Multiple Origins of the Sodium Channel \textit{kdr} Mutations in Codling Moth Populations

Pierre Franck*, Myriam Siegwart, Jerome Olivares, Jean-François Toubon, Claire Lavigne

INRA, UR1115 Plantes et Systèmes de culture Horticoles, Avignon, France

Abstract

Resistance to insecticides is one interesting example of a rapid current evolutionary change. DNA variability in the voltage-gated sodium channel gene (trans-membrane segments 5 and 6 in domain II) was investigated in order to estimate resistance evolution to pyrethroid in codling moth populations at the World level. DNA variation among 38 sequences revealed a unique \textit{kdr} mutation (L1014F) involved in pyrethroid resistance in this gene region, which likely resulted from several convergent substitutions. The analysis of codling moth samples from 52 apple orchards in 19 countries using a simple PCR-RFLP confirmed that this \textit{kdr} mutation is almost worldwide distributed. The proportions of \textit{kdr} mutation were negatively correlated with the annual temperatures in the sampled regions. Homozygous \textit{kdr} genotypes in the French apple orchards showed lower P450 cytochrome oxidase activities than other genotypes. The most plausible interpretation of the geographic distribution of \textit{kdr} in codling moth populations is that it has both multiple independent origins and a spreading limited by low temperature and negative interaction with the presence of alternative resistance mechanisms to pyrethroid in the populations.

Introduction

The codling moth, \textit{Cydia pomonella} (L.) (Lepidoptera: Tortricidae), is one of the major insect pests in the orchards (mainly apple, pear and walnut orchards), worldwide distributed in the temperate regions [1]. Chemical insecticides remain the major means used to maintain populations of this pest at a low level. As a consequence of these treatments, \textit{C. pomonella} developed resistance to numerous insecticides in Australia [2], Americas [3,4,5], and Eurasia [6,7] including resistance to synthetic pyrethroids [8,9].

Resistance to pyrethroids is mainly conferred by modification of their primary target site: the voltage-gated sodium channel [10]. Computer-generated 3D models characterized a small number of mutations linked to insecticide-binding sites in the voltage-gated sodium channel, most of them being in the trans-membrane segments 4 to 6 of the domain II region of the protein [11]. L1014F and M918T originally found in the housefly and \textit{kdr} segments 4 to 6 of the domain II region of the protein [11]. L1014F and M918T originally found in the housefly and \textit{kdr} developed resistance to DTT and pyrethroids. The super-\textit{kdr} mutation is usually linked to the \textit{kdr} mutation and increases by tenfold primary pyrethroid resistance due to \textit{kdr} [13,14]. The L1014F mutation is the only voltage-gated sodium channel mutation reported so far in the codling moth [15]. It was detected in few populations over the World [8,16,17]. A resistance ratio of about 80-fold to the pyrethroid insecticide deltamethrin is conferred by this recessive mutation in first-instar codling moth larvae [18,19]. A low level of pyrethroid resistance in the codling moth is also attributed to enhanced detoxification activity notably due to the P450 cytochrome oxidases [18,20].

The evolution of resistance in insect pest populations depends on both historic and current selective processes that should be understood to manage resistance [21]. To shed light on the evolutionary processes linked to the evolution of pyrethroid resistance in \textit{C. pomonella} populations, we analysed genetic variations at the \textit{para} sodium channel gene. We first report countries over the World where \textit{kdr} resistance has been observed to establish origins of resistance alleles and identify factors that may affect their global spreading. Secondly we present a more detailed analysis on populations from South-eastern France to document the impact of local pyrethroid treatments on resistance evolution at the population level.

Materials and Methods

Sampling

The evolution of pyrethroid resistance conferred by the sodium channel gene was investigated on codling moth samples from 52 different apple commercial orchards in 19 countries (Table 1). Codling moths were collected as diapausing larvae using corrugated cardboard traps wrapped around the trunks of apple tree. Among these 52 codling moth population samples, seven populations were previously studied [16,17]. Codling moth populations from 21 orchards showing high larva density in South-eastern France [22] were further analysed to determine the impact of current pyrethroid treatments and interaction between resistance mechanisms on genetic variation in the \textit{para} sodium channel gene (Table 2). Pyrethroid treatments in these French
Table 1. Origins of the codling moth samples, meteorology characteristic (annual mean of the daily minimal temperature in celsius degree, annual number of freezing days) and proportions in each country of alleles (77 and 112) and homozygous genotypes (77/77) detected with the PCR-RFLP test [16].

