Recurrent Mutations Drive the Rapid Evolution of Pesticide Resistance in the Two-spotted Spider Mite *Tetranychus urticae*

Li-Jun Cao [1, #], Jin-Cui Chen [1, #], Joshua A. Thia [2, #], Thomas L. Schmidt [2], Richard Ffrench-Constant [3], Lin-Xi Zhang [1, 4], Yu Yang [1, 5], Meng-Chu Yuan [1, 5], Jia-Yue Zhang [1, 6], Xiao-Yang Zhang [1, 5], Qiong Yang [2], Ya-Jun Gong [1], Hu Li [4], Xue-Xin Chen [7], Ary A. Hoffmann [2, *], Shu-Jun Wei [1, *]

1. Institute of Plant Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China
2. Bio21 Institute, School of BioSciences, University of Melbourne, Parkville, Victoria 3010, Australia
3. Biosciences, University of Exeter, Exeter, EX4 4QD, UK
4. Department of Entomology, College of Plant Protection, China Agricultural University, Beijing 100193, China
5. College of Plant Science and Technology, Beijing University of Agriculture, Beijing 102206, China
6. Hebei North University, College of Agriculture and Forestry Technology, Zhangjiakou city 075000, Hebei Province
7. Institute of Insect Science, Zhejiang University, Hangzhou 310058, Zhejiang Province, China

#, equal contributions

***Correspondence authors:** Shu-Jun Wei (shujun268@163.com) and Ary A. Hoffmann (ary@unimelb.edu.au)

Abstract

The genetic basis of pesticide resistance has been widely studied, but the exact nature of this evolutionary process in the field is often unclear, particularly when a limited number of populations is considered and when there is a lag between the evolutionary event and its investigation. We showed that an unprecedented number of recurrently evolved mutations in an arthropod pest, the two-spotted spider mite *Tetranychus urticae*, drive the rapid evolution of resistance to a recently commercialized acaricide, cyetpyrafen. We first observed high levels of resistance that appeared and became widespread within three years. Genome scans revealed genetic heterogeneity of resistance among populations and identified 15 target mutations, including six mutations on five amino acid residues of subunit *sdhB*, and nine mutations on three amino acid residues of subunit *sdhD* of the pesticide target succinate dehydrogenase, with as many as five substitutions on one residue. No mutations were present in 2666 screened historical specimens, suggesting that mutations arose rapidly through *de novo* substitutions or from very rare segregating mutations. Identical mutations recurrently appeared in different genetic backgrounds, increasing the likelihood of resistance evolution. The high number of mutational options available for the evolution of target site resistance in this pest challenges resistance management practices.

Keywords: population genomics, pesticide resistance, recurrent evolution, selective sweep

1. Introduction

The development of pesticide resistance represents rapid contemporary evolution of organisms in response to human-induced stressors (ffrench-Constant et al., 2004, McKenzie and Batterham, 1994, Tabashnik et al., 2013, Gould et al., 2018). Since the first instance of pesticide resistance in a scale insect was documented in 1914, cases have exponentially increased in arthropods, weeds and pathogens, posing threats to agricultural production, human health, and the environment (Sparks et al., 2021, Melander, 1914, Gould et al., 2018, Fritz, 2022, Peterson et al., 2018). This has led to attempts to stop the evolution of resistance, or at least slow its appearance within populations (Gould et al., 2018). A key task in resistance management is to understand the evolution of resistance in field populations, where a given mutation may have originated once and spread via gene flow or evolved through multiple independent substitutions, and where resistance-associated mutations can pre-date (standing genetic variation) or arise subsequent to (*de novo* mutation) the introduction of pesticide selection pressures (Ffrench-Constant, 2007, Daborn et al., 2002, Kersten et al., 2023).

A challenge in understanding the evolution of resistance is that the genetic basis of pesticide resistance observed in laboratory-generated resistant strains often does not reflect that in field populations, especially for arthropod pests (Chen et al., 2023, Walsh et al., 2022, Ffrench-Constant, 2013, Legan et al., 2024). Field populations are typically much larger and more genetically heterogeneous than laboratory populations. Because of the greater potential availability of rare mutations with large effect sizes in field populations, monogenic resistance to pesticides involving these rare mutations may occur relatively more frequently than in laboratory-generated strains (McKenzie and Batterham, 1994), though small effect loci can also remain important in field resistance (Schmidt et al., 2024, Lucas et al., 2023). In contrast, artificial selection in the laboratory usually takes place in populations with much smaller effective population sizes, as well as a constant intensity and pattern of selection expected to favor the accumulation of multiple changes of small effect (Ffrench-Constant, 2013). Where alleles with large effects are selected in the laboratory, these may not necessarily reflect the full

71 set of resistant mutations in the field (Legan et al., 2024). Such mismatches between
72 laboratory-selected and field-evolved resistance may lead to an inaccurate understanding of
73 resistance evolution (Ffrench-Constant, 2013, Bras et al., 2022, Legan et al., 2024).

