

1 **A single nucleotide polymorphism in the acetylcholinesterase gene of**
2 **the predatory mite *Kampimodromus aberrans* (Acari Phytoseiidae) is**
3 **associated with chlorpyrifos resistance**

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5 **Stefano Cassanelli ^{a, *}, Shakeel Ahmad ^b, Carlo Duso ^b, Paola Tirello ^b, Alberto**
6 **Pozzebon ^b**

7
8 ^a Department of Life Sciences, University of Modena and Reggio Emilia, Via A. Amendola 2, I-42122
9 Reggio Emilia, Italy

10 ^b Department of Agronomy, Food, Natural Resources, Animals and the Environment (DAFNAE),
11 University of Padova, Viale dell'Università 16, I-35020 Legnaro, Padova, Italy

12
13 * Corresponding author at: Department of Life Sciences, University of Modena and Reggio Emilia, Via A.
14 Amendola 2, I-42122 Reggio Emilia, Italy. Fax: +39 0522 522027. E-mail address:
15 stefano.cassanelli@unimore.it (S. Cassanelli)

16
17 **ABSTRACT**

18
19 The predatory mite *Kampimodromus aberrans* (Oudemans) (Acari Phytoseiidae) is one of
20 the most important biocontrol agents for herbivorous mites in European perennial crops.
21 The use of pesticides, such as organophosphate insecticides (OP), is a major threat to the
22 success of biocontrol strategies based on predatory mites in these cropping systems.
23 However, resistance to OP in *K. aberrans* has recently been reported. The present study
24 investigated the target site resistance mechanisms that are potentially involved in OP
25 insensitivity. In the herbivorous mite *Tetranychus urticae*, resistance to OP is due to a
26 modified and insensitive acetylcholinesterase (AChE) that bears amino acid substitution
27 F331W (AChE Torpedo numbering). To determine whether the predators and prey had
28 developed analogous molecular mechanisms to withstand the same selective pressure,
29 the AChE cDNA from a putative orthologous gene was cloned and sequenced from
30 susceptible and resistant strains of *K. aberrans*. No synonymous mutation coding for a
31 G119S substitution was determined to be strongly associated with the resistant phenotype
32 instead of the alternative F331W. Because the same mutation in *T. urticae* AChE was not
33 associated with comparable levels of chlorpyrifos resistance, the role of the G119S
34 substitution in defining insensitive AChE in *K. aberrans* remains unclear. G119S AChE
35 genotyping can be useful in ecological studies that trace the fate of resistant strains after

36 field release or in marker-assisted selection of improved populations of *K. aberrans* to
37 achieve multiple resistance phenotypes through gene pyramiding. The latent complexity of
38 the target site resistance in *K. aberrans* vs. that of *T. urticae* is also discussed by exploiting
39 data from the genome project of the predatory mite *Metaseiulus occidentalis* (Nesbitt).

40

41 **1. Introduction**

42 *Kampimodromus aberrans* (Oudemans) (Acari: Phytoseiidae) is a predatory mite that
43 occurs in various European cropping systems, such as grapevines, apples and hazelnuts
44 (Ivancich Gambaro, 1973; El Borolossy and Fischer-Colbrie, 1989; Tsolakis et al., 2000;
45 Ozman-Sullivan, 2006). This predatory mite is also common on the uncultivated plants that
46 surround crops and represent a potential reservoir for biocontrol agents (Tixier et al., 2002,
47 2006). *K. aberrans* is considered to be generalist predator (McMurtry and Croft, 1997;
48 Kreiter et al., 2002; Broufas et al., 2007; Lorenzon et al., 2012) and an effective biocontrol
49 agent of tetranychid and eriophyoid mites in European vineyards (Duso 1989; Girolami et
50 al., 1992; Duso and Pasqualetto, 1993; Duso et al., 2012). In addition to several ecological
51 factors, insecticide and fungicide applications strongly affect naturally occurring and
52 artificially introduced *K. aberrans* populations (Ivancich Gambaro 1973; Girolami, 1987;
53 Pozzebon et al., 2002; Peverieri et al., 2009). However, a *K. aberrans* strain has been
54 detected in North Italian vineyards under conditions of integrated pest management
55 strategies (IPM) that rely on common ethylene-bis-dithiocarbamate (EBCD) fungicides and
56 organophosphate (OP) insecticides (Posenato 1994). This strain was also successfully
57 released in other vineyards and apple orchards following organic or IPM strategies (Duso
58 et al., 2007; Duso et al., 2009; Ahmad et al., 2013). Recently, laboratory studies have
59 confirmed significant levels of chlorpyrifos resistance in this same strain (Tirello et al.,
60 2012). The biochemical basis of OP resistance in phytoseiid mites depends on the active
61 ingredients involved in the selective pressure and on species/strain-specific genetic
62 backgrounds. The resistance phenotype might rely on high detoxifying enzyme activities
63 and/or on a modified and insensitive target AChE. For example, laboratory selection with
64 methidathion in *Amblyseius womersleyi* (Schicha) leads to increases in monooxygenase
65 activity and CYP4-d isoform overexpression (Sato et al., 2001, 2006, 2007), while in
66 *Phytoseiulus persimilis* (Athias-Henriot), this selection results in an enhancement of
67 glutathione transferase (Fournier et al., 1987). High rate *in-vitro* degradation of azinphos-
68 methyl has been observed under both polygenic and monogenic control in OP-resistant
69 strains of *Amblyseius fallacis* (Garman) (Motoyama et al., 1971; Croft et al., 1976). Target

70 site resistance to OP has also been detected biochemically either in isolation or
71 combination with enhanced OP detoxifying pathways. Resistance to certain OP and
72 carbamate compounds, such as parathion and propoxur, in a Dutch strain of
73 *Typhlodromus pyri* Scheuten has been found to be under monogenic control and to be
74 associated with an insensitive target AChE (Overmeer and van Zon, 1983). In a paraoxon-
75 resistant strain of *Amblyseius andersoni*, (Chant) the resistant phenotype has been
76 revealed to be due to an insensitive AChE coupled with modified carboxylesterases (Anber
77 et al., 1988, 1989).

