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Target-site resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus* F.

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ABSTRACT

Pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is a major univoltine pest of oilseed rape in many European countries. Winter oilseed rape is cultivated on several million hectares in Europe and the continuous use of pyrethroid insecticides to control pollen beetle populations has resulted in high selection pressure and subsequent development of resistance. Resistance to pyrethroid insecticides in this pest is now widespread and the levels of resistance are often sufficient to result in field control failures at recommended application rates. Recently, metabolic resistance mediated by cytochrome P450 monooxygenases was implicated in the resistance of several pollen beetle populations from different European regions. Here, we have also investigated the possible occurrence of a target-site mechanism caused by modification of the pollen beetle *para*-type voltage-gated sodium channel gene. We detected a single nucleotide change that results in an amino acid substitution (L1014F) within the domain IIS6 region of the channel protein. The L1014F mutation, often termed *kdr*, has been found in several other insect pests and is known to confer moderate levels of resistance to pyrethroids. We developed a pyrosequencing-based diagnostic assay that can detect the L1014F mutation in individual beetles and tested more than 350 populations collected between 2006 and 2010 in 13 European countries. In the majority of populations tested the mutation was absent, and only samples from two countries, Denmark and Sweden, contained pollen beetles heterozygous or homozygous for the L1014F mutation. The mutation was first detected in a sample from Denmark collected in 2007 after reports of field failure using *tau*-fluvalinate, and has since been detected in 7 out of 11 samples from Denmark and 25 of 33 samples from Sweden. No super-*kdr* mutations (e.g. M918T) known to cause resistance to pyrethroids were detected. The implications of these results for resistance management strategies of pollen beetle populations in oilseed rape crops are discussed.

1. Introduction

Pollen beetle, *Meligethes aeneus* F. (Coleoptera: Nitidulidae) is one of the major pests in European oilseed rape and can cause significant economic damage without chemical control measures [1]. For almost two decades, pollen beetle populations in Europe have been effectively controlled by synthetic pyrethroid insecticides that act on voltage-gated sodium channels in the insect central nervous system [2,3]. However, the lack of other available insecticide classes with different modes of action and overlapping pyrethroid treatment windows for stem weevil control has resulted in strong pressure for the selection of resistance. As a result, pyrethroid resistance is now widespread among European pollen beetle populations [3–8]. Resistance monitoring initiatives based on adult-vial bioassays using the reference pyrethroid *lambda*-cyhalothrin that were carried out between 2007 and 2010 revealed the presence of pyrethroid resistant populations in almost all European countries sampled, including Germany, France, Poland, UK, Denmark, Sweden and others [7,8]. Fortunately, resistance has not yet been reported for the newer insecticides with different

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modes of action such as thiacloprid, a recently introduced neonicotinoid insecticide for pollen beetle control in winter/spring oilseed rape, or pymetrozine and indoxacarb, known to act as antifeedant and sodium channel blockers, respectively [9].

Resistance to pyrethroids in pollen beetles is relatively broad spectrum across the whole chemical class, however some pyrethroids such as *tau*-fluvalinate and etofenprox were recently shown to be less affected by cross-resistance issues and have therefore become first choice for control in some countries [10]. Bioassays have also shown moderate levels of cross resistance to *tau*-fluvalinate in pollen beetle populations from Denmark and Sweden [10,11], while Heimbach et al. [12] showed a decrease in the susceptibility of German populations to the non-ester pyrethroid etofenprox over a period of 5 years.

The intense use of pyrethroids against chewing and sucking pest species in many agricultural cropping systems, as well as their use to control disease vectors and urban pests, has resulted in numerous cases of resistance over the past 30 years. Two main types of mechanism are known to be responsible for resistance; one based on modification of the pyrethroid target site and the other caused by enhanced metabolic detoxification from elevated levels of esterases and cytochrome P450 monooxygenases [13].

The biochemical mechanisms underlying pyrethroid resistance in pollen beetles have recently been investigated. The synergistic action of piperonyl butoxide (PBO, a metabolic enzyme inhibitor) in combination with pyrethroids provided initial evidence for the involvement of cytochrome P450 monooxygenases [14,15]. Despite the lack of a correlation between pyrethroid resistance level and elevated cytochrome P450 activity based on biochemical assays with the artificial substrate 7-ethoxycoumarin [14,15], the involvement of monooxygenases in pyrethroid resistance has been clearly demonstrated by the hydroxylation of deltamethrin by pollen beetle microsomes [15]. In this study, the maximum rate of deltamethrin hydroxylation by pollen beetle microsomes correlated well with the level of pyrethroid resistance and was inhibited by both PBO and tebuconazole. Furthermore it was demonstrated that *tau*-fluvalinate and *lambda*-cyhalothrin competitively inhibited the formation of 4-hydroxy deltamethrin, thus confirming pyrethroid cross-resistance based on microsomal oxidation [15].