74 A further challenge in understanding resistance evolution in the field is that the genetic
75 basis of resistance is often examined retrospectively well after the emergence of resistance in
76 the field. Retrospective studies can fail to capture processes that have occurred in the
77 intervening period (Andreev et al., 1999, Raymond et al., 1991, Ffrench-Constant et al., 1993b,
78 Adesanya et al., 2021), such as the introduction of new alleles through gene flow or *de novo*
79 mutations, and fitness costs as well as changing selection regimes through pesticide rotations
80 that can lead to resistance alleles being transient (Schmidt et al., 2010, Ffrench-Constant, 2013,
81 Kreiner et al., 2022b, Kreiner et al., 2019, Groeters and Tabashnik, 2000). Preserved specimens
82 before and following the introduction of pesticides can allow for resistance processes to be
83 investigated retrospectively, but samples are often unavailable (Hartley et al., 2006, D'Costa et
84 al., 2011). Ongoing genetic monitoring of populations can help in overcoming some of these
85 issues, particularly if population processes affecting a species are understood and if the genetic
86 basis and new resistant alleles are identified as they evolve in the field (Van Leeuwen et al.,
87 2012, Pélissié et al., 2022, Pezzini et al., 2024, Taylor et al., 2021).

88 Here, we take advantage of the recent evolution of resistance in the two-spotted spider
89 mite (TSSM), *Tetranychus urticae* (Acari: Tetranychidae), to a novel acaricide, cyetpyrafen, to
90 provide a detailed understanding of contemporary resistance evolution. TSSM is a global super
91 pest that has evolved resistance to at least 11 groups of acaricides based on their modes of
92 action and is the most resistant invertebrate pest worldwide (De Rouck et al., 2023, Van
93 Leeuwen et al., 2010). Succinate dehydrogenase inhibitor (SDHi) acaricides (IRAC group 25)
94 were first launched in 2007 for mite control. Four chemicals consisting of cyetpyrafen (first
95 launched in 2017 in China), cyenopyrafen (2009 in Japan), cyflumetofen (2007 in Japan), and
96 pyflubumide (2015 in Japan) fall within this group of acaricides globally (Hayashi et al., 2013,
97 Nakahira, 2011). Laboratory-selected resistance has already been identified to these new

acaricides, and is conferred by target-site mutations of the SDH coding *sdhB* and *sdhD* genes, with mutations identified at three positions (Njiru et al., 2022, Sugimoto et al., 2020). In China, three acaricidal SDH inhibitors have been registered over the last decade: cyflumetofen (2013), cyetpyrafen (2017) and cyenopyrafen (2019). Since its release in 2017, cyetpyrafen has become the most popular pesticide for controlling spider mites, though low-level resistance was detected in the field only one year after its release (Chen et al., 2019).

We have now tracked resistance through repeatedly monitoring field-evolved resistance of TSSM to cyetpyrafen at a time when resistance has developed rapidly to high levels across geographically distant populations. We investigated resistance using a combination of population genomics, laboratory selection, bioassays, and studies on historically preserved specimens before the introduction of cyetpyrafen. We find that multiple and recurrently evolved mutations drive the rapid development of high-level resistance to cyenopyrafen in field populations across a widespread geographical range. We also demonstrate that these mutations likely evolved *de novo* after the introduction of cyetpyrafen, though they may also have originated from extremely rare mutations segregating in populations before cyetpyrafen selection. We emphasize the benefits of integrating genomics with other approaches in longitudinal studies to capture the dynamics of rapid evolutionary changes.

