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1 First report of the *kdr* pyrethroid resistance mutation in a French population of
2 the English grain aphid, *Sitobion avenae*

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10 **ABSTRACT**

11 The lack of recent insecticide innovations and the withdrawal of efficient, but ecotoxic
12 molecules (e.g. neonicotinoids) may lead to a shift to older insecticidal active substances,
13 particularly pyrethroids. The evolution of resistance to this family of insecticides has long been
14 recognized, including in many aphid species such as the grain aphid *Sitobion avenae*. The
15 target-site resistance mechanism involved, named *kdr* (knock-down resistance), was first
16 identified in the United Kingdom in 2014. In this study, we screened for the presence of this
17 mutation in 25 French populations of *S. avenae* sampled between 2017 and 2021 on various
18 cultivated cereals. Aphids were genotyped individually for the *kdr* mutation using PCR-RFLP or
19 partial sequencing of the voltage-gated sodium channel gene. Of the 400 *S. avenae* individuals
20 tested, one carried a heterozygous *kdr* mutation. This *S. avenae kdr* mutant was sampled in
21 Northern France, the French region closest to the United Kingdom. The low frequency of the
22 detected mutation and the geographical proximity to the UK suggest that it arose from a

23 migration event and not an independent convergent mutation event, although this hypothesis
24 remains to be confirmed. Our study also reveals that partial sequencing of the voltage-gated
25 sodium-channel gene can be used for another cereal aphid species, *Rhopalosiphum padi*.

26 **Keywords:** insecticide resistance monitoring, cereals, aphids

27

28 INTRODUCTION

29 The English grain aphid, *Sitobion avenae* (Fabricius, 1775) is one of the major cereal aphid
30 species in the temperate areas of the Northern Hemisphere. This aphid can infest a wide range
31 of cultivated or wild cereals (wheat, barley, oat, maize, rice, cocksfoot grass, etc.). It causes
32 direct damage by sucking sap from the host, which can lead to senescence of the flag leaf and
33 reduced grain weight (Wratten et al. 1975). This species is also a vector of viruses, such as
34 barley yellow dwarf virus (BYDV), which causes a reduction in yield and grain quality (Edwards
35 et al. 2001, Gray et al. 1991). Controlling this pest generally involves the use of insecticides in
36 conventional agriculture. Repeated application of pesticides exerts selection pressure on
37 pests, which can lead to the evolution of resistance within the species with — in the long run
38 — the risk of losing treatment effectiveness (Hawkins et al. 2019). *S. avenae* has great
39 evolutionary potential, as demonstrated by the multiple types of resistance that have evolved
40 against several active substances, including target-site resistance mechanisms (Foster et al.
41 2014) and non-target site resistance mechanisms (Zang et al. 2017, Gong et al. 2021). Some
42 French populations of *S. avenae* can reproduce both sexually and asexually (Simon et al. 1999,
43 Dedryver et al. 2001), thereby allowing genetic recombination and rapid dispersal of
44 competitive clones by asexual multiplication. Although other active substances have since
45 been developed, pyrethroids remain one of the main insecticide families used to control aphid

46 populations on cereal crops in France. In Europe, pyrethroid-resistant individuals of *S. avenae*
47 were first reported in 2014 on populations collected in 2011 in England and Scotland (Foster
48 et al. 2014). The resistance mechanism involves a target-site mutation, resulting in the amino-
49 acid substitution of a leucine by a phenylalanine on codon 1014 (L1014F) of the voltage-gated
50 sodium channel, the pyrethroid target. This mutation, named *kdr* (knock-down resistance), is
51 one of the most common pyrethroid-resistance mechanisms in insects (Dong *et al.*, 2014).
52 Several other mutations affecting the voltage-gated sodium channel have also been described,
53 especially in aphid species. They are generally located at position 918 and they can be found
54 with or without *kdr* (Eleftherianos et al. 2008, Fontaine et al. 2011, Panini et al. 2015, Carletto
55 et al. 2010). Mutations affecting this codon are called *skdr* (super knock-down resistance),
56 because they most often lead to very high level of resistance to pyrethroids (Eleftherianos et
57 al. 2008, Fontaine et al. 2011).

58 Considering the pyrethroid spray failures caused by a resistant *S. avenae* clone in the UK
59 (Foster et al. 2014) and the wide use of pyrethroids on cereals in France, we screened for the
60 presence of *kdr* between 2017 and 2021, as part of the French system for monitoring the
61 adverse effects of plant protection products.