Target site resistance to pyrethroids is caused by point mutations in the gene for the voltage-gated sodium channel, leading to amino acid substitutions within the channel protein that affect the binding of pyrethroids [16–18]. Two mutations, L1014F and M918T, were originally described in pyrethroid resistant *Musca domestica* and linked to strains phenotypically classified as knock-down resistant (*kdr*) and super-kdr (*s-kdr*), respectively [17]. Since then, the L1014F mutation (or variants such as L1014S) has been identified in a range of different pest species and typically confers moderate (10–20-fold) levels of resistance to all pyrethroids. M918T and other super-kdr like mutations (e.g. T929I) have also been discovered in a range of pests, and these confer much higher levels of resistance (several 100-fold) [17,18]. These mutations are located in the domain II S4–S5 linker and S5, S6 transmembrane regions of the channel protein and are thought to form part of a hydrophobic binding site for the pyrethroids [18].

The possible role of target site modification in pyrethroid resistance has not yet been investigated in pollen beetles. The objective of this study was to investigate the presence and distribution of *kdr* and *s-kdr* mutations in pyrethroid resistant pollen beetle populations from Europe. To achieve this, a partial sequence of the para-type sodium channel of *M. aeneus* spanning the domain II region containing the *kdr* and *s-kdr* mutation sites was PCR amplified and sequenced. We identified the same nucleotide mutation that causes the *kdr* L1014F substitution in a resistant strain from Denmark and used this to develop a diagnostic assay for the mutation based on SNP-genotyping by pyrosequencing technology. Individuals of several hundred pyrethroid-resistant populations surviving a pyrethroid diagnostic dose (according to [7,8]) in an adult-vial bioassay were subjected to pyrosequencing analysis for *kdr* and the results geographically mapped. The consequence for regional resistance management strategies where target-site resistance was found is discussed.

2. Materials and methods

2.1. Insect populations

More than 350 European pollen beetle populations were collected in oilseed rape fields between April and June of 2006–2010 and bioassayed for pyrethroid resistance using a recently described adult vial test [7,8]. The test is based on two concentrations of the reference pyrethroid *lambda*-cyhalothrin, 75 and 15 ng/cm^2 coated onto the inner glass surface and representing 100% and 20% of the recommended field rate, respectively (for a detailed description of the method refer to [8]). Many of the populations investigated here were also included in two recently published studies on the status of pyrethroid resistance in pollen beetle in Europe [7,8]. At least 30 populations sampled in 2009 and 2010 from Sweden were also tested with *tau*-fluvalinate due to an increased number of reports of reduced field efficacy with this pyrethroid [11]. Resistance to *tau*-fluvalinate was checked by using the same adult vial test design, based on 100% and 20% of the recommended field rates, 480 and 96 ng/cm^2, respectively. Beetles that survived the bioassay after 24 h at 100% of the field-recommended rate of either *lambda*-cyhalothrin or *tau*-fluvalinate were stored at −80 °C for subsequent molecular diagnostics. Populations that did not survive the 100% field-rate of either pyrethroid were discarded.

2.2. Amplification and sequencing of M. aeneus para-type sodium channel gene fragment and kdr-genotyping using Sanger sequencing

Individual adult pollen beetles were ground in liquid nitrogen and genomic DNA (approx. 1 μg per adult) was extracted using DNAzol purification reagent (Invitrogen) according to the supplier's recommended protocol. Domain II sodium channel gene fragments were PCR amplified from 100 ng aliquots of gDNA using primers designed against a partial sequence of the pollen beetle para gene deposited in GenBank (sequence accession AF354457, see primer sequences PB1-4 in Table 1). Two rounds of PCR were carried out using various combinations of primers PB1-4 (0.5 μM) in 25 μL reactions containing 1× Taq enzyme reaction mix (Promega, UK) with standard cycling conditions of 94 °C for 2 min, followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min. The ~510 bp sodium channel gene fragments generated in these PCR reactions were ethanol precipitated to remove excess primer and directly sequenced with internal primers (PB5 and PB6) using ABI big-dye terminator reaction kits on a 310 genetic analyser (Applied Biosystems). This was done for a number of individual beetles collected in Denmark in 2007 in order to double-check the (SS/SR/RR) *kdr* results obtained by pyrosequencing. Sequences were analysed using the VectorNTI software package (Invitrogen). Sequence alignment graphics were done by using Geneious v5.5 (www.geneious.com).

2.3. Pyrosequencing kdr/s-kdr diagnostic assay

Pyrosequencing is a DNA sequencing-by-synthesis technique enabling real-time detection of nucleotides forming base pairs in an amplified DNA template strand using an enzyme-cascade finally resulting in bioluminescence signals [19,20]. Individual adult

Table 1
Details and sequences of primers used to analyse for *kdr* (*s-kdr*) mutation.

