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Significance and interpretation of molecular diagnostics for insecticide resistance management of agricultural pests

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Abstract:	Insecticide resistant pests become increasingly difficult to control in current day agriculture. Due to environmental and health concerns, the insecticide portfolio to combat agricultural pests is gradually decreasing. It is therefore crucial to make rational decisions on insecticide use to assure effective resistance management. However, resistance monitoring programs that inform on pest susceptibility and resistance are not yet common practice in agriculture. Molecular markers of resistance that are turned into convenient diagnostic tools are urgently needed and will only increase in importance. This review investigates which factors determine the strength, diagnostic value and success of a diagnostic marker, and in which cases recent technical advances might provide new opportunities for decision making in an operational meaningful way.
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Manuscript

1 # Significance and interpretation of molecular diagnostics for insecticide
2 resistance management of agricultural pests

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Abstract

Insecticide resistant pests become increasingly difficult to control in current day agriculture. Due to environmental and health concerns, the insecticide portfolio to combat agricultural pests is gradually decreasing. It is therefore crucial to make rational decisions on insecticide use to assure effective resistance management. However, resistance monitoring programs that inform on pest susceptibility and resistance are not yet common practice in agriculture. Molecular markers of resistance that are turned into convenient diagnostic tools are urgently needed and will only increase in importance. This review investigates which factors determine the strength, diagnostic value and success of a diagnostic marker, and in which cases recent technical advances might provide new opportunities for decision making in an operational meaningful way.

51 **Introduction**

52 The control of pests that attack our crops is one of the major challenges and costs in agriculture today, as
53 production losses due to arthropod pests continue to grow and reach up to 20% of the total crop yield
54 [1,2]. The development of insecticide resistance becomes an increasingly important problem: more than
55 500 insect and mite species are now resistant to at least some of insecticides used for their control [3,4].
56 In addition, the availability of new crop protection chemistry becomes more and more challenging due to
57 the increasing costs for discovery, development and registration, in part driven by public concerns on
58 environmental safety and human health [5-9]. This probably results in company decisions not to develop
59 new chemistry if projected revenues are minor, for example when compounds target only specific pests
60 in minor crops.

61 Giving the rising resistance problem and pressure on pesticide portfolio, it is very important to make
62 rational decisions on insecticide use [10]. While a few key mosquito species are controlled by a limited
63 number of insecticide classes, and exposure is mainly via residual contact of sprays and treated bed nets
64 [11], the options in agriculture are more complex. Several classes of insecticides with different modes of
65 action and different arthropod exposure routes (direct or residual contact, ingestion by chewing, sucking,
66 rasping …) are applied in multitude in different cropping systems, giving more options for pesticide choice
67 and insecticide resistance management (IRM).

68 Diagnostic tools that monitor susceptibility in pest populations could play a crucial role in the choice of
69 chemicals, as they allow to manage or avoid incidence and spread of resistance. Diagnostic bioassays have
70 since long been developed for several agricultural pests and disease vectors. While extensive monitoring
71 programs are an integral component of mosquito control programs [12], this is much less the case for the
72 numerous agricultural pests on the diversity of crops. Crop pest monitoring programs seem limited to
73 some of the key pests in major crops like corn, soybean, rice and cotton, yet primarily as a result of
74 spontaneous research programs or industry driven activities for managing and launching their new
75 products, but more rarely as systematic country level activities [13,14].

76 Bioassays are often employed for resistance monitoring [10], however their feasibility in high throughput
77 depends on whether insects can be easily collected, stored, and grown in the lab, and equally, whether
78 the host plant is easily cultivated in the lab or artificial diets for pests are available. For example, resistance
79 screens for Bt toxins in key lepidopteran pests have been largely profited from the ability to mix these
80 toxins with artificial diets [15].

81 As increasing number of molecular markers for resistance are being identified, high throughput fast and
82 accurate molecular diagnostic platforms could be used to overcome the need for time-consuming
83 bioassays. However, using this data in making decisions on insecticide use is the next challenge, because
84 of the potential limited predictive value of the markers and/or lack of clearly established links with
85 operational impact.

86 This review investigates to what extent, and in which cases, molecular diagnostics can be reliably used to
87 manage resistance and inform decisions on insecticide use in time and space in the field. As this subject
88 has been recently reviewed for vectors of human disease [11,16], we focus here on agricultural pests.

89 **Resistance mechanisms and molecular markers**

90 The development of resistance is an evolutionary phenomenon of which the mechanisms are most often
91 described in terms of toxicodynamic and toxicokinetic changes in the physiology and biochemistry of
92 resistant strains. This includes changes in penetration, activation, metabolism, transport and excretion for
93 toxicokinetic mechanisms (any changes that alters the amount of toxin that reaches the target-site), and
94 changes to the pesticide target-site (structural changes, knock-out, amplification) for toxicodynamic
95 mechanisms [17,18]. Although this physiological classification is useful in describing the resistance
96 phenotype that results from genetic changes, and sometimes allows specific field interventions such as
97 the use of synergists in metabolic resistance, the actual type of genetic change (mutation) is more relevant
98 to precisely understand the evolution and spread of resistance genes in populations. In addition, it largely
99 determines whether accurate and sensitive molecular diagnostic markers can be feasibly developed. For
100 example, a simple point mutation in a target-site is much more easily turned into a DNA-based marker
101 than increased expression of a metabolic resistance gene. In the latter case, it is much more likely to
102 develop a diagnostic marker based on RNA or protein abundance, than a marker based on the actual
103 mutation, as *cis* and especially *trans* acting mutations regulating gene expression have remained elusive
104 for most cases of metabolic resistance in most pests. A few studies in mosquitoes are now providing DNA
105 markers for metabolic resistance [19,20], while recently developed mapping tools, such as NGS-based
106 bulked segregant analysis [21], might facilitate the identification of QTL markers for major agricultural
107 pests [22-24].