78 Although reductions in chlorpyrifos susceptibility have been reported in other predatory
79 mites, e.g., *T. pyri* (Fitzgerald and Solomon 1999; Cross and Berrie 1994; Bonafos et al.,
80 2008), little is known about the underlying molecular mechanisms. Among the Acari, high
81 levels of chlorpyrifos resistance in *Tetranychus urticae* Koch have been found to be due to
82 a F331W amino acid substitution in the target enzyme acetylcholinesterase (AChE)
83 (Khajehali et al., 2010). Knowledge of a genetic marker associated with chlorpyrifos
84 insensitivity in *K. aberrans* could be useful for understanding the amplitude of this
85 phenomenon and managing predatory mite populations with IPM strategies. Therefore, we
86 report the cloning and sequencing of a *T. urticae*-like acetylcholinesterase cDNA in *K.*
87 *aberrans* and its genotyping in chlorpyrifos-susceptible and resistant strains. The potential
88 complexity of the target site resistance that occurs in predatory mites was also inferred by
89 inspecting the annotated genome of *M. occidentalis*.

90

91 **2. Materials and Methods**

92

93 *2.1. Kampimodromus aberrans* populations

94

95 This study was performed on seven *K. aberrans* strains collected in North-eastern Italy
96 (Veneto Region). Four strains were collected from commercial vineyards, and three strains
97 were collected from untreated European nettle trees (*Celtis australis* L.) (Table 1).

98 All strains were reared without insecticide exposure in separate rearing units at the
99 Department of Agronomy, Food, Natural Resources, Animals and the Environment of the
100 University of Padova, Italy. Grapevine leaves on pads of wet cotton were used as a
101 substrate for the predatory mites, and small pieces of PVC were placed for shelter and
102 oviposition. *Typha latifolia* L. pollen was provided as food (Lorenzon et al., 2012).

103 Information about the effects of OP was available for only two strains; specifically, the PO
104 strain is resistant to chlorpyrifos, and the LE strain is highly susceptible to this insecticide
105 (Tirello et al., 2012).

106

107 *2.2. Insecticide bioassays*

108

109 Laboratory bioassays were conducted for the preliminary screening for resistant and
110 susceptible phenotypes. The bioassays were performed with a commercial formulate
111 (Dursban® 75WG, 75% a.i., Dow AgroSciences). The discriminant concentration for the
112 resistant and susceptible phenotypes was set at 70 g/hl of formulate, which is the
113 recommended field dose for use in vineyards against grape berry moths and leafhoppers.
114 The pesticide formulate was diluted in distilled water before the toxicological test
115 procedures (Tirello et al., 2013). The latter procedures were performed using rectangular
116 leaf sections (approximately 6 cm²). The sections were immersed in the insecticide
117 solution for 30 s, and distilled water was used in the control treatments. When the
118 pesticide residues completely dried out, the leaf sections were placed on wet cotton pads,
119 and cotton barriers were created along their perimeters to prevent predatory mite escape.
120 Two 12-d-old *K. aberrans* females were gently transferred to each leaf section, and fresh
121 pollen was provided as food. The experimental units were maintained in a climate
122 chamber at 25 ± 2° C and 70 ± 10% relative humidity with a 16L:8D photoperiod. Female
123 mortality was assessed 72 h after the treatments. The females that drowned or escaped
124 were removed from the initial test number. In total, we assessed 40-45 females per strain.
125 The corrected mortalities (Abbott, 1925) were calculated.

126

127 *2.3. Primer design for cloning AChE cDNA in K. aberrans*

128

129 The annotated version of the genome assembly (release Mocc_1.0, March 2012) of the
130 predatory mite *Metaseiulus occidentalis* (Nesbitt) (WOPM genome project) was used to
131 search for putative AChE-like proteins with the tBlastn algorithm using the AChE sequence
132 that was amplified from the susceptible strain of *T. urticae* (GenBank accession n.
133 ADK12697.1) as the query sequence.

134 Transcripts predicted to code for putative AChE-like proteins were extracted from the
135 scaffolds, and their open reading frames (ORFs) were compared to the *T. urticae* AChE

136 protein using Lasergene sequence analysis tools EditSeq and MegAlign 5.0 (DNASTAR,
137 Inc., Madison, WI, USA).

138 Degenerate primers were designed by manual inspection of the conserved domains after
139 the alignments of *T. urticae* AChE and putative orthologous AChE-like proteins in *M.*
140 *occidentalis*. The resulting primers were used to amplify the cDNA core fragments of the
141 orthologous AChE in *K. aberrans*. To complete the cloning, walking steps and 3'-5' RACEs,
142 were performed using no degenerate primers and outlined with PrimerSelect 5.0
143 (DNASTAR, Inc., Madison, WI, USA).

144

145 2.4. mRNA extraction and AChE cDNA cloning

146

147 Total RNA was extracted by homogenising 200 adults in 500 µl Tri-Reagent (Sigma),
148 according to the manufacturer's instructions. The sample integrities were examined by
149 electrophoresis in 1.2% agarose and 2.2 M formamide/formaldehyde denaturing gel.
150 Quality and quantity assessments of the extracted RNA were performed in a Nanodrop
151 ND-1000 Spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). First-
152 strand cDNA was synthesised according to the protocol recommended by the supplier
153 using Improm-II reverse transcriptase (Promega) and random primers. Amplification of a
154 cDNA fragment for a putative AChE in *K. aberrans* was achieved through two consecutive
155 rounds of reverse-transcription PCR (RT-PCR) with degenerate primers. The PCR
156 mixtures (25 µl) contained GoTaq Flexi 1x buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 30 pmol
157 forward and reverse degenerate primers, 1 U GoTaq and 2.5 µl of cDNA. The degenerate
158 primers were based on partially conserved functional domains of homologous AChEs
159 proteins in *T. urticae* and *M. occidentalis*. For the first RT-PCR round, the forward and
160 reverse primers were KaAChEF1d (GIPYAKP domain) and KaAChER1d or KaAChER2d
161 (WVYGGSF motif) (Table 2). The PCR product was then diluted 10-fold and used as the
162 template for a second RT-PCR in which the KaAChEF1 primer was replaced with the more
163 internal primer KaAChEF2d, which was designed based on the PYAKPP domain (Table 2).
164 The two PCR rounds shared the following profile: an initial denaturation step of 3 min at
165 94 °C; 5 cycles at 94 °C for 30 s, 45 °C for 30 s and 72°C for 60 s; 5 cycles at 94 °C for 30
166 s, 45 °C plus +1 °C/cycle and 72°C for 60 s; 25 cycles at 94 °C for 30 s, 50 °C for 30 s and
167 72°C for 60 s; and a final extension step at 72 °C for 10 min. PCR products of the
168 expected size (approximately 300 bp) were purified from 1% (w/v) agarose/TBE 1x gel
169 using a EuroGOLD Gel Extraction Kit (Euroclone) and cloned using a pGEM-T easy vector