Name	Sequence 5′–3′
Py-KDR-Seq1	CACTGTGGTTATCGGTAAT
Py-KDR-Seq2	GCCACTGTGGTTATCGGT
Py-KDR-F	ATGTGTCCTGTATTCCCTTC
Py-KDR-R	[btn]GCTGGATGATCCAAAATTG
Py-s-kdr-Seq	TCTAAATTTACTTATATCCA
Py-s-kdr-F	GGCCGACTCTAAATTTACTTATAT
Py-s-kdr-R	[btn]TCCTTACCCGTATAATTTTTGCC
PB1	TGGCCGACTCTAAATTTACTT
PB2	CTCTAAATTTACTTATATCCAT
PB3	TTGGTGCTGATAAGCTGGATG
PB4	CTGGATGATCCAAAATTGCTC
PB5	GACCACGATCTACCTCGTTG
PB6	ACCAACATACAGTCCCACATC

pollen beetles were ground in liquid nitrogen and genomic DNA (approx. 1 μg per adult) was extracted using DNeasy plant kit (Qiagen) or DNAdvance Tissue Kit (Agencourt) according to the supplier' recommended protocol. The domain II sodium channel gene fragment was amplified by PCR from 50 ng aliquots of gDNA using two primers (Table 1, *kdr*: Py-KDR-F & PyKDR-R; s-kdr: Py-s-kdr-F & Py-s-kdr-R) designed with 'Assay Design Software' (PSQ-Biotage AB, now Qiagen) by utilizing the partial sequence of the pollen beetle para gene detailed above (see primer sequences in Table 1). The pyrosequencing protocol comprised 45 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 50 μL reaction mixture containing 1× Taq enzyme reaction mix (HotstarTaq Master Mix, Qiagen) and cycling conditions of 95 °C for 10 min, followed by 45 cycles of 95 °C for 45 s, 49 °C for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min). The single strand DNA preparation required for pyrosequencing was carried out using the Vacuum Prep Tool (Biotage AB) in combination with streptavidin coated beads (Streptavidin Sepharose) to separate the biotinylated reverse strand of the PCR products. The pyrosequencing reactions were carried out according to the manufacturer's instructions using one of two different sequence-primers

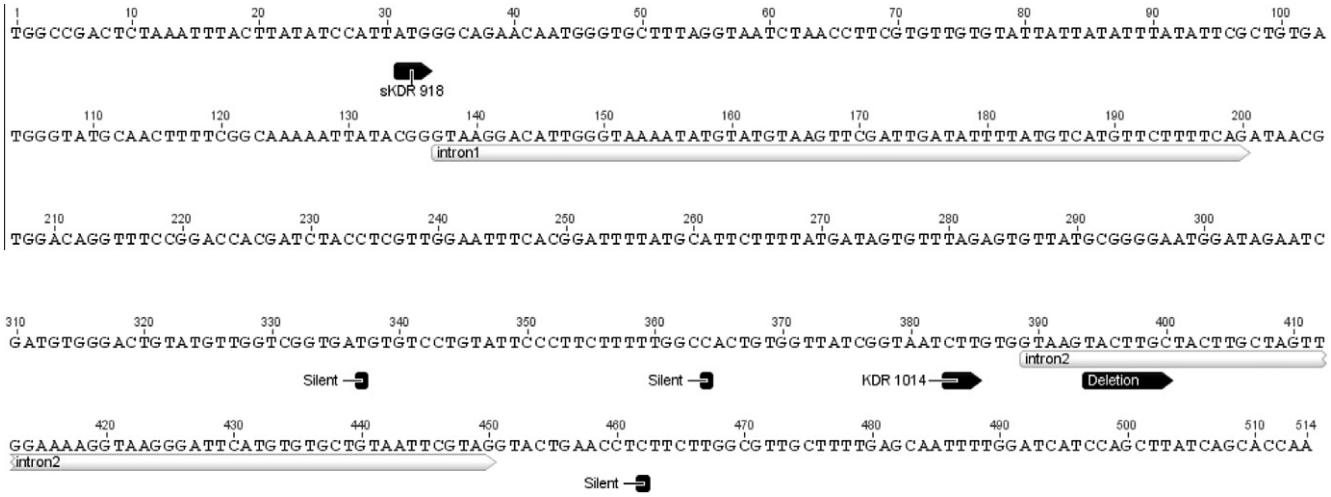

Fig. 1. Partial nucleotide sequence of a 514 bp genomic fragment of the *para*-type sodium channel gene of *Meligethes aeneus*, spanning the region which includes the kdr- and s-kdr mutation sites. The *s-kdr* (M918, ATG) and *kdr* (L1014, CTT) mutation sites are marked below the sequence, as well as three silent mutation sites and one 7 bp indel (TACTTGC) in the intron downstream of the *kdr* site.