108 **Factors affecting the strength and diagnostic value of a molecular marker**

109 One of the issues to consider when developing a diagnostic marker, is the breath of its geographical
110 applicability. As outlined above, pests can develop resistance by multiple mechanisms, and whether

111 different populations of a certain species develop resistance with similar mechanisms is not always clear.
112 For some target-site resistance cases, it is known that similar, if not identical mutations evolve in different
113 populations of the same species, and even among species. For example, resistance to pyrethroids has been
114 associated with kdr and super kdr mutations at domain II of the voltage-gated sodium channel in at least
115 50 different arthropod species [18]. More recent examples include the G4946E mutation in the ryanodine
116 receptor, conferring resistance against diamides, which has been reported in four different lepidopteran
117 species, including *Plutella xylostella* populations spread across 3 continents [25-28], while alterations in
118 the ABCC2 or ABCC3 gene, strongly associated with Cry1-toxin resistance, have been identified in seven
119 different lepidopteran species [29]. Furthermore, mutations at identical position in *chitin synthase 1*
120 (*chs1)*, conferring resistance against benzoylureas, buprofezin and etoxazole, have been reported in three
121 different arthropod species, both insects and mites (*F. occidentalis*, *P. xylostella* and the spider mite
122 *Tetranychus urticae*) [30-32]. *T. urticae* is one of the rare examples where the frequency of a whole panel
123 of different target-site mutations has been investigated worldwide (Table 1 and e.g. [33]), revealing the
124 presence of identical mutations often across continents. The global presence of these and other target-
125 site mutations might be related to functional constraints in pesticide targets, which have been suggested
126 to be considerably high, probably promoting the success of a few amino acid substitution that are
127 constraint-free [34]. Nevertheless, even if conserved target-site mutations are present in populations in
128 broad geographical context, their relative importance in the resistant phenotype needs to be sufficiently
129 high to reliably predict resistance and serve as diagnostic marker. For *T. urticae*, the phenotypic strength
130 of the most common mutations has been determined by repeated back-crossing and marker assisted
131 selection, which is feasible for this species with short generation time. This revealed that in most cases,
132 the presence of the mutation explained the larger part, if not the complete phenotype, suggesting that
133 target-site mutations are a very good predictor of resistance levels in this species [35-37]. In addition, the
134 dominance and fitness cost of certain resistance mutations was determined [31,36,38,39], which further
135 increases the value of a certain molecular marker for IRM [38,40-42]. As introgression of a marker is not
136 feasible for most insect species (however, see [43,44] for exceptions), gene editing in *Drosophila* and/or
137 pest species have also been a very useful tool for validating and measuring the role and effect of certain
138 mutations in resistance against insecticides [30,45-53].

139 The interpretation of metabolic resistance in the context of developing molecular markers is even more
140 complex. This is especially true for many serious pests that are polyphagous. It was previously shown that
141 similar gene-expression responses evolved after both the development of pesticide resistance as
142 adaptation to a new host [54,55]. The ‘pre-adaptation syndrome’, as discussed by Dermauw and

143 colleagues [54], confounds the potential interpretation of some of the key players in metabolic resistance,
144 as candidate metabolic resistance genes might be overexpressed both in relation to pesticide
145 detoxification as well as upon host plant exposure. In addition, although recombinant expression flowed
146 by metabolism assays, reverse genetics by RNAi, or ectopic overexpression have provided different levels
147 of validation for the involvement of detoxification genes in the resistance phenotype, finding appropriate
148 markers has been even more challenging. The complexity of metabolic resistance is also determined by
149 the target marker: while in some insects, such as the pollen beetle *Meligethes aeneus* a single P450
150 (CYP6BQ23) seems to be primarily responsible for pyrethroid resistance [56] indicating a single
151 RNA/protein marker, in others, such as *Helicoverpa armigera* several members of the lepidopteran-
152 specific CYP6AE subfamily can metabolize esfenvalerate [57], moving the target marker at the P450
153 subfamily level. Nevertheless, successful diagnostic assays for P450 based resistance have been developed
154 in some cases, such as the polyphagous white fly *Bemisia tabaci* ([58], Figure 1A), which clearly indicates
155 that this needs to be evaluated case by case.

156 A relevant question for the strength of a marker is also: in how many cases the resistance is caused by the
157 mechanisms under investigation (alone). We need to recognize that the resistance is often polygenic and
158 consists (in many instances) of “major” genes and “minor” genes, and potentially different evolutionary
159 solutions have been selected in different populations. The predictive value of a marker can therefore only
160 be validated in combinations with bioassays in a certain geographical region at a certain time. A validated
161 molecular marker can be subsequently used alone for resistance monitoring but should be used in
162 conjunction with bioassays at certain time intervals, in case new mechanisms evolve.

163 **Methods in molecular diagnostics**

164 The majority of molecular diagnostics used for monitoring insecticide resistance in agricultural pests
165 (described in Table 2 [16,59-64]) are based on nucleic acid detection (DNA and/or RNA). Simple/low-tech
166 versions of PCR- (AS-PCR, PCR-RFLP) and isothermal LAMP are used to detect the presence of known
167 mutations or differentially expressed genes (targeted analysis). In these cases, mutant allelic frequency
168 (MAF) is calculated through screening of several individuals. Sequencing based methods (Sanger,
169 pyrosequencing, next generation sequencing) also allow for the unbiased analysis of the whole genes or
170 transcriptomes revealing potential new SNPs. Improved/High-tech versions of PCR-based methods
171 (rtPASA/SYBR Green qPCR, TaqMan qPCR, ddPCR, lyophilized pellets, LabDisk) and sequencing (Nanopore,
172 NGS transcriptome analysis) allow quantification of MAF within the analyzed sample; thus, samples or
173 populations can be pooled beforehand. More importantly, the exact same technologies can be used for

174 assessing gene expression levels at the RNA level in the same samples used for target-site mutation
175 quantification and thus yield important information regarding metabolic resistance [65,66].