| Country          | Year | Minimal Temperature | Freezing days | N  | n  | 112 | 77  | 77/77 |
|------------------|------|---------------------|---------------|----|----|-----|-----|-------|
| Armenia          | 2002 | n.a.                | n.a.          | 30 | 1  | 0.00| 1.00| 1.00  |
| Argentina        | 2005 | 7.5                 | 45            | 24 | 1  | 0.23| 0.54| 0.25  |
| France           | 2006 | 9.1                 | 56            | 771| 21 | 0.40| 0.24| 0.08  |
| New Zealand      | 2005 | 8.7                 | 5             | 18 | 1  | 0.61| 0.17| 0.00  |
| Turkey           | 2010 | 11.8                | 0             | 42 | 1  | 0.56| 0.14| 0.05  |
| USA              | 2008 | 1.3                 | 177           | 217| 5  | 0.54| 0.09| 0.01  |
| Bulgaria         | 2007 | 5.3                 | 100           | 60 | 3  | 0.54| 0.06| 0.02  |
| Uruguay          | 2005 | 11.8                | 0             | 17 | 1  | 0.32| 0.06| 0.00  |
| Switzerland      | 2003 | 7.2                 | 51            | 35 | 1  | 0.67| 0.01| 0.00  |
| Spain            | 2007 | 10.2                | 21            | 28 | 2  | 0.60| 0.00| 0.00  |
| Italy            | 2007 | 7.9                 | 11            | 329| 5  | 0.36| 0.00| 0.00  |
| Poland           | 2009 | 6.1                 | 64            | 10 | 1  | 0.61| 0.00| 0.00  |
| Czech Republic   | 2005 | 4.2                 | 109           | 31 | 1  | 0.68| 0.00| 0.00  |
| Greece           | 2006 | 8.9                 | 41            | 20 | 1  | 0.55| 0.00| 0.00  |
| Syria            | 2006 | 14.3                | 0             | 53 | 2  | 1.00| 0.00| 0.00  |
| South-Africa     | 2005 | 12.1                | 0             | 13 | 1  | 0.72| 0.00| 0.00  |
| Morocco          | 2010 | 13.1                | 0             | 30 | 2  | 0.45| 0.00| 0.00  |
| Algeria          | 2010 | 0.1                 | 28            | 28 | 1  | 0.86| 0.00| 0.00  |
| Chile           | 2005 | n.a.                | n.a.          | 28 | 1  | 0.70| 0.00| 0.00  |

N and n respectively indicate the number of individuals and the number of orchards analysed per country. 77 and 77/77 respectively correspond to kdr allele [16] and to homozygous kdr genotype [18]. No meteorological data were available for the Armenian and Chilean locations (n.a.).

Detected samples correspond to the population samples A, C and 17 that were analysed in [16].

*These samples correspond to the population samples A, Z, NZ1, U2, S1 that were analysed in [17].

doi:10.1371/journal.pone.0043543.t001

orichards encompassed mainly class II pyrethroids (esfenvalerate, fluvialinate, deltamethrine).

Detection of the Kdr Mutation

Total DNA was extracted from the head of each individual following Wash et al. [23] with 200 μl of 10% Chelex 100 (Biorad) solution and 6 μl (10 mg/ml) of proteinase K (Eurobio). Tissues were digested overnight at 56°C. After boiling for 30 minutes, supernatant was used as DNA template for PCR reaction. A PCR-RFLP test slightly modified from Franck et al. [16] was used to detect the kdr mutation. It was developed based on sequence variations in the para sodium channel gene of susceptible and deltamethrin resistant strains [15]. PCR amplifications were carried out with a Mastercycler thermocycler (Eppendorf) in a 25 μl reaction volume containing 1X reaction buffer (10 mM Tris-HCl, pH = 9, 50 mM KCl, 1.5 mM MgCl2, and 0.1 mg/ml Bovine Serum Albumin), 200 μM of each dNTPs, 0.4 μM of each C6Na-F and C6Na-R primers (Table 3), 1 unit of Go Taq DNA Polymerase (Promega) and 2 μl of DNA template. The PCR conditions were: 3 minutes at 94°C followed by 35 cycles at 94°C for 30s, 55°C for 1 min, and 72°C of elongation for 1 min with a final extension step at 72°C for 2 min. PCR products were digested at 65°C for 16 hours with 2 unit of Tsp509I endonuclease and 1X of NEB1 Buffer (New England Biolabs) in 30 μl of reaction volume. Digested products were separated by electrophoresis on 6.5% polyacrylamide denaturing gel in a Li-Cor 4200 automatic DNA sequencer. The longest digested DNA fragments in acrylamide gels were visualised using 700 IRDye labelled C6Na-F primer with the SAGA software (Li-Cor Biosciences). Each revealed fragment length was defined as an allele. The test was performed on 1784 individuals: 165 individuals were re-analysed according to this modified protocol [16,17] and 1619 individuals were newly investigated (Table 1).

Detoxification Activity by the P450 Cytochrome Oxidases

Enhanced activity of the P450 cytochrome oxidases confers heritable metabolic resistance to pyrethroid insecticides [18]. We assessed the activity of the P450 cytochrome oxidases measuring 7-ethoxycoumarin-O-deethylation (ECOD) activity on 557 moths (out of 771) collected in 19 French orchards (out of 21) to shed light on putative interaction between this resistance mechanism and pyrethroid target mutations in the sodium channel gene. ECOD activity was individually measured on abdomen samples using 0.4 mM ethoxycoumarin in 100 μl Hepes buffer [16,24]. After four hours of incubation at 30°C, the enzymatic reaction was stopped with 100 μl of glycine buffer (10-4 M), pH 10.4/ethanol (v/v) and fluorescence was measured with 380 nm excitation and 465 nm emission filters on a microplate reader (HTS 7000, Perkin Elmer). ECOD activity was estimated for each moth based on the amount of 7-hydroxyocoumarine formed (pg/min).

DNA Sequencing in the Para Gene

DNA sequencing in the para gene (corresponding to transmembrane segments 4 to 6 of the domain II region of the canal sodium protein, Figure 1) was performed on 50 codling moth individuals from various geographic origins and two Grapholita
molestata (Lepidoptera: Tortricidae), collected in France and Brazil, to be used as an outgroup.