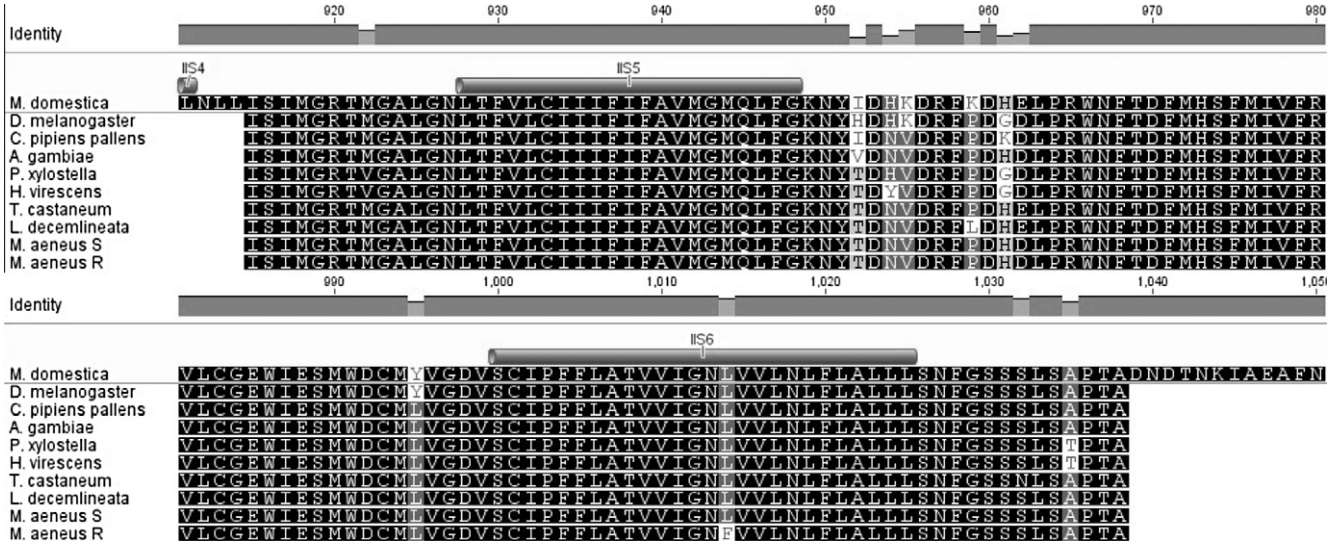

Fig. 2. Multiple sequence alignment of *para*-type sodium channel region DII S4–6 sequences from different insect species. Conserved identical amino acid residues are marked in black boxes. The sequence obtained from *M. aeneus* R (bottom) shows the L1014F mutation known to confer knock-down resistance to pyrethroids.

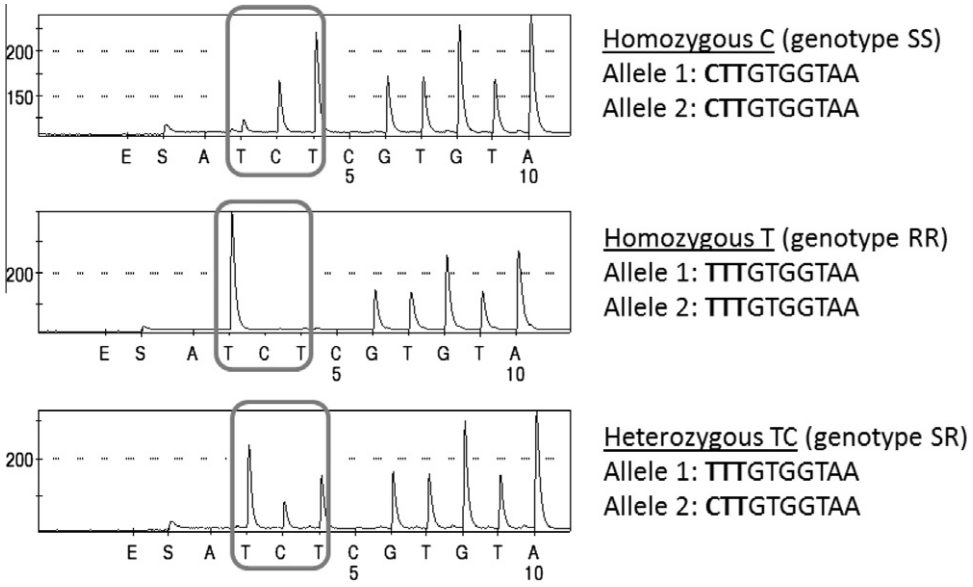

Fig. 3. Pyrograms displaying homozygous SS, RR, as well as heterozygous SR genotypes of the mutation L1014F found in a Danish population of *Meligethes aeneus* resistant to *tau*-fluvalinate (ca. 50% mortality at recommended field rate) and collected in 2007.

for either kdr or s-kdr genotyping (Table 1) and the PSQ 96 Gold Reagent Kit (Biotage AB). The genotypes were analysed using the supplied SNP Software (Biotage AB).