62 MATERIALS AND METHODS

63 Aphid sampling

64 Field samples were done from 2017 to 2021 by harvesting 30 to 40 randomly distributed cereal
65 plants infested with *S. avenae*. Live aphids were collected with a brush and individually placed
66 in 2 mL Eppendorf tubes with a sterile stainless-steel bead of 4.76 mm diameter. Aphids were
67 stored at -80°C until RNA extraction.

68 RNA Purification

69 Samples were ground in a TissueLyser II (Qiagen, Hilden, Deutschland) with two cycles of 2 min
70 at 20 Hz after adding 100 µL of buffer RA1 with 2% TCEP provided in the Nucleospin RNA Mini
71 kit (Macherey Nagel, Düren, Deutschland). RNA was then purified according to the
72 manufacturer's instructions. Final elution was done in 10 µL of RNase-free water. Purified RNA
73 was stored at -80°C until use in a reverse-transcription polymerase chain reaction (RT-PCR).

74 Pyrethroid resistance genotyping

75 PCR-RFLP genotyping for *kdr* mutation

76 From 2017 to 2018, the presence of the *kdr* mutation in the voltage-gated sodium-channel
77 gene was determined using the RT-PCR method described in Foster *et al.* (2014). This method
78 requires reverse transcription of RNA into DNA, followed by PCR with the Sa1 and Sa3 primers.
79 RT-PCR was performed with the Superscript One Step RT-PCR system with Platinum Taq kit
80 (Invitrogen, city, CA, USA). The reaction mix contained 1x reaction buffer, 400 µM of each
81 primer and 0.25 µL of RT/Platinum Taq mix. PCR products were digested with the enzyme
82 *BssSal* (New England Biolabs, Ipswich, United Kingdom) at 37°C for at least 4 h. This enzyme
83 cuts the sequence '5'-CACGAG-3', which can differentiate the wild-type sequence (a single
84 restriction site) from the mutated sequence (no restriction site).

85 Partial sequencing of the voltage-gated sodium channel

86 In addition to the PCR-RFLP method, the voltage-gated sodium-channel mRNA was partially
87 sequenced in 2019–2021 samples. This method has the advantage of including codon 918 and
88 codon 1014 of voltage-gated sodium channels, both known to be implicated in pyrethroid
89 resistance, i.e. the *skdr* and *kdr* mutations, respectively. The sequencing approach also
90 provides the opportunity to investigate the few atypical digestion profiles that were obtained

91 due to incorrect aphid species identification during sampling (see below). RT-PCR products
92 were directly sequenced by Genewiz (South Plainfield, NJ, USA) or Genoscreen (Lille, France)
93 using the Sanger method with the forward primer.

94 RESULTS

95 A total of 25 populations (from 1 to 9 per year) were sampled in small grain cereals (24) and
96 in a maize field (1) (Figure 1). Field sampling was carried out mostly in conventional fields (18
97 vs. 7 from trial fields). The objective was to collect 30 aphids in each sampled field, but this
98 goal was not always reached due to the low infestation rate of some samples. In total 482 out
99 of 501 aphids sampled (mean per sample = 20 +/- 10.75 – Supplementary Data 1) were
100 successfully analyzed. Over the four years of survey, almost two thirds of aphids were sampled
101 in 2019 and 2021. The amplification failure rate of the analyzed aphids was low (3.6%) and
102 related to low-quality RNA extracts.

103 During the two first years of the survey, resistance genotyping was performed using the PCR-
104 RFLP method on 103 aphids; 98 of them showed a digestion profile with two fragments (one
105 of 74 bp and one of 397 bp) corresponding to susceptible individuals. Five aphids showed
106 atypical band profiles consisting of two bands at approximately 230 bp and 240 bp. These PCR
107 fragments were sequenced. A BLAST analysis showed that the highest identity score (98.97%)
108 of these fragments matched with the *Rhopalosiphum padi* sodium channel 1 mRNA sequence
109 (accession number: MN972451.1). The result of the Blast analysis as well as the frequent co-
110 occurrence of *R. padi* and *S. avenae* on straw cereals in France make us think that an error of
111 identification took place during the sampling in the field. Interestingly, this result reveals that
112 the primers used in this study can also amplify the voltage-gated sodium channel from RNA
113 extracted from *R. padi*. In these five aphids, no mutations were identified at codon 918 or

114 1014, known to be implicated in pyrethroid resistance. The sequence was deposited in
115 GenBank (accession number OM585630).

