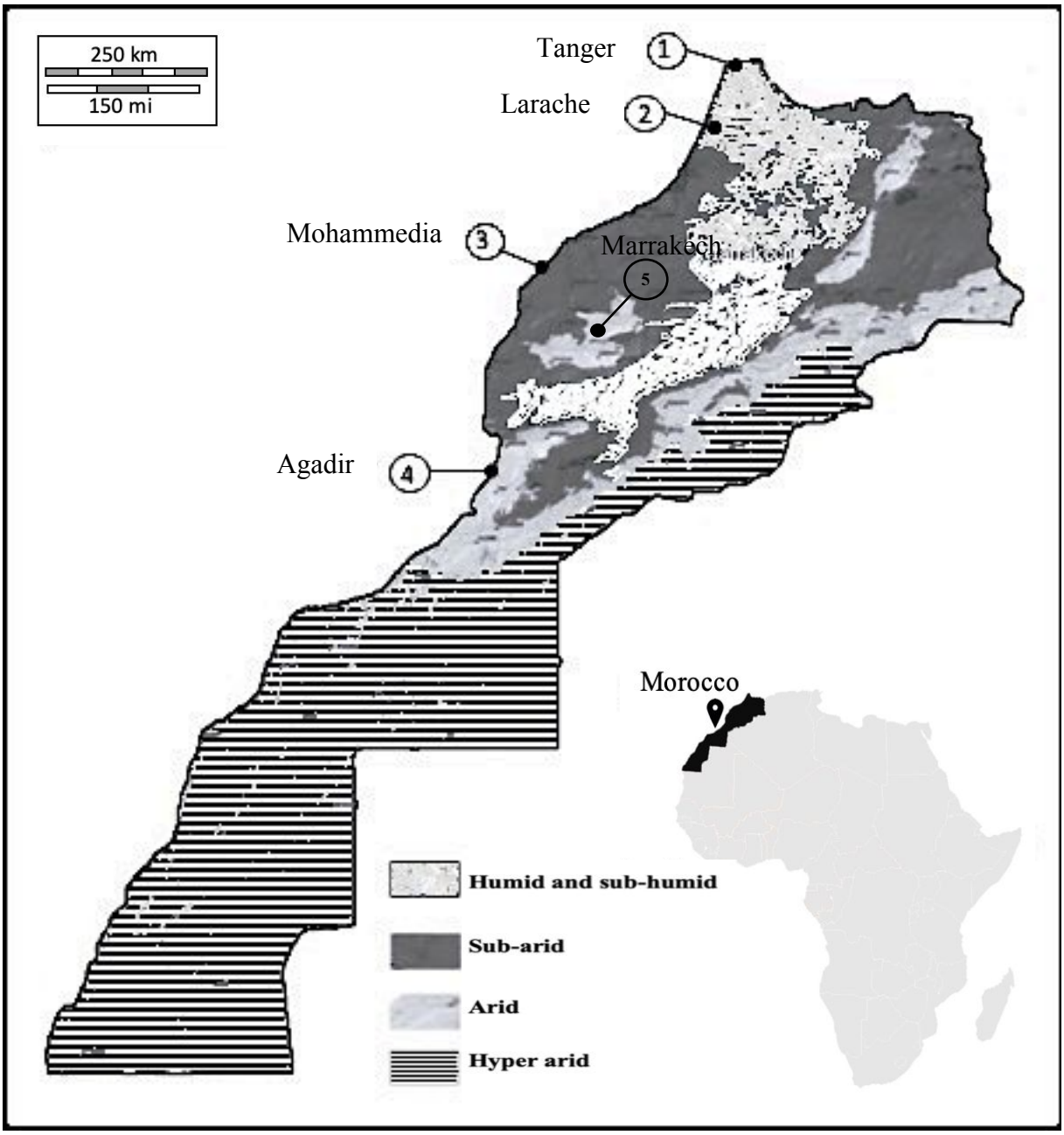

Figure 1: Localities sample during this study in Morocco. The various climatic regions are represented by gray shades (see legend)