The whole region was sequenced for 38 codling moths displaying different homozygous genotypes according to the PCR-RFLP test (Table 4). The whole region was amplified in two independents PCRs with respectively the SKdr-F/ SKdr-R3 and the Kdr-F/Kdr-R primer pairs (Table 3). These PCR were performed in the same conditions as above but at annealing temperatures of 56°C and 54°C respectively. The PCR products were purified from agarose gel [25] then sequenced (GATC Biotech) using, as sequencing primers, SKdr-R1, SKdr-R2 and the four PCR primers described above (Table 3). For twelve additional codling moths collected in orchard treated with pyrethroid (Table 2), the first exon coding for the transmembrane segments 4 and 5 was partially sequenced using the SKdrF and SKdr-R1 primers in order to check for the presence of the super-kdr mutation.

### Table 2. Proportions of alleles (112 and 77) and of homozygous genotypes (77/77), and expected and observed heterozygosities (H_e/H_o) in codling moth samples from 21 commercial apple orchards in South-eastern France.

| Orchard | N | Protection | Pyrethroid treatment | Cytochrom P450 activity | 112 | 77 | 77/77 | H_e/H_o |
|---------|---|------------|----------------------|-------------------------|-----|----|-------|--------|
| 154     | 36 | Conventional | 5                   | 77                       | 0.26| 0.64| 0.42  | 0.52/0.53 |
| 149     | 20 | Conventional | 4                   | 687                      | 0.43| 0.38| 0.10  | 0.66/0.60 |
| 122     | 37 | Conventional | 4                   | 513                      | 0.18| 0.23| 0.05  | 0.57/0.57 |
| 75      | 43 | Conventional | 4                   | 328                      | 0.48| 0.17| 0.02  | 0.63/0.67 |
| 65      | 40 | Conventional | 4                   | 652                      | 0.48| 0.14| 0.02  | 0.61/0.58 |
| 55      | 39 | Conventional | 3                   | 635                      | 0.45| 0.24| 0.10  | 0.65/0.56 |
| 68      | 21 | Conventional | 3                   | 507                      | 0.60| 0.10| 0.00  | 0.55/0.57 |
| 132     | 49 | Conventional | 3                   | 862                      | 0.46| 0.06| 0.00  | 0.56/0.67 |
| 35      | 33 | Conventional | 2                   | n.a.                     | 0.41| 0.38| 0.15  | 0.65/0.70 |
| 84      | 23 | Conventional | 2                   | 141                      | 0.30| 0.37| 0.04  | 0.68/0.87 |
| 17      | 27 | Conventional | 2                   | 542                      | 0.30| 0.35| 0.11  | 0.56/0.68 |
| 140     | 36 | Conventional | 2                   | 208                      | 0.32| 0.31| 0.11  | 0.67/0.61 |
| 134     | 16 | Conventional | 2                   | 340                      | 0.31| 0.16| 0.00  | 0.62/0.81 |
| 10      | 29 | Conventional | 1                   | 406                      | 0.57| 0.17| 0.03  | 0.59/0.66 |
| 42      | 43 | Conventional | 1                   | n.a.                     | 0.52| 0.14| 0.02  | 0.60/0.56 |
| 145     | 41 | Organic     | 0                   | 188                      | 0.28| 0.45| 0.22  | 0.65/0.63 |
| 51      | 59 | Organic     | 0                   | 32                       | 0.38| 0.25| 0.05  | 0.66/0.66 |
| 125     | 52 | Organic     | 0                   | 70                       | 0.41| 0.23| 0.04  | 0.66/0.71 |
| 124     | 45 | Organic     | 0                   | 90                       | 0.39| 0.17| 0.02  | 0.63/0.76 |
| 119     | 58 | Organic     | 0                   | 34                       | 0.45| 0.16| 0.03  | 0.62/0.57 |
| 126     | 24 | Organic     | 0                   | 265                      | 0.40| 0.13| 0.04  | 0.61/0.54 |

References to crop protection practices, numbers of annual pyrethroid treatments, and cytochrom P450 oxidase activity were reported for each orchard and linked population sample. N was the number of individuals analysed per orchard. H_e and H_o were calculated with all the three alleles (77, 101 and 112) detected with the PCR-RFLP. Values reported for cytochrom P450 oxidase activities were estimated as the average ECOD activity (pmol/min/individual) among the individuals collected at each orchard location. No ECOD measure was done on the individuals collected in orchards 35 and 134 (n.a.).

doi:10.1371/journal.pone.0043543.t002

### Table 3. Primers used to amplify and to sequence domain II S4-S6 region of the codling moth para sodium channel gene.

| Primer | Sequence (5’-3’) |
|--------|------------------|
| SKdr-F | GGGCCGACCTTAATTACTCATC |
| SKdr-R1 | TTCCCCGAAAGTGCATACC |
| SKdr-R2 | GGGTTAACGAGCTAAACGTCCAA |
| SKdr-R3 | GCAATCCCACATGCTCTCTA |
| CpNa-F | TAGAGGACATGTGGGATTGC |
| CpNa-R | AATTTCGTAGCCCTTGATCG |
| Kdr-F | GGGTTAACGAGCTAAACGTCCAA |
| Kdr-R | GCAAGGCTAAGAAAGGTTAAG |

Primer positions are indicated in Figure1. Kdr-F and Kdr-R are slightly modified from CgD1 and CgD2, respectively [15]. SKdr-R3 is the reverse of CpNa-F.