170 (Promega). The plasmids were purified with a EuroGOLD Plasmid Miniprep Kit
171 (Euroclone) and sent for sequencing at BMR genomics (Padua, Italy). The sequences
172 were assembled and analysed using SeqMan 5.0 (DNASTAR, Inc., Madison, WI, USA).
173 Identification of the AChE-like sequences was performed via a BLASTX search in
174 GenBank (<http://www.ncbi.nlm.nih.gov>) using the ORFs deduced from the cloned cDNA
175 fragments. The cDNA clones were further extended in the 3' direction by performing an
176 RT-PCR that used a forward primer that was designed based on the first cloned cDNA
177 fragment in *K. aberrans* (KaAChEF3) and a reverse primer (KaAChER3) that was
178 designed based on the sequence coding for the conserved domain CAFWKNFL in both of
179 the AChE transcripts found in *M. occidentalis* without any primer degeneration (Table 2).
180 The RT-PCR mixture had the same composition described above except that the primer
181 concentration was reduced to 15 pmol. The PCR was performed as follows: 1 cycle of
182 94 °C for 2 min; 5 cycles that included the three steps of 94°C for 30 s, 50 °C for 30 s and
183 72 °C for 60 s; 5 cycles of 94 °C for 30 s, 50 °C for 30 s (+ 1 °C/cycle) and 72 °C for 60 s;
184 20 cycles of 94°C for 30 s, 55 °C for 30 s and 72 °C for 60 s; and a final extension step at
185 72 °C for 10 min. The PCR product was purified, sequenced and analysed as described
186 above. Three prime and 5' rapid amplification of cDNA ends reactions (RACEs) were
187 performed to complete the *AChE* cDNA sequences. In the RACE reactions, the first strand
188 cDNAs were synthesised using total RNA and polyT-adaptor primer for 3' RACE or
189 KaAChE-R4 for 5' RACE (Table 2) according to the manufacturer's protocol (5' RACE
190 System for Rapid Amplification of cDNA Ends, Invitrogen). The 3' RACE product spanning
191 across the unknown 3'-end of the *AChE* cDNA was amplified in two consecutive PCR
192 rounds with the KaAChEF4-Adaptor1 and KaAChEF5-Adaptor2 primer pairs. To obtain
193 the 5' end of the AChE transcript, the cDNA was subjected to polyC-tailing of its 3'-end
194 with terminal deoxynucleotidyl-transferase (TdT) following the protocol of the kit (5' RACE
195 System for Rapid Amplification of cDNA Ends, Invitrogen). The upstream cDNA sequence
196 encompassing the 5' untranslated region was amplified with two PCR rounds using the
197 coupled primers KaAChER5-TS-primer and KaAChER6-TS-PCR (Table 2). The first 5'
198 RACE round was performed as follows: 94 °C for 2 min (1 cycle); 5 cycles at 94 °C, 56 °C
199 for 30 s and 72 °C for 60 s; 5 cycles at 94 °C, 57 °C for 30 s and 72 °C for 60 s; and 20
200 cycles at 94 °C for 30 s, 58 °C for 20 s and 72 °C for 60 s. The second 5' RACE round
201 consisted of the following: 1 cycle at 94 °C for 2 min; and 30 cycles at 94 °C for 30 s,
202 55 °C for 30 s and 72 °C for 60 s. The 5' RACE fragment was purified from the agarose gel
203 and sequenced as previously described.

204

205 *2.5. Full length AChE cDNA sequencing*

206

207 Total RNA was extracted from adults of both sensitive (LE) and resistant (PO) strains with
208 TRI-Reagent described for the cDNA cloning. First-strand cDNA was synthesised from
209 total RNA with Improm-II reverse transcriptase (Promega) and random primers as
210 indicated by manufacturer's protocol. To sequence the ORF of the cloned cDNA, three RT-
211 PCR fragments that partially overlapped were generated using the following primer
212 couples: KaAChEF6-R7, KaAChEF7-R8, and KaAChEF8-R9 (Table 2). The PCR reaction
213 (25 µl) included 2 µl of cDNA, a final concentration of GoTaq Flexi 1x buffer, 1.5 mM MgCl₂,
214 0.2 mM dNTPs, 0.6 µM of each primer and 0.625 U/µl GoTaq (Promega). The thermal
215 profile adopted was as follows: 94°C for 2 min (1 cycle); 30 cycles of 94 °C for 30 s, 56 °C
216 for 30 s and 72 °C 60 s; and a final extension step at 72 °C for 10 min. The PCR products
217 were checked by electrophoresis on 1% agarose in TBE 0.5x buffer, purified with the
218 EuroGOLD Cycle-Pure Kit (Euroclone) and sent to BMR genomics (Padua, Italy) for
219 sequencing. To this aim, the same primers used for the RT-PCR amplifications and new
220 internal primers (KaAChEF9, R10, and F10) were used (Table 2). Chromatograms were
221 assembled with SeqMan tools (DNASTAR, Lasergene), and the alignments of the cDNA
222 consensus sequences from sensitive and resistant strains were manually inspected for
223 non-synonymous SNPs with the MegAlign program (DNASTAR, Lasergene).