176 At the protein level, most technologies have been developed to monitor metabolic resistance, with the
177 exception of few target enzyme assays. This is achieved either by assessing the enzymatic activity
178 (cytochrome P450 monooxygenases, glutathione-S-transferases, carboxyl/choline esterases) via general
179 or more specific substrates, or the quantification of protein expression levels via specific antibodies [67].

180 Today, target-site mutations are usually assayed by Sanger sequencing for small sample sizes and TaqMan
181 qPCR for higher throughput needs in which case the cost per sample drops significantly. Metabolic
182 resistance is most frequently determined at the mRNA level by singleplex SYBR Green RT-qPCR at relatively
183 low cost and high throughput. Finally, in situations where large sample screening is required for known
184 resistance mutations, including searching for low frequency/rare mutations, Droplet Digital PCR (ddPCR)
185 could be a valuable tool [66]. It can be used to assess MAF in bulk samples with a detection limit of at least
186 1 mutated individual in a pool of 1000. The same pooled sample can also be used quantify the number of
187 metabolic gene transcripts with very high accuracy, when working with RNA/cDNA templates. Current
188 ddPCR cost may be too high, but prices are expected to drop for already available and new platforms.

189 **Conclusions and future perspectives**

190 Due to concerns on environmental safety and human health, the portfolio of synthetic insecticides is
191 gradually diminishing. To prevail the efficacy of current and future insecticides, the development and
192 application of molecular markers for evidence based IRM will become more crucial. Although resistance
193 monitoring is not common practice yet, this will surely change when the efficacy of a particular insecticide
194 becomes even more crucial in a context where alternative crop protection strategies will rely on a 'last
195 resort' chemical intervention. Robust molecular markers are of great value for IRM. However, in many
196 cases, such strong markers are not available/known and more correlation studies between resistance and
197 molecular markers alone or in combination across geographical regions should be performed, to validate
198 the strength and value of a marker in place and time. Furthermore, while in the past the development of
199 molecular markers was focused on functional markers (e.g. target-site resistance mutation), hypothesis-
200 free approaches (e.g. QTL mapping) and third generation sequencing technologies might generate markers
201 regardless of underlying mechanisms. This will become more and more feasible with the advent of high-
202 quality genome sequences for many if not most pests. Last, distribution of marker-based resistance
203 information in an operationally meaningful way, is challenging but will remain crucial. The development

204 of modern interactive databases and ICT platforms that support such decision making, need to be further
205 developed and implemented.

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210 References

211 1. Oerke EC: **Crop losses to pests**. *The Journal of Agricultural Science* 2006, **144**:31-43.
212 2. Culliney TW: **Crop Losses to Arthropods**. In *Integrated Pest Management: Pesticide Problems, Vol.3*.
213 Edited by Pimentel D, Peshin R: Springer Netherlands; 2014:201-225.
214 3. Mota-Sanchez D, Wise JC: **Arthropod Pesticide Resistance Database. Michigan State University. On-**
215 **line at: http://www.pesticideresistance.org**. Edited by; 2019.
216 4. Gould F, Brown ZS, Kuzma J: **Wicked evolution: Can we address the sociobiological dilemma of**
217 **pesticide resistance?** *Science* 2018, **360**:728-732.
218 5. Isman MB: **Challenges of Pest Management in the 21st Century: New Tools and Strategies to Combat**
219 **Old and New Foes Alike**. *Frontiers in Agronomy* 2019:doi: 10.3389/fagro.2019.00002
220 6. Hillocks RJ: **Farming with fewer pesticides: EU pesticide review and resulting challenges for UK**
221 **agriculture**. *Crop Protection* 2012, **31**:85-93.
222 7. Keulemans W, Bylemans D, Deconinck B: *Farming without plant protection products. Can we grow*
223 *without using herbicides,fungicides and insecticides?* Brussels, European Union: Panel for the
224 Future of Science and Technology (STOA); 2019.
225 8. EEA: **Supplementary information to Priority Objective 3 of the Seventh Environment Action**
226 **Programme - Pesticide sales**. In *Environmental Indicator Report 2018; In Support to the Monitoring*
227 *of the Seventh Environment Action Programme. Vol. 19/2018*. Edited by: European Environment
228 Agency (EAA); 2018:67-78, Available online at:
229 https://www.eea.europa.eu/airs/2018/environment-and-health/pesticides-sales.
230 9. Sparks TC: **Insecticide discovery: An evaluation and analysis**. *Pesticide Biochemistry and Physiology*
231 2013, **107**:8-17.
232 10. Network RP: **Trends and Challenges in Pesticide Resistance Detection**. *Trends in Plant Science* 2016,
233 **21**:834-853.
234 11. Donnelly MJ, Isaacs AT, Weetman D: **Identification, Validation, and Application of Molecular**
235 **Diagnostics for Insecticide Resistance in Malaria Vectors**. *Trends in Parasitology* 2016, **32**:197-
236 206.
237 12. WHO: *Global plan for insecticide resistance management in malaria vectors (GPIRM)*. Geneva,
238 Switzerland: WHO Press; 2012.
239 13. Castañera P, Farinós GP, Ortego F, Andow DA: **Sixteen Years of Bt Maize in the EU Hotspot: Why Has**
240 **Resistance Not Evolved?** *PLOS ONE* 2016, **11**:e0154200.
241 14. Dennehy TJ, DeGain BA, Harpold VS, Brown JK, Morin S, Fabrick JA, Byrne FJ, Nichols RL: *New Challenges*
242 *to Management of Whitefly Resistance to Insecticides in Arizona*: College of Agriculture and Life
243 Sciences, University of Arizona (Tucson, AZ); 2005.