doi:10.1371/journal.pone.0043543.t003

Pyrethroid Resistance in Codling Moth Populations

The whole region was sequenced for 38 codling moths displaying different homozygous genotypes according to the PCR-RFLP test (Table 4). The whole region was amplified in two independents PCRs with respectively the SKdr-F/SKdr-R3 and the Kdr-F/Kdr-R primer pairs (Table 3). These PCR were performed in the same conditions as above but at annealing temperatures of 56°C and 54°C respectively. The PCR products were purified from agarose gel [25] then sequenced (GATC Biotech) using, as sequencing primers, SKdr-R1, SKdr-R2 and the four PCR primers described above (Table 3). For twelve additional codling moths collected in orchard treated with pyrethroid (Table 2), the first exon coding for the transmembrane segments 4 and 5 was partially sequenced using the SKdr-F and SKdr-R1 primers in order to check for the presence of the super-kdr mutation.

### Data Analysis

The genetic variation at the sodium channel detected with the PCR-RFLP test was first analysed. In each orchard population sample, observed (H_O) and expected (H_E) heterozygosities were calculated and departure of genotype frequencies from Hardy-Weinberg proportions tested using the Genepop software [26] considering either all the detected alleles or only the kdr and the susceptible allele groups. Generalised linear models were used (genmod procedure, SAS version 9.1) to explain the proportions of kdr allele, and of homozygous kdr genotypes in the orchard population samples. These proportions were modelled as binomial variables with a logit link function considering the orchard as a random variable. First, the proportions of kdr allele or of homozygous kdr genotypes were modelled for the French population samples as functions of two factors (ECOD activity...
in moths and number of pyrethroid treatments in orchard) and their interactions (Table 2). Second, the proportions of kdr allele or homozygous kdr genotypes were modelled considering all the sampled populations that displayed kdr polymorphism as functions of either the annual mean of the daily minimal temperatures or the annual number of freezing days at each orchard. Meteorological data were obtained from the National Climatic Data Center website [http://www.ncdc.noaa.gov/oa/ncdc.html] for each orchard location and sampling date.

Furthermore, sequence variation at the sodium channel gene was investigated. DNA sequences were manually aligned with the Bioedit software [27]. Recombination between DNA sequences was tested using six different methods implemented in the RDP3 software [28]: RDP [29], GENECONV [30], Chimaera [31], MaxChi [31,32], BootScan [33], and SiScan [34]. Minimum evolution trees were computed with the MEGA software [35] using a p-distance between the sequences that takes into account both substitution and indel polymorphisms. To assess the reliability of the tree, standard error tests were performed for every interior branch by resampling variable sites (1,000 bootstraps).

**Table 4.** Statistical results about six generalized linear models of the proportions of kdr allele and of kdr homozygote genotypes in the orchard population samples.

| Statistical models          | Observed data | Statistical test |
|-----------------------------|---------------|------------------|
| Dependent variables         | Covariables    | Orchard origins  | N   | n   | chi2  | (df) | P-value |
| Proportion of kdr allele    | Pyrethroid treatments | France         | 1114| 19  | 0.16  | (1)  | 0.687   |
|                             | ECOD activity  |                 |     |     | 1.00  | (1)  | 0.317   |
|                             | Pyrethroid × ECOD |               |     |     | 0.92  | (1)  | 0.338   |
| Proportion of kdr allele    | Minimal temperature | World        | 1830| 30  | 5.05  | (1)  | 0.025   |
| Proportion of kdr allele    | Freezing days  | World          | 1830| 30  | 4.57  | (1)  | 0.033   |
| Proportion of kdr homozygote| Pyrethroid treatments | France       | 557 | 19  | 0.09  | (1)  | 0.768   |
|                             | ECOD activity  |                 |     |     | 9.16  | (1)  | 0.003   |
|                             | Pyrethroid × ECOD |               |     |     | 2.63  | (1)  | 0.105   |
| Proportion of kdr homozygote| Minimal temperature | World       | 915 | 30  | 3.13  | (1)  | 0.077   |
|                             | Freezing days  | World          | 915 | 30  | 3.54  | (1)  | 0.060   |

N and n respectively indicate the number of individuals and the number of orchards observed for each statistical model.

doi:10.1371/journal.pone.0043543.t004
Results

Detection and Distribution of the Kdr Mutation in Populations

To detect the kdr mutation (L1014F) in codling moth populations we amplified a 170 bp region with the CpNa-F and CpNa-R primers (Figure 1 and Table 3), then digested it with the Tsp509I endonuclease that specifically cut ↓ AATT sites. Two out of five restriction sites in the 170 bp region were polymorphic (Figure 1, Table 4). This polymorphism was summed up by DNA fragments of three different detectable lengths (77, 101 and 112 bp), hereafter designed as three different alleles (Figure 2). Restriction at position 1417 generated the 77 bp fragment, which was interpreted as corresponding to the kdr allele [16]. Restrictions at positions 1441 and 1452 in intron II respectively generated 101 and 112 bp fragments, which were interpreted as corresponding to two different susceptible alleles.