224

225 *2.6. DNA extraction and exon-intron junction amplification*

226

227 DNA extraction was performed according to the methods described by Tixier et al. (2008)
228 while scaling up the reagents. Two hundred frozen adults of each strain were
229 homogenised in 150 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol,
230 100 mM EDTA, and 100 mM Tris-HCl at pH 8.0) using a micro tissue grinder (Wheaton,
231 Millville, NJ). The homogenate was transferred to a 0.5-ml test tube and incubated for one
232 hour at 65°C with periodic hand mixing. One hundred and fifty microliters of a
233 chloroform:isoamyl alcohol mixture (24:1) was added, the solution was mixed by inversion,
234 and tubes were centrifuged at 6°C for 5 min at 1000 g. The aqueous solution was collected
235 in a new test tube, and 80 µl of isopropanol was added to the decanted aqueous phase,
236 which was then chilled at -20 °C for 20 min for DNA precipitation. After centrifugation (15
237 min, 6 °C, 1000 g), the pellet was suspended in 100 µl of 96% alcohol at 4 °C. After a final

238 centrifugation of 10 min (6 °C, 1000 g), the dried pellet was suspended in 30 µl of de-
239 ionised water. The quality and quantity of the extracted DNA were assayed by
240 spectrophotometric analyses with a Nanodrop ND-1000, and the integrities were verified
241 through electrophoresis on 1% agarose/TBE 0.5x gel. Exon-intron boundary predictions
242 were made by aligning the AChE cDNA sequence cloned in *K. aberrans* with the scaffold
243 form the *M. occidentalis* genome project from which the transcript XR_145413 had been
244 predicted (Genbank accession n. AFFJ01003151.1). Relying on hypothetical gene
245 structure conservation, the primers were designed on the exon sequences to generate
246 partially overlapping PCR fragments that encompassed the putative introns in the *K.*
247 *aberrans* AChE gene. The PCR products were purified and sequenced as described for
248 the cDNA sequencing using the same primers that were employed for the DNA
249 amplifications.

250

251 **3. Results**

252

253 *3.1. Insecticide bioassays*

254

255 Laboratory trials confirmed the findings reported by Tirello et al. (2012). At the discriminant
256 dose, 100% corrected mortalities were observed for the LE, PA and PD strains, which
257 originated from untreated nettles, and high survival rates were observed for the PO, ME,
258 SF and BX strains (4.08%, 9.57%, 7.69% and 4.26% corrected mortalities, respectively),
259 which were collected from commercial vineyards.

260

261 *3.2. T. urticae AChE-like gene in the M. occidentalis genome*

262

263 The tBlastn search on the annotated genome of the predator mite *M. occidentalis* using
264 the AChE cloned from *T. urticae* as the query sequence resulted in two predicted mRNAs
265 that codes for putative AChEs and had sequences that were significant similar to that of
266 the query (XR_145413 and XR_145279; identity 54%, positive 69%, e-value 0.0). These
267 mRNAs originated from genes in partially overlapping contigs (AFFJ01003151 and
268 AFFJ01002402). The corresponding open reading frames differed primarily in the amino
269 terminal due to a diverse prediction of the first splicing site, while they shared the
270 remaining five, which resulted in only 8 mismatches out of the 593 conserved amino acid
271 residues. These mismatches arose from indels in the coding regions of the two genes,

272 which did not differ in the intronic sequences with the exception of the first intron that
273 originated from alternative splicing paths. Because the algorithms used for automatic
274 splicing site predictions often fail to identify splicing sites at the 5' end of putative
275 transcripts and because of the low level of sequence divergence between the two genes, it
276 was unclear whether there were two copies per genome or if they were derived from *in*
277 *silico* mis-assembling of the high-throughput sequencing reads. In any case, when the *M.*
278 *occidentalis* transcriptome shotgun assembly was interrogated with Blastn with the two
279 putative transcripts, a pair of cDNA fragments were retrieved that covered both mRNAs
280 (JL046593.1 and JL050556.1; identities 99% and 98%), which confirmed that they were
281 actually transcribed. Altogether, these findings suggested that the two very similar
282 predicted mRNAs could be informative for cloning *T. urticae*-like AChE cDNA in *K.*
283 *aberrans*.

284

285 3.3. AChE cDNA in the susceptible strains of *K. aberrans*

286

287 cDNA of 2329 was isolated from the susceptible LE strain (Genbank accession number:
288 HF934042). The deduced precursor was composed of 655 amino acids (Fig. 1) with a
289 signal peptide that was predicted to encompass the first 32 amino acids from the amino
290 terminal (Shen et al. 2007). The cloned KaAChE displayed most of the amino acids
291 responsible for the functional integrity of the enzyme that are typically well conserved both
292 in insect and mite AChEs; i.e., the KaAChE residues involved in the intramolecular
293 disulphide bonds (C139, C166, C325, C336, C471, and C593), the catalytic triad (S271,
294 E395, and H509), the anionic subsite (W156), the oxianion-hole (G189, G190, and A274),
295 and the acyl pocket (W304, F360, and F399) (Fig. 1). The highest identity (> 93%) was
296 observed for the AChE that was annotated in *M. occidentalis* from the transcript
297 XR_145413 because the first splicing path was consistent with that predicted in this
298 putative mRNA. No alternative cDNA sequence similar to the *M. occidentalis* transcript
299 XR_145279 was detected in *K. aberrans*. As expected, the greatest divergences in the
300 amino acid sequences between the KaAChE and XR_145413 predicted AChEs were
301 restricted to the amino and carboxy terminals of the protein outside of the functional
302 domains. The amino acid identities with the other cloned and predicted AChEs in the Acari
303 genomes that carry multiple AChE loci ranged from 61% (*Ixodes scapularis* putative AChE,
304 XP_002413212) to 33% (*Rhipicephalus microplus*, AChE3, AAP92139). The amino acid
305 identity was 52% between the AChEs coded by single copy genes in the *T. urticae* and *T.*

306 *evansi* that carry mutations associated with reduced chlorpyrifos sensitivity (GQ461344,
307 ADK12694, and AFS60097) This divergence was compatible with that observed in the
308 AChEs from different species of Acari and even between AChEs from multiple loci in the *I.*
309 *scapularis* or *R. microplus* genomes. AChEs of insects are divided in two groups, i.e.,
310 those orthologous and those paralogous to the *D. melanogaster* AChE (Kim et al., 2012),
311 and KaAChE exhibited a high level of similarity to the paralogous AChEs found in
312 *Nephotettix cincticeps* (Hemiptera: Deltocephalidae) and *Blattella germanica* (Blattodea:
313 Blattellidae) with an amino acid identity of approximately 57% (ADZ15146; ABB89946).