3. Results

3.1. Detection of mutations in para-type sodium channel fragments

A 514 bp fragment of the *M. aeneus para*-type sodium channel gene was PCR amplified from genomic DNA extracted from individual pollen beetles collected in Denmark in 2007 (Fig. 1). This fragment encodes the domain IIS4–IIS6 region of the sodium channel *alpha* subunit which contains five of the putative mutation sites previously associated with *kdr/s-kdr*-type pyrethroid resistance in a range of insect species, i.e. M918, L925, T929, L932 and L1014 [16]. This fragment also contains two short intron sequences (64 and 62 bp), the positions of which are also conserved across species [18]. The amino acid sequence of this fragment of the para-type sodium channel of *M. aeneus* shows close homology to that of other insects, with over 90% direct amino acid identity in this region of the protein (Fig. 2). Based on the comparison of the aligned coleopteran sequences including those from *Leptinotarsa decemlineata* and *Tribolium castaneum* the amino acid identity in this region of the protein is close to 100%.

A total of 14 adult beetles from the obtained Danish sample were sequenced. Those beetles were part of a population which shows only 50% mortality at 100% of the field-recommended rate of *tau*-fluvalinate (47.5 g/ha). The sequencing revealed two distinct alleles of the sodium channel in the *M. aeneus* population that had been sampled; one corresponding to the 514 bp sequence shown in Fig. 1, and a second allele with a 7 bp indel downstream of the kdr-site in the second intron (the deleted bases are shown by an arrow in Fig. 1). Both alleles also carry up to three silent nucleotide polymorphisms in the coding sequence 54 bp upstream of the second intron (also marked in Fig. 1). Of the 14 adults tested, eight were homozygous for the sequence with the 7 bp insertion (allele A), four were homozygous for the second allele with the 7 bp deletion (allele B), and the other two beetles were heterozygotes carrying both allele types.

Five of the 14 beetles tested were homozygous for the kdr mutation (F1014; Fig. 2), eight were homozygous wild-type (L1014), and the remaining individual was a heterozygote (L/F1014). None of the beetles sequenced contained any of the other mutations (s-*kdr*) mentioned above and known to confer resistance to pyrethroids. Interestingly, the *kdr* mutation was not confined to one of the two allele types described above (A and B), but instead was found in both types. Of the eight allele A homozygotes, five were also homozygous for L1014 (susceptible), two were homozygous for the F1014 mutation (kdr), and one was a heterozygote. Similarly, of the four allele B homozygotes, two were L1014 homozygotes and two were F1014 homozygotes. This result was surprising as it suggests that the kdr-like mutation has arisen independently in two different allele types.

3.2. Validation of target-site resistance diagnostics by pyrosequencing

The pyrosequencing diagnostic assay identifies all three kdr genotypes in individual beetles, designated SS (homozygous L1014), SR (heterozygous L/F1014) and RR (homozygous F1014). The PCR carried out to amplify the template DNA for kdr-pyrosequencing produced a ~160 bp fragment of the *M. aeneus* para-type sodium channel (from genomic DNA). For SNP analysis 10 nucleotides starting upstream the putative kdr-like polymorphism site (codon CTT at position 1014) were pyrosequenced using the gene specific sequence-primer Py-KDR-Seq1.

Similar to the Sanger sequencing approach described above (3.1), pollen beetles of the same Danish sample collected in 2007 were used to validate the pyrosequencing method. In total 16 adult beetles were individually analysed. Four of the 16 beetles tested were homozygous for the *kdr* mutation (F1014; Fig. 3), seven were homozygous wild-type (L1014), and the remaining five individuals were heterozygotes (L/F1014). As shown in the pyrograms in Fig. 3 the assay successfully detects the polymorphism (C/T) at the first coding position of the triplet in position 1014. The nucleotide sequences experimentally obtained for SS, SR, and RR are 5′-CTT GTGGTAA-3′, C/TTTGTGGTAA-3′ and TTTGTGGTAA-3′, respectively, thus based on the sequential reaction of the nucleotides with the template DNA in the order of which the individual nucleotides were dispensed (Fig. 3). Taking the results of both sequencing approaches