116 From 2019 to 2021, to avoid genotyping failure due to incorrect species identification, RT-PCR
117 was directly followed by sequencing. The analysis revealed that one aphid had been wrongly
118 identified and belonged to *R. padi*. Among 385 *S. avenae* successfully analyzed during the last
119 three years of the survey, only one aphid was heterozygous for the *kdr* mutation (L1014F)
120 (Figure 2). This *S. avenae* individual was sampled in 2020 in a straw cereal crop field, located
121 in the located in one of the northernmost plots in this study (Figure 3). None of the analyzed
122 aphids showed a mutation at codon 918. The partial the voltage-gated sodium-channel gene
123 sequences of a wild and mutant clone were deposited in GenBank (accession numbers
124 OM585631 and OM585632 for the wild and *kdr*-mutated sequence, respectively).

125

126 DISCUSSION

127 This is the first report of *S. avenae* with a *kdr* mutation in France. Using bioassays,
128 heterozygous *kdr* shows resistance level that is 30–40 times greater than that of the
129 homozygous wild-type and is associated with reports of pyrethroid spray failure (Foster *et al.*,
130 2014). In the UK, only heterozygous aphids have been detected and all of them have belonged
131 to a unique clonal lineage (Sa3). In French *S. avenae* populations, the *kdr* mutation appears to
132 be present at a very low frequency, which contrasts strongly with the situation observed in
133 the UK, where more than 50 % *S. avenae* collected were resistant (Walsh *et al.* 2020). We
134 could not determine whether the clone identified in France is identical to that detected in the
135 UK. Given the geographical proximity between the UK and Northern France where the
136 resistant aphid was detected, a migratory event (natural or related to human activity) is the

137 most likely origin of the mutation. However, we cannot completely rule out independent
138 emergence of the *kdr* mutation. Genotyping neutral markers is required to determine whether
139 the resistant French aphid is related to the Sa3 clone. Our study was conducted on RNA and
140 not on DNA, precluding DNA analysis. Recent studies have reported a fitness trade-off in
141 *S. avenae* clones with *kdr*, such as lower population growth and lower individual relative
142 growth as well as a greater rate of parasitization by *Aphidius ervi* (Jackson et al. 2020) and
143 later onset of male forms (Walsh et al. 2021). Although the very low *kdr* allele frequency in
144 our survey (0.2%) can be suggestive of fitness trade-offs, this finding must be interpreted with
145 caution. The situation in the UK has shown how a resistant clone can spread rapidly across a
146 large area due to selection pressure in the presence of pyrethroids.

147 Interestingly, the primers published by Foster et al. (2014) and used in this study were able to
148 amplify the sodium channel from RNA of another aphid species living on cereal crops. Cereals
149 are often co-infected with different aphid species at different stages of development, which
150 can complicate aphid identification. Adult aphids can be easily identified, but species
151 determination can be more difficult at the larval stage. Therefore, some identification errors
152 (1%) can occur during aphid sampling of infested cereals. Both aphid species experience
153 comparable insecticide treatments, and pyrethroid resistance involving voltage-gated
154 sodium-channel mutations has been recently reported in Chinese populations of *R. padi*
155 (Wang et al. 2020). Therefore, the availability of a tool for the characterization of the *kdr*
156 mutation compatible with *S. avenae* and *R. padi*, two aphids widespread on cereal crops,
157 offers the advantage of detecting resistance-related genotypes even if the exact identification
158 of the species is uncertain. This tool could be easily used in the monitoring framework planned
159 to early detection and following of pyrethroid resistance involving target site modification

160 specially, in countries where the withdrawal of neonicotinoids has occurred or where cases of
161 resistance are suspected in these species of cereals.

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231 Figure 1: Map of fields sampled from 2017 to 2021. The pie charts indicate the proportion of
232 small-grain cereal (yellow) and maize (green) crop fields (populations) sampled in each
233 administrative division (“*département*”) included in the survey. The size of the pie charts is
234 proportional to the number of populations sampled (i.e.: field plot sampled).

235 Figure 2: Chromatographs of wild type and *kdr* heterozygous *S. avenae* sequences around the
236 position 1014 of the para-type sodium channel gene. The 1014th codon is indicated by a red
237 rectangle. Y corresponding to T and C pics according the IUPAC code. CTC and TTC are coding
238 for leucine and phenylalanine, respectively.

239 Figure 3: Map of the distribution of *kdr*, the target site of pyrethroid resistance, in populations
240 of *Sitobion avenae* sampled in French cereal crop fields from 2017 to 2021. The pie charts
241 indicate the proportion of populations (i.e.: field plot sampled) in which the *kdr* mutation
242 (L1014F) was detected (red) or not detected (blue) in each administrative division
243 (“*département*”) included in the survey. The size of pie charts is proportional to the number
244 of populations sampled.

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