314

315 3.4. Organisation of the clone AChE locus in *K. aberrans*

316

317 The intron-spanning amplifications of the *K. aberrans* AChE locus confirmed the exon-
318 intron junctions that were predicted *in silico* in the *M. occidentalis* genome scaffold
319 AFFJ01003151, which lead to XR_145413 transcript annotation and coding for a putative
320 *M. occidentalis* AChE (MoAChE). However, the first 106 nucleotides of the 5' UTR region
321 of the KaAChE cDNA did not match with any portion of the scaffold sequence
322 AFFJ01003151. In contrast, the unmatched 5'UTR portion of the KaAChE cDNA exhibited
323 an 81% identity with segments of two partially overlapping scaffolds in the *M. occidentalis*
324 genome (Genbank accession n. AFFJ01002403 and AFFJ01002403). The GT-AG
325 consensus rule for donor and acceptor splice sites was also respected using the KaAChE
326 cDNA sequence to guide the joining of the putative and still unannotated 5'UTR portion of
327 MoAChE on the scaffolds AFFJ01002403 and AFFJ01002403 to the 5' end of the
328 remaining open reading frame relying on the AFFJ01003151 scaffold. Because the
329 AFFJ01002403 and AFFJ01002403 scaffolds do not overlap with AFFJ01003151, a long
330 intronic sequence has to be envisaged in the MoAChE locus and is likely excluded from
331 the assembly step. Assuming intron size conservation between the two phytoseiids, this
332 hypothesis was supported by the unsuccessful amplification of this intron in the *K.*
333 *aberrans* AChE locus. Although we were able to characterise 5 introns experimentally and
334 an additional putative splicing site bioinformatically, we suggest that the KaAChE gene
335 includes seven exons (I-VII) that are separated by 6 introns (Table 3, (Genbank accession
336 n. HG328327). Exon I is non-coding, whereas exon II contains the initiation codon (ATG),
337 which is similar to the observations of the majority of the AChE gene loci that have been
338 annotated in insects and mites. Exons III-VI formed the catalytic domain and exhibited
339 partially amino acid conservation across the AChEs that were cloned from the mites. Exon

340 exon seven contains the stop codon (TAG) and the 3' UTR region. All intron-exon
341 boundaries followed the GT-AG rule (Breathnach et al., 1978); furthermore, these
342 boundaries contained the YTNAN consensus sequence for lariat formation at the branch
343 point close to the 5' end of the acceptor-splicing site. In addition to the positions, the
344 lengths of the amplifiable introns were also conserved in the homologous AChE loci from
345 the two phytoseiidae species with the exception of the third intron, which was slightly
346 longer in the *K. aberrans* than in the *M. occidentalis* AChE gene (1162 bp vs. 936 base
347 pairs, respectively). Sequence inspection of the third intron in the *K. aberrans* AChE locus
348 revealed the presence of short microsatellite repeats and a long inverted repeat (LIR)
349 (Wang et al., 2006). These nucleotide motifs can cause sliding of the intron sequences
350 during DNA replication and might account for the different sizes of the third intron in the
351 KaAChE gene.

352

353 3.5. Comparison the AChE cDNA sequences across different strains

354

355 Full-length sequencing of the KaAChE cDNA of the susceptible (LE) and resistant (PO)
356 strains revealed a non-synonymous G to A mutation at position 687 that led to a G191S
357 substitution in the protein sequence (G119S AChE *Torpedo* numbering; Fig. 1). This
358 residue is involved in the oxianion hole, which is one of the functional domains of AChE
359 activity (Zhang et al. 2002). The susceptible and resistant strains also differed in another
360 single nucleotide polymorphism (SNP) at position 1499 of the cloned cDNA; this C to T
361 transition did not affect the codon for the D461 residue. The resistant strain was
362 homozygous at this site and carried only the T allele, while the sensitive strain exhibited
363 both SNPs with a preference for C over T as indicated by the electropherograms. The
364 phenylalanine residue (F339) that was replaced by a tryptophan in the chlorpyrifos-
365 resistant stains of *T. urticae* (F439W mutation, or F331W AChE, *Torpedo* numbering) was
366 still conserved both in the susceptible and resistant strains of *K. aberrans*. The same was
367 true for the glycine residue (G336) that was found to be replaced with alanine (G328A) in
368 the F331W-bearing strains of *T. urticae*. The cDNA KaAChE sequences of two additional
369 susceptible (PA and PD) and three resistant (ME, SF and BX) strains of *K. aberrans* were
370 also examined. The resistant strains were all homozygous for the G191S substitution,
371 while the susceptible strains carried only the G191 allele. The resistant BX strain
372 sequence differed from the other strains in a SNP in the 3' UTR that consisted of a G to A
373 substitution.

374

375 **4. Discussion**

376

377 Resistance to pesticides can be a desirable feature in *K. aberrans* because this predatory
378 mite is an effective biocontrol agent for spider mites in perennial crops. Indeed, strains of
379 this predatory mite that are apparently resistant to OP have successfully been released in
380 vineyards and apple orchards in which the pest control strategies included chlorpyrifos and
381 many other pesticides (Duso et al. 2009; Duso et al., 2012; Ahmad et al., 2013). The
382 resistance to chlorpyrifos of these strains has been definitively demonstrated (Tirello et al.,
383 2012), but the underlying molecular mechanisms remain poorly understood. An initial clue
384 about this issue came from the chlorpyrifos-resistant strain of *T. pyri* that exhibited a lower
385 level of AChE activity than the susceptible strain, which suggests that the reduced
386 substrate affinity observed in the biochemical assay might be associated with a modified
387 AChE (Fitzgerald and Solomon, 1999). In Acari, target site resistance due to a modified
388 AChE that confers high levels of insensitivity to OPs, including chlorpyrifos, has been
389 described in *T. urticae* and *Tetranychus kanzawai* Kishida (Aiki et al., 2004; Van Leeuwen
390 et al. 2010; Khajehali et al., 2010). A G119S substitution (AChE torpedo numbering) in the
391 single copy AChE gene has been associated with the moderate decreases in chlorpyrifos
392 susceptibility between the resistant compared to the sensitive strains of *T. urticae*
393 (resistance ratio at LD₅₀, RR₅₀ = 31), and a greater resistance ratio has been detected in
394 cases of F331W replacement (RR₅₀ > 400). *In vitro* expression of the AChE isoforms of *T.*
395 *urticae* that carry F331W and/or G119S substitutions has revealed a reduction in
396 sensitivity to another organophosphate (monocrotophos) and a decrease in the catalytic
397 efficiency of the enzyme (Kwon et al., 2012); however, no data had been reported for
398 chlorpyrifos. Although these features appeared much more evident in the F331W-mutated
399 AChE, the two substitutions acted synergistically when they were associated *in vitro* and
400 thus were favourably co-selected in the resistant strains *in vivo* (Kwon et al., 2010b; Ilias et