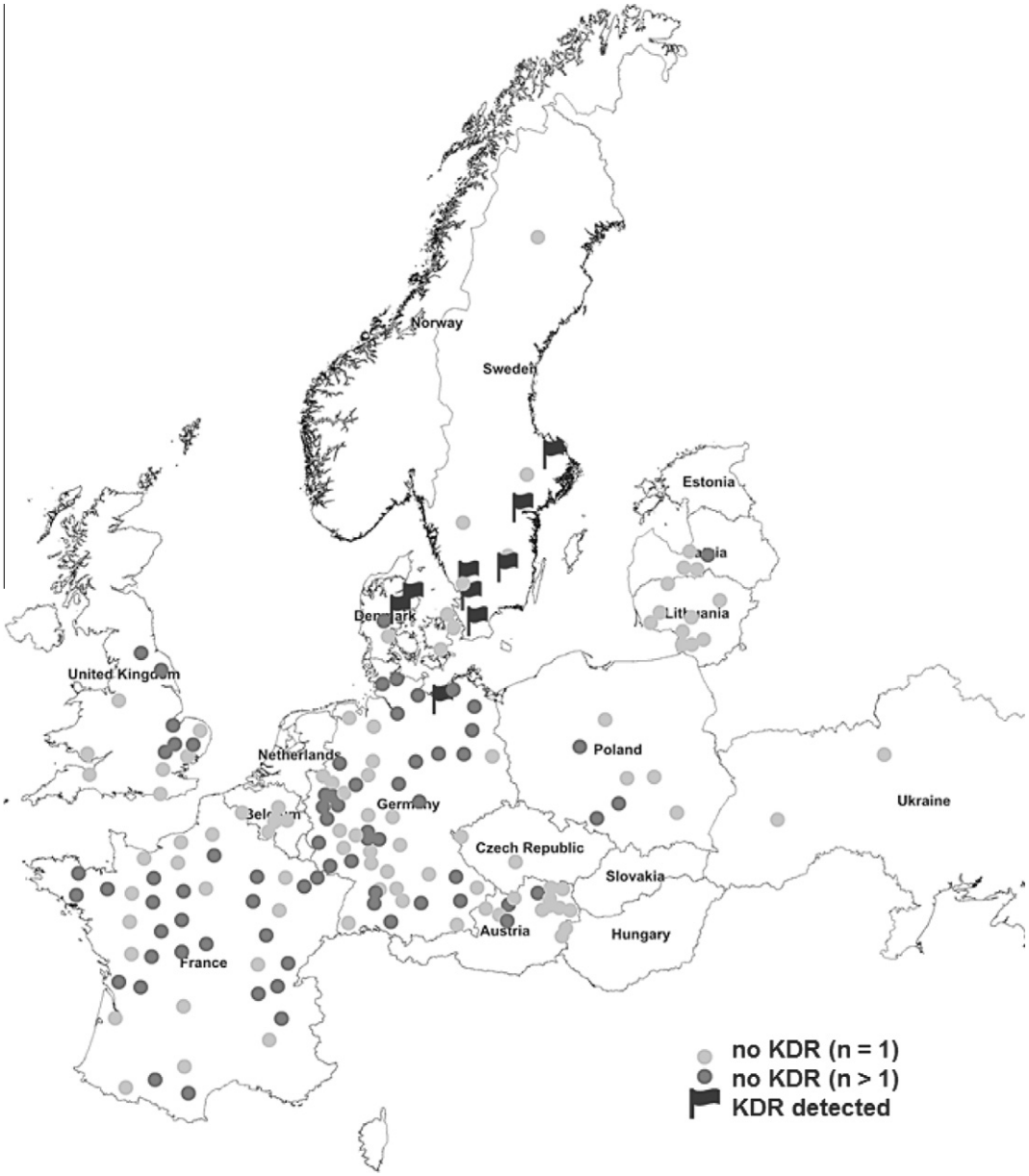

Fig. 4. Geographical mapping of *kdr*-based target-site resistance in European populations of pollen beetles. In total 400 collected populations were screened between 2006 and 2010 (Table 2). Individual flags displaying the presence of *kdr* genotypes may include several positively tested populations. *Abbreviations:* (*n*) refers to the number of populations tested per spot.

Table 2
Number of pyrethroid-resistant populations genotyped for *kdr* resistance (L1014F) and collected in different European countries between 2006 and 2010. Only those beetles which survived 100% of the field-recommended rate of either *lambda*-cyhalothrin or *tau*-fluvalinate were genotyped. Those populations listed to include SR and RR genotypes (in bold) always also contained SS individuals, except one from Sweden.

Genotype	A	B	CZ	DK	F	FIN	G	LT	LV	PL	SE	UA	UK
SS	24	6	2	7	97	3	141	9	5	16	33	2	24
SR	0	0	0	**6**	0	0	**1**	0	0	0	**12**	0	0
RR	0	0	0	**7**	0	0	0	0	0	0	**9**	0	0
Total	24	6	2	7	97	3	141	9	5	16	34	2	24

A = Austria, B = Belgium, CZ = Czech Republic, DK = Denmark, F = France, FIN = Finland, G = Germany, LT = Lithuania, LV = Latvia, PL = Poland, SE = Sweden, UA = Ukraine, UK = United Kingdom.

together, the Danish sample collected in 2007 contained 15 (50%), 6 (20%) and 9 (30%) individuals of the genotypes SS, SR and RR, respectively.

The primers designed to detect mutations at the M918T *s-kdr* site were used for parallel sequencing with each individual tested, but no mutation was observed at this site.