A total of 1784 codling moths were genotyped using this PCR-RFLP test (Table 1). The 77 allele was observed in population samples from all the continents except Africa and from 9 out of the 19 countries analysed. It was observed in all the samples from South-eastern France in variable proportions (Table 2). It was the only observed allele in the Armenian sample. The 101 and 112 alleles were both observed at relatively high proportions in all the other samples, except the one from Syria that was monomorphic for the 112 allele (Tables 1 and 2). No departure from Hardy-Weinberg equilibrium was detected in any sample from the 52 orchards analysed when the 101 and 112 susceptible alleles were grouped (Fisher’s exact test, P>0.28). Significant heterozygote excesses were detected in two French samples (Fisher’s exact test, P=0.03 and P=0.04 in the orchard sample 84 and 132, respectively) when all three alleles were considered (Table 2). The proportions of 77 allele and 77/77 genotype in the population samples from South-eastern France were slightly higher in orchards sprayed with pyrethroid insecticides (Tables 2 and 4), but these proportions did not depend on the number of pyrethroid treatments according to the generalized linear models (chi2, df = 1, P=0.077) in the codling moth samples were positively correlated to the annual number of freezing days in the sampled regions were highly correlated (r = −16.6 minimal temperature +184, R² = 0.897). Consequently, generalized linear models were performed independently with these two climatic variables (Table 4). The proportions of 77 allele (chi2 = 5.05, df = 1, P = 0.025) and, to a lesser extent, the proportions of 77/77 genotype (chi2 = 3.13, df = 1, P = 0.077) in the codling moth samples were positively correlated with temperature in the sampled regions. Consistently, very similar negative correlations were observed with the annual number of freezing days (Table 4).

DNA Sequencing in the Para Gene and Haplotype Divergences

Partial DNA sequences of the para gene (domain II, trans-membrane segments 5 and 6, Figure 1) were obtained for two G. molesta (accession number GU082359 and GU082360) and 38 C. pomonella individuals (GU082334-GU082358 and JQ946336–JQ946348): These 38 sequenced codling moths displayed homozygous genotypes according to the PCR-RFLP test; eleven were 77/77, ten 101/101 and seventeen 112/112. Introns I and II were

Figure 2. Detection of the kdr mutation by PCR-RFLP. PCR were conducted with the CpNa-F and CpNa-R primers (Table 3), and then digested with Tsp509I (700 IRDye labelled CpNa-F primer). Lengths of the restricted fragments were determined by electrophoresis in a Li-Cor 4200 automatic DNA sequencer (6.5% polyacrylamide denaturing gel) and visualised using the SAGA software (Li-Cor Biosciences). The 77 bp fragment corresponds to the kdr allele. The 101 and 112 bp fragments correspond to two different susceptible alleles. A–F corresponds to the different PCR-RFLP genotypes: 77/77, 101/101, 112/112, 77/101, 112/112 and 77/112. G is the length of the non-restricted PCR product (170 bp). 50–350 pb sizing standard (Biosciences) is in the left well. doi:10.1371/journal.pone.0043543.g002
both shorter in *G. mola* than in *C. pomonella* (Figure 3) and we were not able to align and compare *G. mola* and *C. pomonella* intron sequences. Twelve different haplotypes were identified among the 38 codling moth sequences. The sequences differed by their lengths (1649 to 1756 bp) because of the presence of three indels in intron I and one in intron II (Figure 3, Table 5). In addition, 13 substitutions were observed. Only one substitution at position 1420 was non-synonymous (*L1014F*). All the eleven addition, 13 substitutions were observed. Only one substitution at

indels in intron I and one in intron II (Figure 3, Table 5). In their lengths (1649 to 1756 bp) because of the presence of three among the 38 codling moth sequences. The sequences differed by

DNA ladder (Promega) was in the central well.

**Discussion**

DNA sequences and SNPs analyses in a gene involved in insecticide resistance are complementary tools to shed light on recent evolutionary changes [13,36]. Proximal evolutionary processes can be assessed by analysing the DNA sequence that contains the mutations involved in resistance. SNPs analyses are convenient molecular tools that allow following the dynamic of insecticide resistance in natural populations and understanding selective processes that may enhance or delay insecticide resistance evolution [21].

Although useful, SNP detection methods may fail to detect some genetic variations involved in insecticide resistance at a selected gene [37,38]. The L1014F replacement in the voltage-gated sodium channel was primarily observed in a deltamethrin resistant codling moth strain [15]. In the present study, a rapid PCR-RFLP test was developed to monitor this *kdr* mutation in codling moth populations. However, at least two additional mutations were reported in insect pest populations at this locus: *L1014S* in *Culex pipiens* [39] and *Anopheles gambiae* [40] and *L1014H* in *Heliothis virescens* [41] and Musca domestica [42]. As for the F1014 variant, the S1014 but not the H1014 variants would have produced a 77 bp restriction fragment in *C. pomonella* with the developed PCR-RFLP test. However, it is unlikely that these two additional mutations are present in the codling moth: L1014F was the only non-synonymous variation in the *para* gene observed along transmembrane segments 5 and 6 in domain II in individuals from various origins in the World. In absence of other proof, we assumed that L1014F was the only *kdr* mutation in this species.

A total of 1784 individuals collected all over the World were analysed using this PCR-RFLP test to estimate the distribution of *kdr* within and among populations. The 77 allele was observed almost worldwide and it is heterogeneously distributed among the codling moth populations. High proportions of 77/77 homozygous genotypes that are physiologically resistant to pyrethroid were only observed in Armenia, Argentina, Turkey and South-eastern France. These results confirm and extend previous observations [8,16,17,43].