401 al., 2014). In mosquitoes, two acetylcholinesterase genes are present, both substitutions
402 affect the paralogous AChE in the highly OP- and carbamate-resistant strains of *Culex*
403 *pipiens* L. and *Anopheles gambiae* Giles (G119S; Weill et al., 2004a) and in *Culex*
404 *tritaeniorhynchus* Giles (F331W; Alout et al., 2007). The role of these mutations in
405 reducing the AChE sensitivity to OP was confirmed via inhibition analysis of the expression
406 of AChE from mutated mosquito S2 cells (Weill et al., 2003; Oh et al., 2006). The F331W
407 substitution has also been detected in AChE1 of a chlorpyrifos-resistant strain of the sweet
408 potato whitefly *Bemisia tabaci* Gennadius (Alon et al., 2008). The *K. aberrans* strain with
409 the highest level of insensitivity to chlorpyrifos (PO strain) described by Tirello et al. (2012)
410 has a $RR_{50} = 539,602$, and this ratio is even higher than that found in the *T. urticae* and *B.*
411 *tabaci* populations with the F331W AChE genotype. There a target site resistance might
412 be present in that strain. Because no AChE sequences for *K. aberrans* are stored in
413 databases, the annotated genome project of the predatory mite *M. occidentalis* was
414 inspected. More than a dozen AChEs-like sequences were predicted by the curators of the
415 *M. occidentalis* genome project using an automated computational analysis, although
416 some of the transcripts represented uncompleted open reading frames or differed only in
417 their splicing paths. To identify a suitable AChE candidate that is potentially responsible for
418 target site resistance in *K. aberrans*, the AChE protein sequence from *T. urticae* was used
419 to probe probing the annotated genome of *M. occidentalis*. Once a putative homologous
420 AChE in *M. occidentalis* was found, its sequence was used to speed up the cloning of the
421 corresponding AChE cDNA in *K. aberrans*. Full sequencing of the cloned AChE cDNA
422 revealed that the resistant strain (PO) differed from the susceptible strain (LE) in terms of
423 non-synonymous G to A mutation that introduced a G191S substitution in the AChE open
424 reading frame. That mutation corresponds to the aforementioned G119S substitution in
425 AChE Torpedo numbering. Strangely, the corresponding amino acid position in the
426 homologous AChE that was found in the annotated genome of *M. occidentalis* is occupied

427 by a serine. Unfortunately no information is available concerning the chlorpyrifos
428 susceptibilities of the *M. occidentalis* strains employed for the genome project. Notably, the
429 G119 in the *K. aberrans* AChE is encoded by a GGC codon, which could easily be
430 converted to the AGC codon for serine. This substitution in the mosquito paralogous
431 AChE seems not to be neutral under the selective pressure produced by organophosphate
432 and carbamate treatments (Weill et al. 2004b). Indeed, when KaAChE cDNA from an
433 additional two chlorpyrifos-susceptible and three chlorpyrifos-resistant unrelated strains of
434 *K. aberrans*, the G191S substitution was absent only in the resistant strains in the in
435 homozygous condition. The silent nucleotide polymorphisms found in the KaAChE cDNA
436 of the resistant strains might may also suggest that different G119S mutation events
437 occurred independently. Although, in *Culex quinquefasciatus* Say, a chlorpyrifos inhibition
438 study of a paralogous AChE bearing the G119S substitution revealed a reduced sensitivity
439 to the insecticide (Liu et al., 2005) that likely resulted from the reduced accessibility of the
440 catalytic site (Weill et al., 2004a). *In vivo*, *T. urticae* strains with the same mutated AChE
441 genotype display only a moderate resistance to chlorpyrifos. In contrast, all examined
442 resistant strains of *K. aberrans* are highly resistant to the insecticide (Tirello et al., 2012).
443 Thus, the role of the G119S remains unclear, although the possibility that the same
444 mutation has different effects on chlorpyrifos AChE sensitivity in the predatory mite cannot
445 be ruled out. Nevertheless, the non-silent nucleotide polymorphism responsible for the
446 G119S substitution represents a reliable molecular marker that is associated with the
447 detected resistant phenotype. Alternative target site resistance mechanisms not explored
448 in this study might also rely on the amplification of modified AChEs as occurs in *T. urticae*
449 and *T. evansi* (Kwon et al., 2010a; Carvalho et al., 2012) or on mutations that affect
450 multiple AChE loci with additive effects such as has been observed in *R. microplus*
451 (Temeyer et al., 2009, 2010, 2012). In either case, the co-expression of sensitive and
452 insensitive AChEs might contribute to reducing the fitness costs associated with OP

453 resistance (Carvalho et al., 2012, Temeyer et al. 2013a). Alternatively, the overexpression
454 of distinct AChEs from different loci it is thought to result in to bio-scavenging due to the
455 supply of excess targets for xenobiotics, including OP and carbamate insecticides (Lee et
456 al. 2014, in press). While the detection of homozygosity for the G119S substitution argues
457 against the co-existence of duplicated sensitive and insensitive AChEs in chlorpyrifos-
458 resistant strains, the role of multiple AChE loci in *K. aberrans* cannot be not excluded.
459 Intriguingly, the genome of the closely related phytoseiides *M. occidentalis* harbours at
460 least six AChE-like candidates (GenBank accession numbers XP_003743019,
461 XP_003747509, XP_003739584, XP_003738701, XP_003744479, and XP_003745369).
462 Although these putative AChEs have lower amino acid identities (< 34%) to those found in
463 *T. urticae*, they exhibit conserved functional residues for acetylcholinesterase activity (i.e.,
464 the form the catalytic triad and the acetylcholine binding pocket), exhibit conserved amino
465 acid positions, are potentially involved in substitutions that affect AChE sensitivity to
466 organophosphates and carbamates, have and received support from transcriptomics
467 analyses (Hoy et al., 2013). Together, these findings suggest that, in predatory mites,
468 multiple AChEs resemble the composite picture observed in ticks (Temeyer et al. 2013b).
469 Thus, herbivorous and predatory mites can differ not only in detoxification pathways
470 (Mullin et al., 1982; Grbic et al., 2001; Dermauw et al., 2012) but also in AChE repertoires,
471 which potentially offers alternative solutions for the development of target site resistance.
472 However, chlorpyrifos inhibition assays of AChE activity should be performed to support
473 this hypothesis in examined OP resistant strains. Although, the contribution of enhanced
474 detoxifying activities to chlorpyrifos resistance in predatory mites has not yet been reported
475 as it has for other organophosphates (Sato et al., 2001, Fournier et al., 1987; Motoyama et
476 al., 1971; Anber et al., 1988), the use of synergists in bioassays should be combined with
477 detoxification enzyme assays to confirm or deny the involvement of metabolic resistance.