244 15. Ludwick DC, Meihls LN, Huynh MP, Pereira AE, French BW, Coudron TA, Hibbard BE: **A new artificial diet for western corn rootworm larvae is compatible with and detects resistance to all current Bt toxins**. *Scientific Reports* 2018, **8**:5379.
247 16. Vontas J, Mavridis K: **Vector population monitoring tools for insecticide resistance management: Myth or fact?** *Pesticide Biochemistry and Physiology* 2019, **161**:54-60.
249 17. Kennedy C, Tierney K: **Xenobiotic Protection/Resistance Mechanisms in Organisms**. In *Environmental Toxicology*. Edited by Laws EA: Springer 2013:689-721.
251 18. Feyereisen R, Dermauw W, Van Leeuwen T: **Genotype to phenotype, the molecular and physiological dimensions of resistance in arthropods**. *Pesticide Biochemistry and Physiology* 2015, **121**:61-77.
253 19. Mugenzi LMJ, Menze BD, Tchouakui M, Wondji MJ, Irving H, Tchoupo M, Hearn J, Weedall GD, Riveron JM, Wondji CS: **Cis-regulatory CYP6P9b P450 variants associated with loss of insecticide-treated bed net efficacy against *Anopheles funestus***. *Nature Communications* 2019, **10**:4652.
256 20. Weedall GD, Mugenzi LMJ, Menze BD, Tchouakui M, Ibrahim SS, Amvongo-Adjia N, Irving H, Wondji MJ, Tchoupo M, Djouaka R, et al.: **A cytochrome P450 allele confers pyrethroid resistance on a major African malaria vector, reducing insecticide-treated bednet efficacy**. *Science Translational Medicine* 2019, **11**:eaat7386.
260 21. Kurlovs AH, Snoeck S, Kosterlitz O, Van Leeuwen T, Clark RM: **Trait mapping in diverse arthropods by bulked segregant analysis**. *Current opinion in insect science* 2019, **36**:57-65.
262 22. Snoeck S, Kurlovs AH, Bajda S, Feyereisen R, Greenhalgh R, Villacis-Perez E, Kosterlitz O, Dermauw W, Clark RM, Van Leeuwen T: **High-resolution QTL mapping in *Tetranychus urticae* reveals acaricide-specific responses and common target-site resistance after selection by different METI-I acaricides**. *Insect Biochemistry and Molecular Biology* 2019, **110**:19-33.
266 23. Wybouw N, Kosterlitz O, Kurlovs AH, Bajda S, Greenhalgh R, Snoeck S, Bui H, Bryon A, Dermauw W, Van Leeuwen T, et al.: **Long-Term Population Studies Uncover the Genome Structure and Genetic Basis of Xenobiotic and Host Plant Adaptation in the Herbivore *Tetranychus urticae***. *Genetics* 2019, **211**:1409-1427.
270 24. Park Y, González-Martínez RM, Navarro-Cerrillo G, Chakroun M, Kim Y, Ziarsolo P, Blanca J, Cañizares J, Ferré J, Herrero S: **ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis**. *BMC Biology* 2014, **12**:46.
273 25. Boaventura D, Bolzan A, Padovez FE, Okuma DM, Omoto C, Nauen R: **Detection of a ryanodine receptor target-site mutation in diamide insecticide resistant fall armyworm, Spodoptera frugiperda**. *Pest Management Science* 2019:doi: 10.1002/ps.5505.
276 26. Troczka BJ, Williamson MS, Field LM, Davies TGE: **Rapid selection for resistance to diamide insecticides in *Plutella xylostella* via specific amino acid polymorphisms in the ryanodine receptor**. *NeuroToxicology* 2017, **60**:224-233.
279 27. Steinbach D, Gutbrod O, Lümmen P, Matthiesen S, Schorn C, Nauen R: **Geographic spread, genetics and functional characteristics of ryanodine receptor based target-site resistance to diamide insecticides in diamondback moth, *Plutella xylostella***. *Insect Biochemistry and Molecular Biology* 2015, **63**:14-22.
283 28. Roditakis E, Steinbach D, Moritz G, Vasakis E, Stavrakaki M, Ilias A, García-Vidal L, Martínez-Aguirre MdR, Bielza P, Morou E, et al.: **Ryanodine receptor point mutations confer diamide insecticide resistance in tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae)**. *Insect Biochemistry and Molecular Biology* 2017, **80**:11-20.
287 29. Wu C, Chakrabarty S, Jin M, Liu K, Xiao Y: **Insect ATP-Binding Cassette (ABC) Transporters: Roles in Xenobiotic Detoxification and Bt Insecticidal Activity**. *International Journal of Molecular Sciences* 2019, **20**:2829.
290 30. Douris V, Steinbach D, Panteleri R, Livadaras I, Pickett JA, Van Leeuwen T, Nauen R, Vontas J: **Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of**