At the orchard level, neither *kdr* selection by current pyrethroid treatments nor *kdr* counter-selection in absence of pyrethroid treatment was evident. The proportions of *kdr* allele were not significantly correlated with the number of pyrethroid treatments in the French apple orchards. Distributions of *kdr* genotypes did not significantly depart from Hardy-Weinberg proportions in any orchard population samples. The low impact of pyrethroid treatments observed on *kdr* proportions at the orchard level seems to be a general feature whatever the within-year generation of the codling moth [44]. These results contrast with observations in *Haematobia irritans* or in *Musca domestica* populations that clearly showed seasonal variations in the proportions of *kdr* allele as a function of pyrethroid treatments [45,46]. Three non exclusive hypotheses may explain such lack of structure of *kdr* in codling moth populations according to current insecticide applications. First, resistance management guidelines recommend alternation of pyrethroids with other insecticides among codling moth generations. Non-continuous use of pyrethroids largely limits the selection of sodium channel target mutations. This could explain why the *super-kdr* mutation is apparently absent in codling moth populations, a result also found in wild populations of horn flies [47]. Second, the usage of a large spectrum of insecticides selected various resistance mechanisms in the codling moth populations. Metabolic resistance associated with enhanced activity of the P450 cytochrome oxidases is largely spread over the World [16,17] and confers cross-resistance to numerous insecticides including pyrethroids [8,20]. In the present study, the activity of the P450 cytochrome oxidases was negatively correlated with the proportion of pyrethroid treatment [48]. Third, insignificant fitness cost associated with *kdr* was measured in laboratory codling moth strains [49], which could explain the maintenance of high *kdr* proportions in absence of pyrethroid selection as observed in
populations from organic orchards in South-eastern France (the granulosis virus was the only insecticide used to control codling moth in these orchards). However, fitness costs are difficult to predict when several resistance mechanisms interact [50] and may depend on the environmental conditions [51].

The lack of population structure of the 

kdr

resistance evolved in interaction with other resistance mechanisms. Second, 

kdr

proportions were negatively correlated with temperature in codling moth populations displaying 

kdr

polymorphism in agreement with the hypothesis that fitness cost associated with sodium channel target mutations depends on temperature [51]. Consequently, fitness cost associated with 

kdr

could be not equally distributed genetically and differently expressed along seasons in 

C. pomonella

as previously noted in house fly populations [42,52]. It is to note that such temperature-dependent cost could also explain the latitudinal variation in the proportion of 

kdr

allele previously observed in codling moth populations from France [16]. Finally, in the absence of strong resistance cost, ancient insecticide treatments may explain current 

kdr

distribution [53]. Resistance to DTT was reported in some codling moth populations in the early 1950s [54]. The first cases of resistance to pyrethroid in the 1990s in some codling moth populations in the early 1950s [54]. The first cases of resistance to pyrethroid in South-eastern France (the granulosis virus was the only insecticide used to control codling moth in these orchards). However, fitness costs are difficult to predict when several resistance mechanisms interact [50] and may depend on the environmental conditions [51].

Table 5. Sequence variability in the codling moth voltage gated sodium channel gene with variable positions numbered according to Figure 1.

| Haplotype | Syria 1 | Armenia 1 | Bulgaria 1 | Turkey 1 | Turkey 2 | Czech 1 | Czech 2 | Argentinia 1 | Argentina 2 | Argentina 3 | Chile 1 |
|-----------|---------|-----------|------------|----------|----------|---------|---------|-------------|------------|-------------|--------|
|           | A 0 0   | G 0 0 0   | A 0 0 0   | G 0 0 0  | G 0 0 0  | G 0 0 0 | G 0 0 0 | A 0 0 0 0  | G 0 0 0 0 | G 0 0 0 0  | G 0 0 0 |
|           | T T T A | T A T A   | T A T A   | T A T A  | T A T A  | T A T A | T A T A | T A T A    | T A T A    | T A T A    | T A T A |
|           | 0 0 0 C | 0 0 0 C   | 0 0 0 C   | 0 0 0 C  | 0 0 0 C  | 0 0 0 C | 0 0 0 C | 0 0 0 C    | 0 0 0 C    | 0 0 0 C    | 0 0 0 C |
|           | G G G G | G C G C   | G G G G   | G C G C  | G C G C  | G C G C | G C G C | G C G C    | G C G C    | G C G C    | G C G C |
|           | C C C C | C A A T   | C C C C   | C A A T  | C A A T  | C A A T | C A A T | C A A T    | C A A T    | C A A T    | C A A T |
|           | 0 T T T | 0 T T T   | 0 T T T   | 0 T T T  | 0 T T T  | 0 T T T | 0 T T T | 0 T T T    | 0 T T T    | 0 T T T    | 0 T T T |
|           | 116 C 77 1106 305 | 116 C 112 1106 305 | 116 C 101 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 | 116 C 112 1106 305 |
|           | G A T   | T A T A   | T A T A   | T A T A  | T A T A  | T A T A | T A T A | T A T A    | T A T A    | T A T A    | T A T A |

Substitutions are indicated by the observed nucleotides and indels by the number of inserted or deleted base pairs using the Syria1 sequence as reference. Nucleotides in bold characters refer to TspS091 restriction sites. The three last columns respectively refer to lengths in base pairs of the largest TspS091 digested fragment, and of introns I and II. Twelve different haplotypes were recognized among 38 sequences. The distribution of these twelve haplotypes among countries was reported in figure 4.

doi:10.1371/journal.pone.0043543.t005

Pyrethroid Resistance in Codling Moth Populations
Acknowledgments

We thank the EPI team for its help collecting codling moth larvae in the Basse-Durance Valley, France. We thank Maritza Reyes, Wilson Barros, Jim Walker, Claudio Ioriatti, Damian Gorzka, Costas Voudouris, John Margaritopoulos, Frantisek Marec, Oldrich Pultar, Alan Knight, Azya Ter-Hovhannesyan, Nadia Lombarkia, Salma Iraqui, Jesus Avilla, Marcella Rodriguez, Mohanad Ismail, and Recep Ay who kindly provided the codling moth samples from the other countries. We also thank Juliette Gousopoulos and Mathilde Willerval for their help extracting DNA and performing biochemistry analyses. Finally, we thank Benoit Mouy for his suggestion about testing recombination and anonymous reviewers for their relevant comments and their corrections.