2. Results

2.1 Strong resistance evolves rapidly in field and lab-selected populations

To monitor the evolution and spread of cyetpyrafen resistance in the field, we conducted bioassays on 46 mite population samples collected from various regions in China between 2020 and 2024 (Figure 1, Tables S1-S2). The susceptibility of these population samples to cyetpyrafen was compared with previously reported data for populations collected between 2017 and 2018 during the early release stage of cyetpyrafen in China (Chen et al., 2019, Gong et al., 2017). In 2017-2018, all seven tested populations remained susceptible, with LC_{50} values ranging from 1.4 to 21.6 mg/L (Figure 1a). In 2020, one population from Shouguang City, Shandong Province

124 (SDSG2), showed resistance with an LC_{50} > 32,000 mg/L, while the other 13 populations
125 remained susceptible (Figure 1b). By 2021, high levels of resistance to cyetpyrafen quickly
126 became widespread in field populations: eight of 10 tested populations had an LC_{50} higher than
127 1606.2 mg/L (Figure 1c). In 2024, 11 of the 13 tested populations evolved resistance with an
128 LC_{50} higher than 1498.6 mg/L (Figure 1d). Overall, the susceptibility of all populations was
129 bimodal, with populations being highly susceptible (LC_{50} ranges from 0.61 to 30.1 mg/L, $\bar{x}$ = 6.72
130 mg/L) or highly resistant (LC_{50} > 1498.6 mg/L, $\bar{x}$ > 5892.5 mg/L) and an 877-fold difference
131 between mean LC_{50}s of the susceptible and resistant groups. While only a single sample was
132 resistant in 2020, by 2021 resistance was observed across the entire range of China (Figure 1c).
133 This pattern indicates that field populations of TSSMs have rapidly developed high levels of
134 resistance to cyetpyrafen across a wide geographical area.

135 We conducted artificial selection to further investigate resistance evolution, using two
136 laboratory strains derived from the same field population and reared in parallel. The field
137 population for selection was collected from Hangzhou, Zhejiang province (ZJXS1), with an LC_{50}
138 of 21.6 mg/L. One laboratory strain, 'LabR', was exposed repeatedly to cyetpyrafen, while the
139 other, 'LabS', was not. LabR underwent a gradual increase in cyetpyrafen resistance for the first
140 15 selection rounds (31 generations), but a much sharper increase over the remaining 18
141 selection rounds (35 generations) (Table S3). By the end of the experiment, LabR possessed an
142 LC_{50} of 54,336 mg/L, a 2,521-fold increase in resistance relative to the ZJXS1 field population. In
143 contrast, the LC_{50} of the LabS strain decreased to 1.21 mg/L over the same period (Figure 1e,
144 Table S3), due to unknown reasons, perhaps linked to adaptation to the laboratory
145 environment. However, this is a very minor change in resistance compared to the large increase
146 seen in the selected strain.

147 Additional bioassays were used to test whether the selected LabR strain showed cross-
148 resistance to other pesticides that target proteins in the mitochondrial electron transport chain.
149 For the other two mitochondrial complex II inhibitors (IRAC group 25), the LC_{50} of cyenopyrafen
150 increased 21,254-fold (3.76 to 79918 mg/L), and the LC_{50} of cyflumetofen increased 69,861-fold

151 (6.47 to 452,003 mg/L). For the mitochondrial complex III inhibitors, the LC_{50} of bifenazate
152 (group 20D) increased 2-fold (9.87 to 18 mg/L), and the LC_{50} of pyridaben (group 21A) increased
153 3-fold (3,763.4 to 10,352 mg/L). The results indicate that cyetpyrafen selection led to high
154 cross-resistance to other mitochondrial complex II inhibitors but weak cross-resistance to the
155 mitochondrial complex III inhibitors (Table S3).