292 **action on chitin biosynthesis**. *Proceedings of the National Academy of Sciences of the United*
293 *States of America* 2016, **113**:14692-14697.
294 31. Van Leeuwen T, Demaeght P, Osborne EJ, Dermauw W, Gohlke S, Nauen R, Grbic M, Tirry L,
295 Merzendorfer H, Clark RM: **Population bulk segregant mapping uncovers resistance mutations**
296 **and the mode of action of a chitin synthesis inhibitor in arthropods**. *Proceedings of the National*
297 *Academy of Sciences of the United States of America* 2012, **109**:4407-4412.
298 32. Suzuki Y, Shiotsuki T, Jouraku A, Miura K, Minakuchi C: **Benzoylurea resistance in western flower thrips**
299 ***Frankliniella occidentalis*** **(Thysanoptera: Thripidae): the presence of a point mutation in chitin**
300 **synthase 1**. *Journal of pesticide science* 2017, **42**:93-96.
301 33. Ilias A, Vontas J, Tsagkarakou A: **Global distribution and origin of target site insecticide resistance**
302 **mutations in *Tetranychus urticae***. *Insect Biochemistry and Molecular Biology* 2014, **48**:17-28.
303 34. ffrench-Constant RH: **The molecular genetics of insecticide resistance**. *Genetics* 2013, **194**:807-815.
304 35. Riga M, Bajda S, Themistokleous C, Papadaki S, Palzewicz M, Dermauw W, Vontas J, Leeuwen TV: **The**
305 **relative contribution of target-site mutations in complex acaricide resistant phenotypes as**
306 **assessed by marker assisted backcrossing in *Tetranychus urticae***. *Scientific Reports* 2017, **7**:9202.
307 36. Fotoukkiaii SM, Tan Z, Xue W, Wybouw N, Van Leeuwen T: **Identification and characterization of new**
308 **mutations in mitochondrial cytochrome b that confer resistance to bifenazate and acequinocyl**
309 **in the spider mite *Tetranychus urticae***. *Pest Management Science* 2019:doi: 10.1002/ps.5628.
310 37. Bajda S, Dermauw W, Panteleri R, Sugimoto N, Douris V, Tirry L, Osakabe M, Vontas J, Van Leeuwen T:
311 **A mutation in the PSST homologue of complex I (NADH:ubiquinone oxidoreductase) from**
312 ***Tetranychus urticae*** **is associated with resistance to METI acaricides**. *Insect Biochemistry and*
313 *Molecular Biology* 2017, **80**:79-90.
314 38. Bajda S, Riga M, Wybouw N, Papadaki S, Ouranou E, Fotoukkiaii SM, Vontas J, Van Leeuwen T: **Fitness**
315 **costs of key point mutations that underlie acaricide target-site resistance in the two-spotted**
316 **spider mite *Tetranychus urticae***. *Evolutionary applications* 2018, **11**:1540-1553.
317 39. Van Leeuwen T, Vanholme B, Van Pottelberge S, Van Nieuwenhuyse P, Nauen R, Tirry L, Denholm I:
318 **Mitochondrial heteroplasmy and the evolution of insecticide resistance: Non-Mendelian**
319 **inheritance in action**. *Proceedings of the National Academy of Sciences* 2008, **105**:5980-5985.
320 40. ffrench-Constant RH, Bass C: **Does resistance really carry a fitness cost?** *Current Opinion in Insect*
321 *Science* 2017, **21**:39-46.
322 41. Bourguet D, Genissel A, Raymond M: **Insecticide Resistance and Dominance Levels**. *Journal of*
323 *Economic Entomology* 2000, **93**:1588-1595.
324 42. Jin L, Wang J, Guan F, Zhang J, Yu S, Liu S, Xue Y, Li L, Wu S, Wang X, et al.: **Dominant point mutation**
325 **in a tetraspanin gene associated with field-evolved resistance of cotton bollworm to transgenic**
326 **Bt cotton**. *Proceedings of the National Academy of Sciences* 2018, **115**:11760-11765.
327 43. Zuo Y-Y, Ma H-H, Lu W-J, Wang X-L, Wu S-W, Nauen R, Wu Y-D, Yang Y-H: **Identification of the**
328 **ryanodine receptor mutation I4743M and its contribution to diamide insecticide resistance in**
329 ***Spodoptera exigua*** **(Lepidoptera: Noctuidae)**. *Insect Science* 2019:doi: 10.1111/1744-
330 7917.12695.
331 44. Yang YH, Yang YJ, Gao WY, Guo JJ, Wu YH, Wu YD: **Introgression of a disrupted cadherin gene enables**
332 **susceptible *Helicoverpa armigera* to obtain resistance to *Bacillus thuringiensis* toxin Cry1Ac**.
333 *Bulletin of Entomological Research* 2009, **99**:175-181.
334 45. Zuo Y, Wang H, Xu Y, Huang J, Wu S, Wu Y, Yang Y: **CRISPR/Cas9 mediated G4946E substitution in the**
335 **ryanodine receptor of *Spodoptera exigua* confers high levels of resistance to diamide**
336 **insecticides**. *Insect Biochemistry and Molecular Biology* 2017, **89**:79-85.
337 46. Yang X, Chen W, Song X, Ma X, Cotto-Rivera RO, Kain W, Chu H, Chen Y-R, Fei Z, Wang P: **Mutation of**
338 **ABC transporter ABCA2 confers resistance to Bt toxin Cry2Ab in *Trichoplusia ni***. *Insect*
339 *Biochemistry and Molecular Biology* 2019, **112**:103209.