Author Contributions

Conceived and designed the experiments: PF MS CL. Performed the experiments: JO JFT. Analyzed the data: PF MS. Wrote the paper: PF MS.

References

1. Shel'Deshova GG (1967) Ecological factors determining distribution of the codling moth Lepespisia pomonella L. in the northern and southern hemispheres. Entomological Review 46: 349–361.

2. Thwaite WG, Williams DG, Hatley AM (1993) Extent and significance of Azinphos-Methyl resistance in codling moth in Australia. Pest Control and Sustainable Agriculture: 166–168.

Figure 4. Minimum evolution trees established with the p-distance among 38 partial DNA sequences of the codling moth para gene. The trees were computed either with all the variable sites (left: 4 indels and 13 substitutions) or all the variable sites except the kdr locus (right: 4 indels and 12 substitutions). The 112 and 101 sequences (L1014) were represented by unfilled circles and squares, respectively. The 77 sequences (F1014) were represented by filled diamonds. Bootstraps values above 60% were reported.
doi:10.1371/journal.pone.0043543.g004
Pyrethroid Resistance in Codling Moth Populations

3. Fuentes-Cotranes E, Reyes M, Barros W, Sauphanor B (2007) Evaluation of azinphos-methyl resistance and activity of detoxifying enzymes in coddling moth (Leptodectra : Tortricidae) from central Chile. Journal of Economic Entomology 100: 551–556.

4. Solano J, Anguiano L, De’Angelo AP, Cichon L, Fernandez D, et al. (2008) Toxicological and biochemical response to azinphos-methyl in Culex pipiens L. (Leptodectra : Tortricidae) among orphans from the Argentinian Patagonia. Pest Management Science 64: 964–970.

5. Varela LG, Wolter SC, Jones VP, Brunner JF, Rodol H (1993) Monitoring and characterization of insecticide resistance in coddling moth (Leptodectra : Tortricidae) in 4 Western States. Journal of Economic Entomology 86: 1–10.

6. Reuveny H, Cohen E (2004) Evaluation of mechanisms of azinphos-methyl resistance in the coddling moth Culex pipiens (L). Archives Of Insect Biochemistry And Physiology 57: 92–100.

7. Sauphanor B, Bouvier JC, Brosse V (1998) Spectrum of insecticide resistance in Cydia pomonella (Lepidoptera : Tortricidae) in southeastern France. Journal of Economic Entomology 91: 1225–1231.

8. Reyes M, Franck P, Charlemiell P.J, Ioriatti C, Olivares J, et al. (2007) Diversity of insecticide resistance mechanisms and spectrum in European populations of the coddling moth, Cydia pomonella. Pest Management Science 63: 890–902.

9. Sauphanor B, Brosse V, Bouvier JC, Sprey P, Micoud A, et al. (2000) Monitoring resistance to deltamethrin and deltamethrin in French coddling moth populations (Cydia pomonella). Pest Management Science 56: 74–82.

10. Zlotkin E (1999) The insect voltage-gated sodium channel as target of insecticides. Annual Review of Entomology 44: 429–455.

11. O'Reilly AO, Khambay BPS, Williamson MS, Field LM, Wallace BA (2006) A modified BOOTSCAN algorithm for automated identification of recombination events and recombination breakpoints. AIDS Research and Human Retroviruses 21: 98–102.

12. Martin DP, Posada S, Cranack KA, C W (2005) A molecular phylogenetics approach for identifying recombination in evolutionarily divergent DNA sequences. Bioinformatics 21: 355–362.

13. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

14. Lynd A, Weeneman D, Barbosa S, Egyi Yassoun A, Mitchell S, et al. (2010) Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in Anopheles gambiae s.s. Molecular Biology and Evolution 27: 1117–1125.

15. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berger JB, et al. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. Insect Molecular Biology 7: 179–184.

16. Kanson H, Jensen B, Vihale JM, Wang X, Heningway J, et al. (2000) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Molecular Biology 9: 491–497.

17. Martinez-Torres D, Chevillon C, Brun-Barale A, Berger JB, Pasteur N, et al. (1999) Voltage-dependent Na+(V) channels in pyrethroid-resistant Culex pipiens L. mosquitoes. Pesticide Science 55: 1012–1020.

18. Pinto J, Lynd A, Vicente JL, Santalacussa F, Randle ND, et al. (2007) Multiple origins of knockdown resistance mutations in the Afrotopical mosquito vector Anopheles gambiae PLoS One 2:.

19. Park Y, Taylor MEJ (1997) A novel mutation L1019F in sodium channel gene bap associated with pyrethroid resistance for Helicoverpa zea (Lepidoptera: Noctuidae). Insect Biochemistry and Molecular Biology 27: 9–13.