156 ***2.2 Selective sweep signals are clear but heterogeneous***

157 To identify genes potentially associated with cyetpyrafen resistance, we conducted pooled
158 whole-genome resequencing on 22 TSSM population samples, including historic samples of
159 eight geographical populations collected in 2017 before the first field releases of cyetpyrafen,
160 12 samples (10 resistant and two susceptible) collected afterwards, and two laboratory
161 populations (LabS and LabR) which originated from the same field population (Tables S1, S4). To
162 allow for variation in the genetic basis of resistance and genetic backgrounds among
163 populations, we chose the Population Branch Excess (PBE) analysis (Yassin et al., 2016) method
164 to scan selection signals across the genome; we used two susceptible populations (NMHS2 and
165 LabS) as non-focal populations to correct for the varied genetic backgrounds, and one resistant
166 population as the focal population to identify population-specific signals of pesticide selection.
167 No clear and coincident selection signal was identified among historical population samples
168 (Figure 2a). The laboratory-selected resistant LabR population had clear selection signals at two
169 genes, *sdhB* and *sdhD,* that encode two subunits of succinate dehydrogenase, the target of
170 SDHi acaricides (Figure 2b). None of the 10 field-collected resistant populations had selection
171 signals at both of these genes, but four (BJDX6, ZJHZ1, ZJHZ2, and LNDD) had selection signals
172 at *sdhB*, and one (BJCP4) had a selection signal at *sdhD*. Five of the resistant populations
173 (QHHD, GXNN, SDSG3, SDQZ and YNYX) showed no selection signal at either gene; this same
174 pattern was observed in the one population sample (HNCS2) that remained susceptible after
175 cyetpyrafen was introduced. Additional PBE peaks were observed at other genomic regions in
176 historical, resistant, and susceptible populations (Figure 2a, 2b); these could reflect
177 demographic processes or other selective forces not considered in this study.

178 Selection for cyetpyrafen resistance could impact patterns of genetic diversity in the
179 regions of interest or more widely across the genome if selection decreases overall population
180 size. We investigated these impacts focusing on genome-wide patterns of nucleotide diversity
181 (π) and on Tajima's D. Five resistant populations with selection signals identified by PBE analysis
182 (Figure 2b) showed corresponding decreases in π and Tajima's *D* near the two SDH genes
183 (Figure S1). The overall genetic diversity and Tajima's *D* across the genome were not
184 significantly associated with resistance level, genetic structure or geographic coordinates ($P >$
185 0.05) (Figure S2, Table S4), suggesting that resistant populations had not experienced strong
186 bottlenecks. Five resistant populations without selection signals did not show obvious reduced
187 genetic diversity or altered Tajima's *D* values around the SDH genes (Figure S1) suggesting that
188 these had not experienced strong selection or selective sweeps.

189 ***2.3 Multiple mutations have arisen in the field***

190 To identify specific mutations conferring resistance, we examined the two SDH genes, *sdhB* and
191 *sdhD*, where selective sweep signals were detected in some populations. We used whole-
192 genome resequencing data from pooled individuals; we also conducted Sanger sequencing of
193 partial *sdhB* (216 bp of 1001 bp) and *sdhD* (155 bp of 1,008 bp) genes to validate the pool-seq
194 data and examined mutation frequencies in additional populations; this involved 1268
195 individuals from 52 populations, including samples collected before and after the release of
196 cyetpyrafen (Table S1). Allele frequencies identified by pool-seq and Sanger sequencing were
197 correlated (Figure S4). A total of 15 amino acid substitutions were identified, including six
198 mutations on five amino acid residues of *sdhB*: H146Q (CAT to CAA), S212I (AGC to ATC), H258Y
199 (CAT to TAT), I260T (ATC to ACC), I260V (ATC to GTC), and A285S (GCA to TCA), and nine
200 mutations on three amino acid residues of *sdhD*: D116G (GAC to GGC), D116E (GAC/T to GAA),
201 D116N (GAC to AAC), R119C (CGT to TGT), R119L (CGT to CTT), R119G (CGT to GGT), R119P
202 (CGT to CCT), R119H (CGT to CAT), and P120L (CCC to CTC) (Figure 3b, Table S2). The residue
203 R119 on *sdhD* was a notable extreme case, involving five amino acid changes. The second
204 codon position of R119 (G) was substituted by the other three nucleotides, while the first codon

205 position (C) was substituted by two other nucleotides (T and G), indicating that the first two
206 codon positions of R119 are almost saturated in terms of possible nucleotide substitutions of
207 these codon positions.

208 Multiple mutations were commonly found in a single population, while none of the
209 mutations were fixed in any field population (Figure 2b, Table S2). Populations sampled in 2020
210 had at most two mutations, while those sampled in 2021 and 2024 had at most five.
211 Populations sampled in 2020, 2021 and 2024 had on average 1.17, 2.46 and 2.55 mutations,
212 respectively. We also examined the other two SDH subunit genes, *sdhA* and *sdhC*, using pool-
213 seq data, but no amino acid replacements were identified.