340 47. Wang X, Ma Y, Wang F, Yang Y, Wu S, Wu Y: **Disruption of nicotinic acetylcholine receptor α6 mediated**
341 **by CRISPR/Cas9 confers resistance to spinosyns in *Plutella xylostella***. *Pest Management Science*
342 2019:doi: 10.1002/ps.5689.
343 48. Guo Z, Sun D, Kang S, Zhou J, Gong L, Qin J, Guo L, Zhu L, Bai Y, Luo L, et al.: **CRISPR/Cas9-mediated**
344 **knockout of both the PxABCC2 and PxABCC3 genes confers high-level resistance to *Bacillus***
345 ***thuringiensis* Cry1Ac toxin in the diamondback moth, *Plutella xylostella* (L.)**. *Insect Biochemistry*
346 *and Molecular Biology* 2019, **107**:31-38.
347 49. Guest M, Goodchild JA, Bristow JA, Flemming AJ: **RDL A301S alone does not confer high levels of**
348 **resistance to cyclodiene organochlorine or phenyl pyrazole insecticides in *Plutella xylostella***.
349 *Pesticide Biochemistry and Physiology* 2019, **158**:32-39.
350 50. Douris V, Papapostolou K-M, Ilias A, Roditakis E, Kounadi S, Riga M, Nauen R, Vontas J: **Investigation**
351 **of the contribution of RyR target-site mutations in diamide resistance by CRISPR/Cas9 genome**
352 **modification in *Drosophila***. *Insect Biochemistry and Molecular Biology* 2017, **87**:127-135.
353 51. Samantsidis G-R, O'Reilly AO, Douris V, Vontas J: **Functional validation of target-site resistance**
354 **mutations against sodium channel blocker insecticides (SCBIs) via molecular modeling and**
355 **genome engineering in *Drosophila***. *Insect Biochemistry and Molecular Biology* 2019, **104**:73-81.
356 52. Zimmer CT, Garrood WT, Puinean AM, Eckel-Zimmer M, Williamson MS, Davies TGE, Bass C: **A**
357 **CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine**
358 **receptor confers resistance to spinosad in *Drosophila melanogaster***. *Insect Biochemistry and*
359 *Molecular Biology* 2016, **73**:62-69.
360 53. Wang J, Zhang H, Wang H, Zhao S, Zuo Y, Yang Y, Wu Y: **Functional validation of cadherin as a receptor**
361 **of Bt toxin Cry1Ac in *Helicoverpa armigera* utilizing the CRISPR/Cas9 system**. *Insect Biochemistry*
362 *and Molecular Biology* 2016, **76**:11-17.
363 54. Dermauw W, Wybouw N, Rombauts S, Menten B, Vontas J, Grbić M, Clark RM, Feyereisen R, Van
364 Leeuwen T: **A link between host plant adaptation and pesticide resistance in the polyphagous**
365 **spider mite *Tetranychus urticae***. *Proceedings of the National Academy of Sciences of the United*
366 *States of America* 2013, **110**:E113–E122.
367 55. Zhu F, Moural TW, Nelson DR, Palli SR: **A specialist herbivore pest adaptation to xenobiotics through**
368 **up-regulation of multiple Cytochrome P450s**. *Scientific Reports* 2016, **6**:20421.
369 56. Zimmer CT, Bass C, Williamson MS, Kaussmann M, Wölfel K, Gutbrod O, Nauen R: **Molecular and**
370 **functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to**
371 **pyrethroids in European populations of pollen beetle, *Meligethes aeneus***. *Insect Biochemistry*
372 *and Molecular Biology* 2014, **45**:18-29.
373 57. Shi Y, Wang H, Liu Z, Wu S, Yang Y, Feyereisen R, Heckel DG, Wu Y: **Phylogenetic and functional**
374 **characterization of ten P450 genes from the CYP6AE subfamily of *Helicoverpa armigera* involved**
375 **in xenobiotic metabolism**. *Insect Biochemistry and Molecular Biology* 2018, **93**:79-91.
376 58. Nauen R, Wölfel K, Lueke B, Myridakis A, Tsakireli D, Roditakis E, Tsagkarakou A, Stephanou E, Vontas
377 J: **Development of a lateral flow test to detect metabolic resistance in *Bemisia tabaci* mediated**
378 **by CYP6CM1, a cytochrome P450 with broad spectrum catalytic efficiency**. *Pesticide Biochemistry*
379 *and Physiology* 2015, **121**:3-11.
380 59. Black IV WC, Vontas JG: **Affordable assays for genotyping single nucleotide polymorphisms in insects**.
381 *Insect Mol Biol* 2007, **16**:377-387.
382 60. Blaser S, Diem H, von Felten A, Gueuning M, Andreou M, Boonham N, Tomlinson J, Muller P, Utzinger
383 J, Frey B, et al.: **A Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Identification**
384 **of *Bemisia tabaci***. *J Vis Exp* 2018, **140**:e58502.
385 61. Bronzato Badial A, Sherman D, Stone A, Gopakumar A, Wilson V, Schneider W, King J: **Nanopore**
386 **Sequencing as a Surveillance Tool for Plant Pathogens in Plant and Insect Tissues**. *Plant Dis* 2018,
387 **102**:1648-1652.

388 62. Phelan S, Barthe MS, Tobie C, Kildea S: **Detection of the cytochrome b mutation G143A in Irish**
389 ***Rhynchosporium commune* populations using targeted 454 sequencing**. *Pest Manag Sci* 2017,
390 **73**:1154-1160.
391 63. Shu C, Su H, Zhang J, He K, Huang D, Song F: **Characterization of cry9Da4, cry9Eb2, and cry9Ee1 genes**
392 **from *Bacillus thuringiensis* strain T03B001**. *Appl Microbiol Biotechnol* 2013, **97**:9705-9713.
393 64. Zink FA, Tembrock LR, Timm AE, Farris RE, Perera OP, Gilligan TM: **A droplet digital PCR (ddPCR) assay**
394 **to detect *Helicoverpa armigera* (Lepidoptera: Noctuidae) in bulk trap samples**. *PLoS One* 2017,
395 **12**:e0178704.
396 65. Mavridis K, Wipf N, Medves S, Erquiaga I, Müller P, Vontas J: **Rapid multiplex gene expression assays**
397 **for monitoring metabolic resistance in the major malaria vector *Anopheles gambiae***. *Parasites*
398 *& Vectors* 2019, **12**:9.
399 66. Mavridis K, Wipf N, Müller P, Traoré MM, Muller G, Vontas J: **Detection and Monitoring of Insecticide**
400 **Resistance Mutations in *Anopheles gambiae*: Individual vs. Pooled Specimens**. *Genes* 2018,
401 **9**:479.
402 67. Moores GD, Ffrench-Constant RH, Devonshire AL: **Immunoassay for detecting insecticide resistance**
403 **in aphids**. *Pesticide Science* 1989, **26**:324-326.

404

405 **Annotated references**

406 ***Vontas and Mavridis 2019** (ref 16)

407 A critical review on the true value of molecular diagnostic tools for tracking insecticide resistance
408 in mosquito vectors.