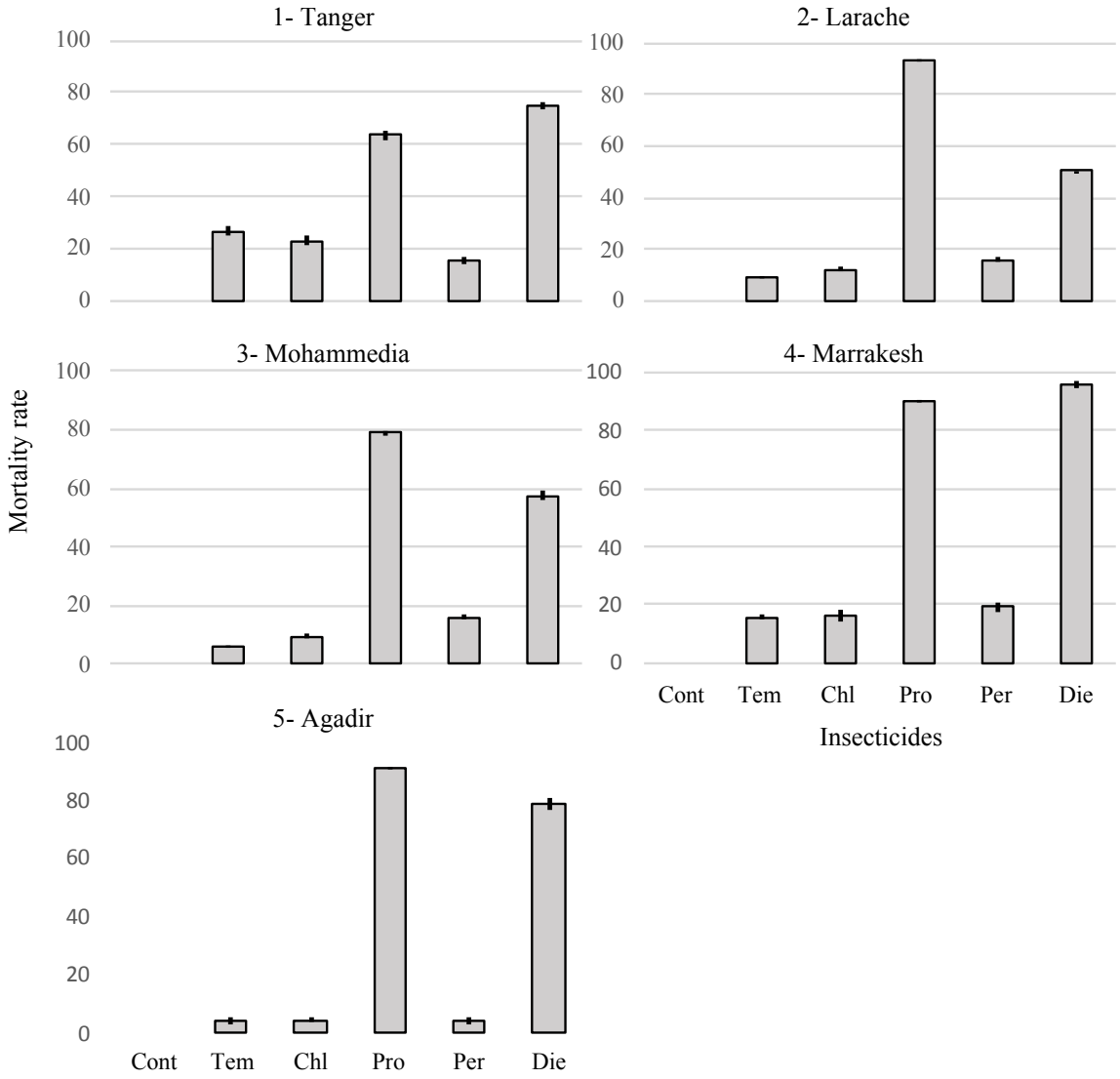

Figure 2 : Larval mortality after exposure in the different sampled localities. For each locality, the mortality rate after 24h exposure of fourth instar larvae in single-diagnostic-dose bioassay (see methods), to various insecticides, temephos (Tem, 0.0003 mg.l-1), chorpyrifos (Chl, 0.003 mg.l-1), propoxur (Pro, 2 mg.l-1), permethrin (Per, 0.005 mg.l-1) and dieldrin (Die, 0.01 g.l-1), or in the unexposed controls (Cont).