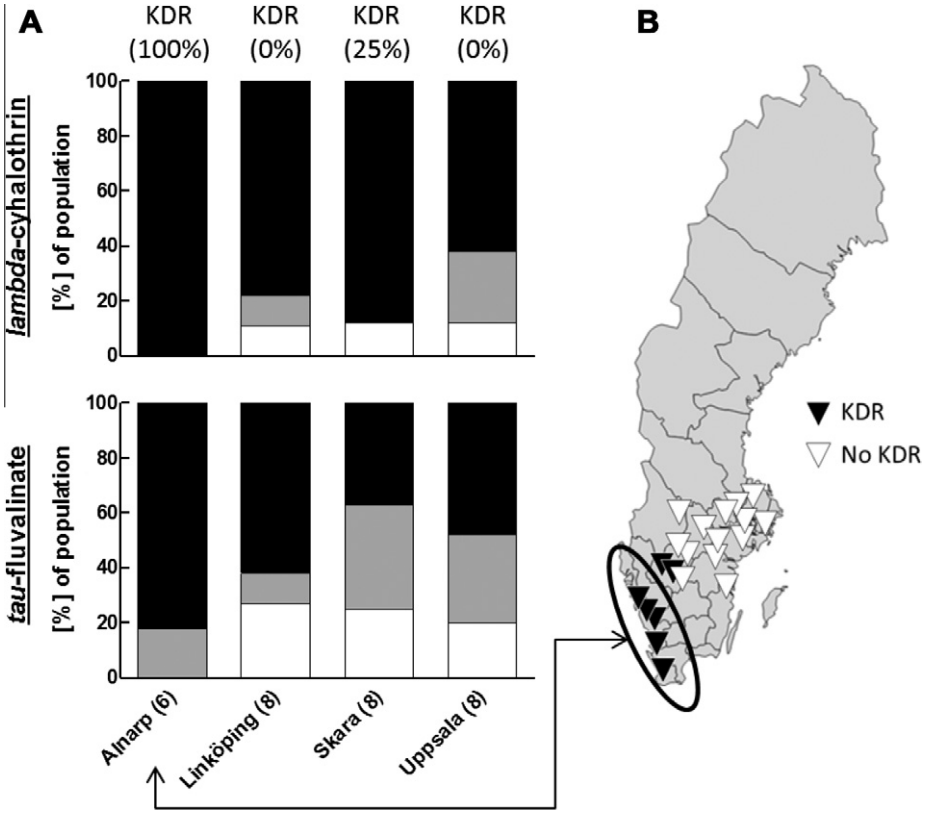

Fig. 5. (A) Stacked-bar chart showing the proportion of 2009 collections of Swedish pollen beetle populations resistant (black), moderately resistant (grey) and susceptible (white) to *lambda*-cyhalothrin and *tau*-fluvalinate in discriminating dose bioassays. Surviving beetles were genotyped for knock-down resistance (*kdr*) and the proportion homozygous or heterozygous for the L1014F mutation is given in %. (B) Geographical mapping of the populations tested reveals a strong presence of the *kdr* resistance allele in populations sampled in southern regions.

3.3. Monitoring for target-site resistance and geographical distribution of the L1014F mutation in Europe

In total, more than 350 populations of *M. aeneus* were SNP-genotyped for polymorphisms at both the *kdr*- and *s-kdr* sites by pyrosequencing and mapped according to their geographic location (Fig. 4). We analyzed individuals of 45, 37, 83, 86 and 99 pyrethroid-resistant populations collected in 2006, 2007, 2008, 2009 and 2010, respectively. The populations were collected from oilseed rape in 13 countries and interestingly only samples from two Scandinavian countries, Denmark and Sweden, were found to contain the *kdr* (L1014F) mutation that confers target-site resistance. None of the pyrethroid-resistant samples collected between 2006 and 2010 in Austria, Belgium, Czech Republic, Finland, Lithuania, Latvia, Poland, Ukraine and UK contained this mutation (Table 2). Only one population collected in 2010 out of 141 sampled in Germany between 2006 and 2010 contained a single heterozygote (SR) individual; all other beetles tested were SS genotypes (Table 2). In 2007 we detected *kdr* for the first time in three field-collected samples from Denmark and subsequently in four samples collected in 2010, confirming the spread of the mutation in Danish populations. In 2009, numerous populations from different regions of Sweden were collected that showed high levels of resistance in bioassays with *lambda*-cyhalothrin and *tau*-fluvalinate, particularly those from the south of Sweden (Malmö) (Fig. 5A). A lower proportion of resistant individuals were found in the populations collected in the middle of Sweden (Uppsala). All samples from the southern part of Sweden showed a high frequency of the *kdr* allele, but in many cases also contained susceptible genotypes. In contrast, we never found the *kdr* allele in the more northern populations, including those that were resistant to pyrethroids and able to survive 100% of the recommended field-rate (Fig. 5B).