478 In summary, the potential target site resistance to chlorpyrifos in *K. aberrans* has barely
479 been dissected compared to that in *T. urticae*. The F331W substitution that is responsible
480 for AChE that is highly insensitive to chlorpyrifos in Tetranychidae was absent in a putative
481 homologous gene that was cloned from the resistant strain *K. aberrans*. However, a
482 G119S mutation that was detected in the same gene appeared to be associated with the
483 resistant phenotype. Because pesticide treatments strongly affect the success of predatory
484 mites release (Ahmad et al., 2013), this polymorphism might be useful as a molecular
485 marker for tracing the resistant phenotype in ecological studies or in gene pyramiding and
486 marker-assisted selection of desirable traits for multiple insecticide resistance.

487

488 **References**

489 Not included

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Table 1. Strains of *Kampimodromus aberrans* and their origins.

| Strain | Origin |
|--------|---|
| PO | Commercial vineyard at Monteforte d'Alpone (Verona province) |
| SF | Commercial vineyard at S. Pietro Incariano (Verona province) |
| BX | Commercial vineyard at Soave (Verona province) |
| ME | Commercial vineyard at Valdobbiadene (Treviso province) |
| LE | Untreated European nettle trees (<i>Celtis australi</i> L.) at Legnaro (Padova province) |
| PD | Untreated European nettle trees (<i>Celtis australi</i> L.) at Padova |
| PA | Untreated European nettle trees (<i>Celtis australi</i> L.) at Paese (Treviso province) |

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Table 2. Primer sequences

| | Forward primer | Sequence 5' → 3' | Reverse primer | Sequence 5' → 3' |
|---------------------------|---|---|---|---|
| <i>Degenerate primers</i> | KaAChEF1d KaAChEF2d | GGNATHCCNTAYGCNAARCC CCNTAYGCZAARCCNCC | KaAChER1d KaAChER2d | RAANSWNCCNCCRTANACCCA RAARCTNCCNCCRTANACCCA |
| <i>3' cDNA extension</i> | KaAChEF3 | TGGAATGCCAACACTAATA | KaAChER3 | AGGAAGTTCTTCCAAAACGCGCA |
| <i>3' RACE</i> | | | oligdTadapter | ACAGCAGGTCAGTCAAGCAGTAGC AGCAGTTCGATAAGCGGCCGCAT GGAT ₁₂ DN |
| <i>5' RACE</i> | KaAChEF4 KaAChEF5 | AGGTGGTGAACGAAGCCATCAT TTGAGTACACGGACTGGTTGAACCCT | Adapter1 Adapter2 | ACAGCAGGTC AAGTCAAG AGCAGTAGCAGCAGTTCGATA |
| | TS-Primer | CACCATCGATGTCGACACGCGTCGGG IGGIG | KaAChER4 KaAChER5 | CTCCGTA AACCCATAGAAGGA CCACATTGTTGAACCCTCAAAGTCG |
| <i>cDNA sequencing</i> | TS-PCR | CATCGATGTCGACACGCGTC | KaAChER6 | TGTTGAACCCTCAAAGTCGTCG |
| | KaAChEF6 KaAChEF7 KaAChEF8 KaAChEF9 KaAChEF10 | ACCTTGATAAACTGTCGCTGTGGC CAATGCAGGCATGATGGACCAAGT GTTGAACCCTGACGATCCGATCAA AAATGCGATTTTCGACATCCTGTGCC GCGCTATCGGGCAACAACACAAACA | KaAChER7 KaAChER8 KaAChER9 KaAChER10 | AAAGTCACGTTGTTTCGGGTTGCCT AGGAAATTCTTCCAGAATGCGCA TCCTAGTTCGCTCCTTCAGTTGGA CCAACAATCTTGTGCGACGGCATCT |
| <i>G119S screening</i> | KaAChEF11 | AATGCGATTTTCGACATCCTGTGCC | KaAChER11 | AAAGTCCGTTGTTTCGGGTTGCC |
| <i>F331W screening</i> | KaAChEF12 | AAACTCGCGGAGGAAGTCAAGTGT | KaAChER12 | CCAACAATCTTGTGCGACGGCATCT |

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Table 3. Genome organisation of the *K. aberrans* ace locus

| Exon | Position (nt) ^a | Exon size (bp) | Splice junction | Intron size (bp) | phase | amino acid involved |
|-------------------|----------------------------|----------------|---|------------------|-------|---------------------|
| Exon I (inferred) | (-106) - (-11) | 106 | TCGGAGGACTGGgt ... | unknown | - | 5' UTR |
| Exon II (ATG) | (-10) - 310 | 320 | agATGATTGGATAT ... ACGCATTCCCTCGgt ... | 113 | I | G104 |
| Exon III | 311 - 876 | 566 | agGTATTCCGTATG ... CTATTTTCTCAAgt ... | 1162 | 0 | Q292 |
| Exon IV | 877 - 1302 | 426 | agGCCGTGCTGCAA ... TTTACCTCGCAGgt ... | 194 | 0 | Q434 |
| Exon V | 1303 - 1494 | 192 | agGTGGTGAACGAA ... CGCTCGTCTCAGgt ... | 402 | 0 | Q498 |
| Exon VI | 1495 - 1822 | 328 | agAATAAATGGCCG ... TCGCGCTATCGGgt ... | 145 | I | G608 |
| Exon VII (TAA) | 1823 - 2213 | 391 | agGCAACAACACAA ... | | | 3'UTR |