409 ****Riga et al. 2017** (ref 35)

410 Using marker-assisted inbreeding, a large number of target-site resistance mutations was
411 introgressed in a susceptible genetic background of *T. urticae*. This allowed to assess the
412 phenotypic strength of a single resistance mutation, not confounded by additional resistance
413 mechanisms, and partly determines its diagnostic value. It also allowed to determine associated
414 fitness costs in a follow-up study, see **Bajda et al.** (ref 38)

415 **Zuo et al. 2017** (ref 45)

416 The use of CRISPR-Cas9 gene editing to validate (the strength of) a SNP marker, G4946E conferring
417 diamide resistance, in a lepidopteran pest species. The mutation was previously validated in the
418 genetic model organism *Drosophila* by **Douris et al.** (ref 50)

419 ***Dermauw et al. 2013** (ref 54)

420 Adaptation to host plants and pesticides select for similar responses in the polyphagous mite
421 *Tetranychus urticae*, potentially confounding the predictive value of a metabolic marker such as
422 overexpression of a detoxification gene.

423 ****Nauen et al. 2015** (ref 58)

424 Reports a test kit based on an lateral flow test for the detection of CYP6CM1-based neonicotinoid
425 resistance in while flies. The kit is as easy to use as a pregnancy test and is validated to provide a
426 reliable estimate of resistance in populations across the globe.

427 ***Bronzato et al. 2018** (ref 61)

428 The application of nanopore sequencing with the portable MinION variant as a tool for monitoring
429 pathogens in plants and agricultural pests

430 ***Zink et al. 2017** (ref 64)

431 One of the first studies documenting the application of ddPCR for monitoring molecular markers
432 in pooled samples of agricultural pests.

433 **Figure Legends**

434 **Figure 1 - Current and future diagnostic assays**

435 (A) Test kit box based on lateral flow assay for the detection of CYP6CM-based neonicotinoid resistance in
436 *B. tabaci*. Test line intensity provides reliable estimation of the presence and approximate levels of
437 resistance. The major advantage of such a test is its user-friendly format allowing its application under
438 field conditions without specialized equipment or training, and the quick availability of the test result
439 within minutes. The test has been successfully validated against a number of neonicotinoid resistant *B.*
440 *tabaci* strains and field populations around the globe [58]. (B) Droplet Digital PCR (ddPCR) and (C) Oxford
441 Nanopore: two of the most promising technologies for future use in monitoring insecticide resistance in
442 agricultural pests. Both can be used for pooled samples. Major additional advantages for ddPCR is that it
443 can be used to accurately assay known mechanisms in large bulks of samples with high sensitivity and
444 specificity. Additional advantages for Oxford Nanopore are the deep sequencing capabilities, the
445 identification of potential novel mutations and the practicality of portable, “field-friendly” variants
446 (MinION Nanopore).

447 ## Tables

448 **Table 1 - Geographical distribution of major target-site resistance mutations across *T. urticae***
449 **populations**

450 **Table 2 - Current and future molecular diagnostic methods for assessing agricultural pest resistance**

451

452 ## Supplementary Tables

453 **Table S1 - Table 1 with references**

454

Manuscript

No conflict of interest

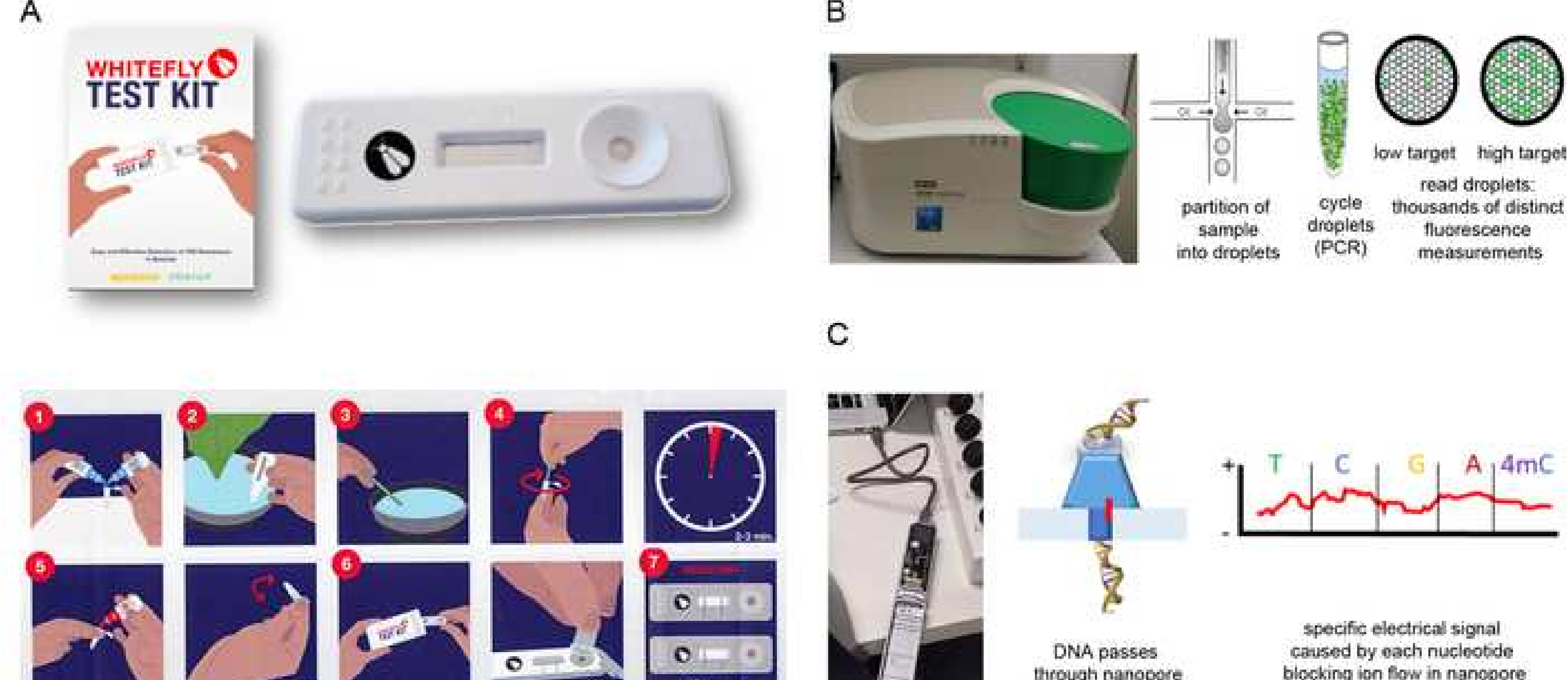
A
WHITEFLY
TEST KIT
B
low target
high target
partition of
sample
into droplets
cycle
droplets
(PCR)
read droplets:
thousands of distinct
fluorescence
measurements
C
T
C
G
A
4mC
DNA passes
through nanopore
specific electrical signal
caused by each nucleotide
blocking ion flow in nanopore