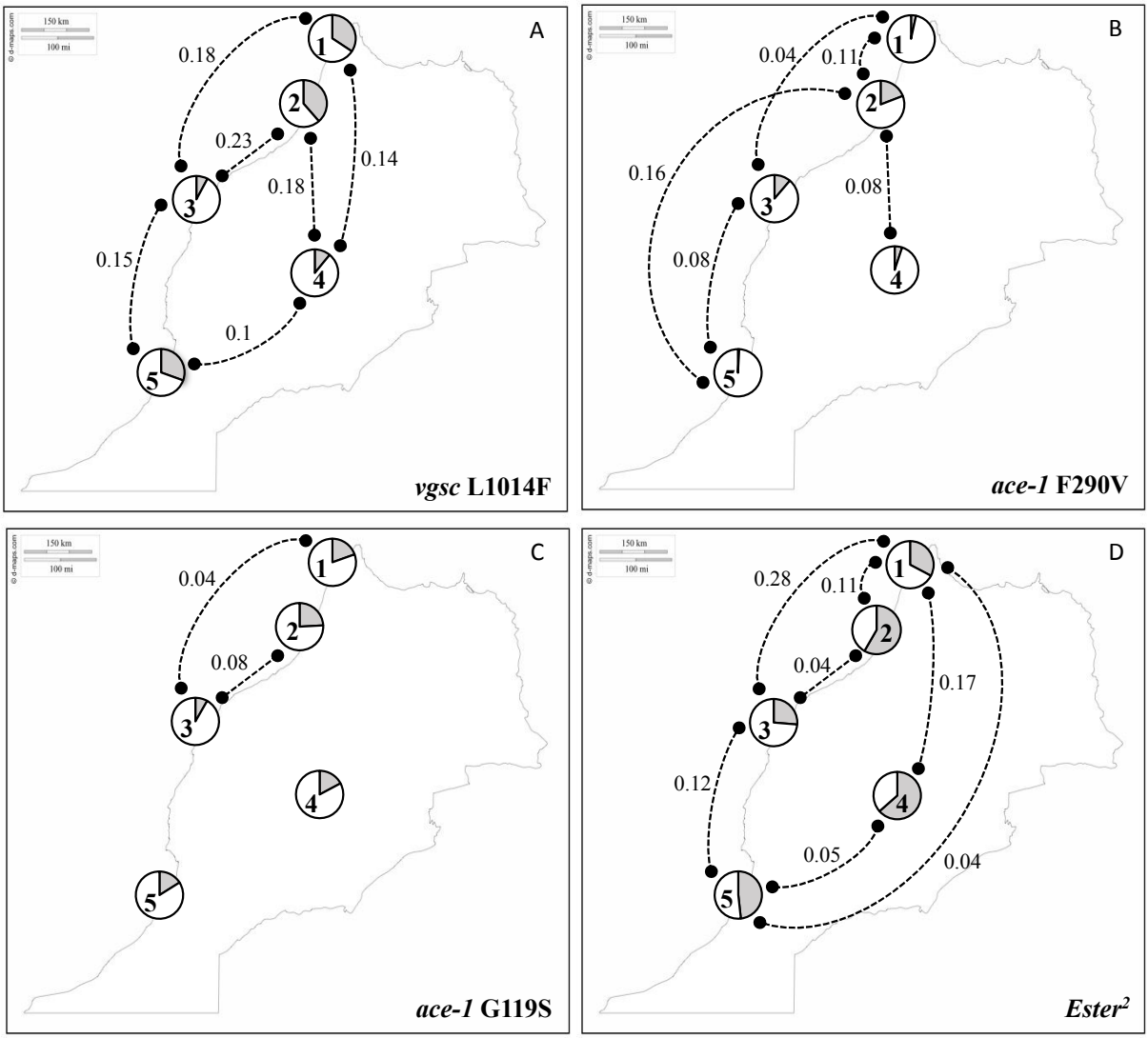

Figure 3: Distribution of the resistance mutations frequencies in Morocco. For each locus and each mutation (A, B, C and D), and for each population sampled (numbers as in Fig. 1), the frequencies of the resistant allele are represented by the grey sectors in each circle. The genetic differentiation between two populations, *i.e.* the F_{st} value, is indicated by the links: all pairs of populations have been tested, but only pairs with statistically significant genic differentiation (exact G-test, genepop, $p < 0.05$) are indicated. NB: the *Rdl* mutation is not represented as only one population was analyzed.