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The numbering of nucleotides is based on the *K. aberrans* AChE cDNA in which +1 corresponds to start codon.

| | | | |
|----|--------|---|-----|
| 1 | KaAChE | MRLGVSGGASGLAK-LALCRSMAATVRSLSLTRA\LF AFLIHLWLVVTL CVGRVDARAAHL | 58 |
| 2 | MoAChE | MWLGVS GGASGLAKCKLALCRSMATILRSMSTRTLFAFLINTWLVVTL CVGRVDARAAHL | 60 |
| 3 | | * *****: :*:***:*****: ***** | |
| 4 | | ▽ | |
| 5 | KaAChE | LHHHRHRTAGSAQS QGDPLL VHTTKGPV RGITLQASNGKLVDAFLGIPYAKPPV GKMRFR | 118 |
| 6 | MoAChE | LHHHRHRTAGNAQS QGDPLV VHTTKGPV RGITLQASNGKLVDAFLGIPYAKPPV GKMRFR | 120 |
| 7 | | *****.*****:*****:*****:*****:*****:***** | |
| 8 | | 1 + 1 | |
| 9 | KaAChE | HPVPM DPWEKPLNVTERPATCVQVVDTYFDDFEGSTMWNANTNMS EDCLNMLVWVPRPRP | 178 |
| 10 | MoAChE | HPVPM DPWEKPLNVTEPPATCVQVVDTYFDDFEGSTMWNANTNMS EDCLNMLVWVPRPRP | 180 |
| 11 | | ***** ***** | |
| 12 | | ++ | |
| 13 | KaAChE | TNAAVLLWVYGG G FYSGCATLDVYDGKILASEENVIVVSFN YRVGSLGFLYLDHADAPGN | 238 |
| 14 | MoAChE | TNAAVLLWVYGG S FYSGCATLDVYDGKILASEENVIVVSFN YRVGSLGFLYLDHADAPGN | 240 |
| 15 | | *****.***** | |
| 16 | | + + | |
| 17 | KaAChE | AGMMDQVMALRWVQDNIHLFGGNPNNVTLFGES S AGAVSVAYHLLSPLSRDLFSQAVLQSG | 298 |
| 18 | MoAChE | AGMMDQVMALRWVQDNIHLFGGNPNNVTLFGES S AGAVSVAYHLLSPLSRDLFSQAVLQSG | 300 |
| 19 | | ***** | |
| 20 | | + 2 2 | |
| 21 | KaAChE | GATVPWGYNERQTAMTNGYKLAEEVKCPTDDVEATVKCLRLQDPDLLVKSEIFATGVVDF | 358 |
| 22 | MoAChE | GATVPWGYNERQTAITNGYKLAEEVKCPTDDVEATIKCLRLQDPDLLVKSEIFATGVVDF | 360 |
| 23 | | *****.*****.***** | |
| 24 | | + + | |
| 25 | KaAChE | SFVPVVDGAFILTERPEDTMNSGNFKKCKILLGNSNRD E GTYFIIYYLTQLFKRDENVYLTR | 418 |
| 26 | MoAChE | SFIPVVDGAFILTERPEDSMSSGNFKKCKILLGNSNRDEGTYFIIYYLTQLFKRDENVYLTR | 420 |
| 27 | | ** :*****:*.***** | |
| 28 | | ▽ | |
| 29 | KaAChE | EDFVDAVQALSPFTSQVVNEAII FEYTDWLNPD DPKNRDAVDKIVGDYFTCPVIDTAH | 478 |
| 30 | MoAChE | EDFVDAVQALSPFTSSVVNEAII FEYTDWLNPD DPKNRDAVDKIVGDYFTCPVIDMAH | 480 |
| 31 | | *****.***** ** | |
| 32 | | ▽ | |
| 33 | KaAChE | YYSSAGLDVYMYYYVYRSSQNKWPEW MGVI H ADEIAYVFGEPLNQ TWSYRQDEQMFSRRI | 538 |
| 34 | MoAChE | YYFISGLDVYMYYYVYRSSQNKWPEW MGVI H ADEIAYVFGEPLNQ TWSYRQDEQMFSRRI | 540 |
| 35 | | ** :***** | |
| 36 | | 3 | |
| 37 | KaAChE | MRYWANFARMGNPSLNP DGNWEKTYWPAHTAFGKEFLILDVNSTQVGYGNRAKHCAFWKN | 598 |
| 38 | MoAChE | MRYWANFARMGNPSLNP DGNWEKTYWPAHTAFGKEFLILDVNSTQVGYGNRAKHCAFWKN | 600 |
| 39 | | ***** | |
| 40 | | ▽ + | |
| 41 | KaAChE | FLPNLIALSGNNTNKAEEGCRDGASSQSSS IMLLCSLAASIVVTGRILSQPPATAA | 655 |
| 42 | MoAChE | FLPNLIALSGNNTNKADESKDGASTQSSS LTLCSLAASMIVTGRLLSSSTARAA | 657 |
| 43 | | *****:*.***:*****: *****: :*****:***.*** ** | |
| 44 | | | |

Fig. 1 Alignment of the AChEs cloned in from the chlorpyrifos -susceptible strain of *Kampimodromus aberrans* (KaAChE) and predicted from *Metaseiulus occidentalis* transcript XR_145413 (MoAChE). Identical amino acids are indicated by asterisks, and conservative substitutions are indicated by dots. The cleavage site of signal peptide is indicated by a slash. The mutated residue (G191S) in the chlopyrifos-resistant strain is in reverse in the background, the cysteine residues that form the intramolecular disulphide bonds are numbered and on the light-gray background (C139-C166, C325-C336, C471-C593), the catalytic triad residues are boxed (S271, E395, H509), and the following conserved residues are indicated with plus signs: anionic subsite (W156), oxianion-hole (G189, G190, A274), acyl pocket (W304, F360, F399), and cysteine residue forming

- 1 intermolecular disulphide bond (C634). The inverted triangles indicates the exon
- 2 boundaries in the cDNA open reading frame.