Table 1 - Geographical distribution of major target-site resistance mutations across *T. urticae* populations. Additional references can be found in Table S1.

target-site	resistance mutation	phenotypic strength[1]	fitness cost?[2]	geographical distribution				
				Europe	Asia	North-America	Oceania	Africa
AChE	G119S	n.i.	n.i.	✓	✓	-	✓[3]	-
	F331W/Y	n.i.	n.i.	✓	✓	✓	✓[3]	✓
VGSC	M918L+F1534S	n.i.	n.i.	-	-	✓	-	-
	F1534S+F1538I	n.i.	n.i.	-	-	✓	-	-
	L1024V	strong	no	✓	✓	-	✓[3]	✓
	F1538I	strong	n.i.	✓	✓	✓	-	✓
CHS1	I1017F	strong	yes	✓	✓	✓	✓	✓
cytB	G132A	strong	yes	✓	-	-	-	-
	G126S+A133T	strong	n.i.	✓	-	-	-	-
	G126S+I136T	n.i.	n.i.	✓	-	-	-	-
	G126S+S141F	strong	n.i.	✓	-	-	-	-
	I260V+N326S	n.i.	n.i.	-	✓	-	-	-
	P262T	moderate	no	✓	✓	-	-	-
GluCl1	G314D	weak	yes	✓	✓	-	-	-
GluCl3	G326E	weak	yes	✓	✓	-	-	✓
PSST	H92R	moderate	n.i.	✓	-	-	-	-

[1] based on [35-37]: n.i., not investigated

[2] based on [36,38]: n.i., not investigated; “no” should be interpreted as not detected under the conditions of [38]

[3] mutation was detected in a lab strain

Table2

Table 2 - Current and future molecular diagnostic methods for assessing agricultural pest resistance

Methods	Category	Application	Pro (+) / Contra (-)	Examples
Currently used molecular diagnostics				
AS-PCR PCR-RFLP	'Low-tech' PCR-based	T, D	+ Applicable to basic laboratory settings + Low-cost, simple - Low specificity (AS-PCR) - Low throughput - High protocol run time	[59]
TaqMan HRM analysis	'Hi-tech' PCR-based	T, Q M	+ High-throughput + Easy protocol and result interpretation - High capital cost (machine, equipment)	[59,63]
Direct sequencing/ Pyrosequencing	PCR-Sequencing	T, D	+ Detection of unknown resistance mutations - No quantitative information - High capital and per reaction cost - Multi-step complicated protocol, not suitable for large sample size	[62]
LAMP	Isothermal amplification	T, D	+ No requirement for thermal cycler; Low cost + Easy, rapid one-step protocol; "Naked-eye" result determination + Rugged, field-friendly variants can be developed - Complex and restrictive assay design - No quantitative information/ low specificity for SNPs	[60]
Promising molecular diagnostics for future use				
Direct-in-lysate analysis coupled with lyophilized pellets	Multiplex direct Taqman (RT) qPCR	T, Q M	+ Compatibility with most qPCR platforms + Fast, with minimum handling: all reagents in a single pellet + Multiplexing capability - High capital cost for qPCR machine - Needs calibration for quantification	[16]
Droplet Digital PCR (ddPCR)	Third generation PCR	T, Q M	+ Extremely accurate and sensitive + Simplified analysis and experimental procedure + No calibration or controls needed for quantification - High capital and per-assay cost	[64]
Nanopore sequencing	Third generation sequencing	T, Q M	+ Deep sequencing (RNA-, DNA-seq) capabilities + High-throughput + Identification of potential novel mutations + Portable, "field-friendly" variants (MinION Nanopore) - Requires complicated bioinformatic analysis - High capital cost	[61]

T, D: Target-site, Detection of mutations; T, Q: Target-site, Quantification of mutation frequency (pooled samples); M: Metabolic resistance; AS: Allele Specific; HRM: High Resolution Melting; LAMP: Loop mediated isothermal amplification; RFLP: Restriction Fragment Length Polymorphism

Box 1 - Factors affecting the diagnostic value of a molecular marker for IRM

- Intensity of underlying resistance phenotype associated with the marker (how much is the phenotype determined by a single marker)
- Geographic distribution of the marker (on what scale do resistance mechanisms vary)
- Cross spectrum resistance predictive value of the marker
- Epistasis and how many resistance markers are required for diagnosis in each case.
- Untangle gene expression patterns associated with resistance and host plant (detoxification enzymes can be overexpressed after adaptation to pesticides and plant allelochemicals)
- Dominance and fitness cost of the resistance marker
- Robustness, accuracy and cost effectiveness of diagnostic assay to capture the marker

highlights

Resistance monitoring is not common practice in agriculture

Molecular markers can be a crucial tool in resistance management of agricultural pests

Strength and predictive value of a diagnostic marker depends on many factors

New technologies (MinION, ddPCR) will allow to determine mutation frequency at low levels

suppl table1

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Supplementary Material
Table_S1.docx