4. Discussion

In this study, we demonstrate for the first time the presence of a target-site mutation (L1014F), commonly known as *kdr*, in voltage-gated sodium channels of pyrethroid-resistant pollen beetle collected in oilseed rape in Europe. This mutation has been shown to confer resistance to pyrethroids in a range of insect pests and its effect on the insect sodium channel has been functionally demonstrated [13,16,18]. However, to our knowledge *M. aeneus* is only the second coleopteran species in which this mutation has been detected, following earlier reports in populations of the Colorado potato beetle, *L. decemlineata* [21,22]. An alternative *s-kdr*-like mutation (T929I) has also been described recently in maize weevils, *Sitophilus zeamais* [23]. None of the pyrethroid-resistant pollen beetle populations analyzed in this study carried any of the *s-kdr* mutations at positions M918, L925, T929 and L932 that have been described in other insect pests [16]. Interestingly, we found in some of the amplified fragments a 7 bp deletion (TACTTGC) in the intron downstream of the *kdr*-site, which was not correlated with the presence of the *kdr* mutation. A similar indel (but 5 bp, TCACA) in the intron downstream the *kdr* mutation was recently also described in sodium channel fragments amplified from *Culex quinquefasciatus* [24]. The authors were also unable to link the indel to the presence of the mutation in pyrethroid-resistant mosquitoes.

The pyrosequencing assay developed in this study allows at least 200 beetles to be genotyped per day and is therefore a high-throughput resistance screening methodology for monitoring the spread of kdr-like resistance in pollen beetle. Recently a similar approach was described for dieldrin resistance monitoring in the malaria vector *Anopheles funestus* [25]. During the course of the present study we genotyped thousands of individuals but were

only successful in detecting the L1014F mutation in populations collected in Denmark and Sweden, with the exception of a single heterozygote beetle from the 141 populations tested from northern Germany. Due to high levels of resistance to pyrethroids such as *lambda*-cyhalothrin, many Danish oilseed rape farmers recently switched to another pyrethroid, *tau*-fluvalinate [10]. The decision was based on the fact that *tau*-fluvalinate seemed to retain better efficacy under field conditions, partly because it is used at 6–7 times higher rates than *lambda*-cyhalothrin and also because it was shown to be less affected by the metabolic resistance mechanism of resistance selected by compounds such as deltamethrin, *lambda*-cyhalothrin and cypermethrin and caused by elevated levels of cytochrome P450 that is already widespread in European pollen beetle populations [15]. However, the continued application of this compound several times per season as described by Hansen [10] may have contributed to the selection for target-site resistance in these populations. The situation in Sweden is even more interesting, because resistant *kdr* genotypes were only detected in the very south, whereas *kdr* was not observed in samples from middle Sweden despite the fact that several populations survived 100% of the field rate of *tau*-fluvalinate. Why target-site resistance has only evolved in Scandinavian populations remains unclear, but is perhaps related to low economic infestation thresholds (as low as 0.5 beetles per plant [6]) in these areas, triggering more pyrethroid applications and creating high selection pressure in years when high numbers of beetles migrate into winter and spring oilseed rape fields. Another contributing factor is the fact that between 1985 and 2001, i.e. for 15 years exclusively pyrethroids were used for pollen beetle control without any rotation with other compounds.

Although no target site mutations were found in the other European countries, including France, Germany and Poland, high levels of pyrethroid resistance, with ratios between 500- and 1000-fold, were nevertheless described in hundreds of samples collected from these countries between 2007 and 2010 [7,8]. Many of the samples collected in these countries were shown to have elevated levels of monooxygenases resulting in an enhanced metabolic detoxification of pyrethroids as demonstrated by the formation of 4-OH deltamethrin [15]. It has also been shown in other species that very high resistance ratios to pyrethroids can be explained just by metabolic mechanisms based on the over-expression of cytochrome P450s [13]. Examples include the over-expression of CYP6BQ9 in deltamethrin resistant *T. castaneum* in which confers resistance ratios of up to 4000-fold to deltamethrin [26], and cytochrome P450-based pyrethroid cross-resistance in an isogenic line of *Helicoverpa armigera* confering resistance ratios of >10,000-fold to certain pyrethroids [27]. Such examples demonstrate the effectiveness of metabolic resistance mechanisms, which expressed at high levels would suffice to render maximum application rates of a pyrethroid completely useless for control purposes. In such cases any further selection for other mechanisms such as target-site resistance seems unlikely, unless individuals with such a mechanism exhibit strong fitness advantages sufficient to out-compete less fit individuals. However, this additional target-site mechanism would be advantageous since it affects the entire class of pyrethroid chemistry even in the absence of metabolic mechanisms of resistance. For resistance management purposes it may not be advisable to replace pyrethroids that are most affected by metabolic resistance with others that are less affected, as this may provide stronger selection pressure for rare genotypes carrying mutations in the voltage-gated sodium channel, as seen in pollen beetle populations from Denmark and Sweden. Therefore it is strongly recommended to seek for alternative modes of action for pollen beetle control and to follow the resistance management recommendations given by local experts or published annually by the Insecticide Resistance Action Committee (IRAC) [28].

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