20. Ranson H, Zhang L, Hamml RL, Brady SG, Lazzaro BP, et al. (2006) Frequencies of the pyrethroid resistance alleles of Culex pipiens LCP in house flies from the eastern United States. Insect Molecular Biology 15: 157–167.

21. Voulouris CC, Sauphanor B, Franck P, Reyes M, Marmurzi M, et al. (2011) Insecticide resistance status of the codling moth Cydia pomonella (Lepidoptera: Tortricidae) from Greece. Pesticide Biochemistry and Physiology 100: 229–238.

22. Rinkevich FD, Zhang L, Hamm RL, Brady SG, Lazzaro BP, et al. (2006) Insecticide resistance status of the codling moth Cydia pomonella (Lepidoptera: Tortricidae) from Greece. Pesticide Biochemistry and Physiology 100: 229–238.

23. Bouvier JC, Bues R, Boivin T, Daoudion L, Beslay D, et al. (2001) Deltamethrin resistance in the coddling moth (Leptodectra : Tortricidae): inheritance and number of genes involved. Heredity 87: 436–462.

24. Sauphanor B, Bouvier JC, Brosse V, Monier C, Bouvier JC (1998) Differential oviposition and larvalicidal resistance to benzo[b]thiophens in the coddling moth, Cydia pomonella. Entomologia Experimentalis et Applicata 88: 247–253.

25. Bouvier JC, Cuany A, Monier C, Brosse V, Sauphanor B (1998) Enzymatic diagnostic of resistance to deltamethrin in diapausing larvae of the coddling moth, Cydia pomonella (L). Archives of Insect Biochemistry and Physiology 39: 55–64.

26. Labbé P, Lenomond T, Raymond M (2005) On the worldwide spread of an insecticide resistance gene: a role for local selection. Journal of Evolutionary Biology 18: 1471–1484.

27. Labbé P, Lenomond T, Raymond M (2005) On the worldwide spread of an insecticide resistance gene: a role for local selection. Journal of Evolutionary Biology 18: 1471–1484.

28. Heath L, van der Walt E, Varsani A, Martin DP (2006) Recombination patterns in aphid genomes mirror those found in other poxviruses. Journal of Virology 80: 11827–11832.

29. Martin D, Rybicki E (2000) RDP: detection of recombination amongst aligned sequences. Bioinformatics 16: 562–563.

30. Padidam M, Sawyer S, Faust CM (1999) Possible emergence of new geminiviruses by frequent recombination. Virology 265: 218–225.

31. Posada D, Cranack KA (2001) Evaluation of methods for detecting recombination from DNA sequences: computer simulations. Proceedings of the National Academy of Sciences of the United States of America 98: 13757–13762.

32. Maynard Smith J (1992) Analysing the mosaic structure of genes. Journal of Evolutionary Biology 53: 563–577.

33. Martin DP, Posada D, Crandall KA (2005) A modified BOOTSCAN algorithm for automated identification of recombination events and recombination breakpoints. AIDS Research and Human Retroviruses 21: 98–102.

34. Davies ET, O'Reilly AO, Field LM, Wallace BA (2006) A modified BOOTSCAN algorithm for automated identification of recombination events and recombination breakpoints. AIDS Research and Human Retroviruses 21: 98–102.

35. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

36. Lynd A, Weeneman D, Barbosa S, Egyi Yassoun A, Mitchell S, et al. (2010) Field, genetic, and modeling approaches show strong positive selection acting upon an insecticide resistance mutation in Anopheles gambiae s.s. Molecular Biology and Evolution 27: 1117–1125.

37. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berger JB, et al. (1998) Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s. Insect Molecular Biology 7: 179–184.

38. Kanson H, Jensen B, Vihale JM, Wang X, Heningway J, et al. (2000) Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Molecular Biology 9: 491–497.

39. Martinez-Torres D, Chevillon C, Brun-Barale A, Berger JB, Pasteur N, et al. (1999) Voltage-dependent Na+(V) channels in pyrethroid-resistant Culex pipiens L. mosquitoes. Pesticide Science 55: 1012–1020.

40. Pinto J, Lynd A, Vicente JL, Santalacussa F, Randle ND, et al. (2007) Multiple origins of knockdown resistance mutations in the Afrotopical mosquito vector Anopheles gambiae PLoS One 2:.
56. Alon M, Benting J, Lueke B, Pouge T, Alon F, et al. (2006) Multiple origins of pyrethroid resistance in sympatric biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). Insect Biochemistry and Molecular Biology 36: 71–79.

57. Anstead JA, Williamson MS, Denholm I (2005) Evidence for multiple origins of identical insecticide resistance mutations in the aphid *Myzus persicae*. Insect Biochemistry and Molecular Biology 35: 249–256.

58. Meraner A, Brandstätter A, Thaler R, Aray B, Unterlechner M, et al. (2008) Molecular phylogeny and population structure of the codling moth (*Cydalima perspectalis*) in Central Europe: I. Ancient clade splitting revealed by mitochondrial haplotype markers. Molecular Phylogenetics and Evolution 48: 825.

59. Guillemaud T, Rooker S, Pasteur N, Raymond M (1996) Testing the unique amplification event and the worldwide migration hypothesis of insecticide resistance genes with sequence data. Heredity 77: 535–543.

60. Denholm I, Devine GJ, Williamson MS (2002) Insecticide resistance on the move. Science 297: 2222–2223.