214 We checked the evolutionary conservation of these mutations across animals by aligning
215 homologous sequences from ~700 animal species, encompassing nematodes, insects, fishes,
216 and mammals (Figs. 3a and 3b). The protein sequences of *sdhB* and *sdhD* were highly conserved
217 among various animal species, particularly around the mutant residues H146, S212, I260, D116,
218 and R119 observed in this study. In a structural model of human SDH, eight amino acid residues
219 were predicted at the ubiquinone binding site (Q-site), corresponding to 211, 215 and 260 on
220 mite subunit *sdhB*, 58, 63, 67 and 71 on *sdhC*, and 117 on *sdhD* (Njiru et al., 2022, Du et al.,
221 2023). All detected mutations in the mite are located around or in the Q-site, although
222 differences exist between human and mite sequences (Figure 3d).

223 Although cyetpyrafen only started to be used in 2017, it is possible that these potential
224 resistance mutations were present as standing genetic variation before this time. To investigate
225 this, we obtained Sanger sequencing and pool-seq data from 2317 female mites collected
226 before the cyetpyrafen release and 4631 after its release (Figure 3e, Table S2). Of the 15
227 putative resistance mutations, only one (A285S) was observed in the pre-release specimens,
228 which was present in population SDRZ (unclear resistance status), but was not detected in
229 specimens collected after the cyetpyrafen release. The historical trajectory of resistance alleles
230 revealed extreme selection after release of cyetpyrafen (Figure 3e). These 14 resistance alleles
231 have collectively experienced a selective strength of $\tilde{s}$= 0.707 (Z = 3.372, p = 0.0007) per year.

232 However, based on logistic regression, the estimate of the strength of selection on each
233 resistance allele was only significant for I260V (p = 0.011). Seven mutations were first detected
234 in 2020 (I260T, D116G, D116N, R119C, R119L, R119P, and R119H), three mutations were first
235 detected in 2021 (H146Q, S212I, and I260V), and the remaining four mutations were first
236 detected in 2024 (H258Y, D116E, R119G, and P120L) (Figure 3e, Table S2). Among the target-
237 site mutations, I260V and R119C were the most common, found in 65.9% and 46.3%
238 (respectively) of the populations collected after the release of cyetpyrafen, with their frequency
239 increasing rapidly (Figure 3e). In the laboratory-selected population LabR, two mutations (I260V
240 and R119C) were fixed, but neither was detected in either the field population from which it
241 was sourced (ZJXS1) or the unselected susceptible laboratory population derived from ZJXS1
242 (LabS). These results indicate that the putative resistance mutations were not likely to be
243 present as standing genetic variation unless they were segregating at very low frequencies, and
244 they may have evolved recently through *de novo* substitutions although this cannot be
245 demonstrated definitively.

246 ***2.4 Recurrent evolution of identical mutations***

247 When a specific resistance mutation is found in multiple populations, this can be the result of
248 migration between populations or to multiple independent origins of the same mutation. We
249 evaluated these evolutionary hypotheses using two methods: TreeMix (Pickrell and Pritchard,
250 2012) and haplotype network analysis.

251 We used TreeMix to investigate whether populations with identical alleles were more
252 genetically similar or had recent histories of migration. TreeMix builds maximum likelihood
253 trees from population allele frequencies and then adds optional migration edges between
254 populations. We set the SCCD population from southwest China as the root population as it had
255 the highest genetic diversity, and we built trees that followed a strict drift model (i.e. no
256 migration) and trees with up to 10 migration edges. The strict drift model explained most of the
257 variance in relatedness among populations (mean over 1000 replicates of 0.930). The best

258 estimate of the number of historical migration events was one. The inclusion of a single gene
259 flow event resulted in only a very small increase in explained genetic variance compared to
260 0.930 for the drift model, suggesting that migration among populations is low. The consensus
261 tree over the 1000 replicates assigned most of the populations into northern and southern
262 groups by geography, but not by resistance status (Figure 4a). We also observed several
263 instances where geographically distant populations grouped together. Some southern
264 populations were placed with the northern group (YNYX), and vice versa (BJDX6); these
265 populations may have been recently established via long-distance dispersal, possibly through
266 the transport of seedlings.

267 The above-mentioned mutations in *sdhB* and *sdhD* genes were detected in six populations
268 from the southern group and seven populations from the northern group. No significant
269 isolation-by-distance pattern was detected in resistance allele frequencies across survey years
270 (Mantel test: 2020, $P = 0.73$; 2021, $P = 0.52$; 2023, $P = 0.16$), indicating that the spread of
271 resistant alleles was not linked to geographical factors. The mutation I260V was detected in
272 every field population sampled after the release of cyetpyrafen (Figure 4a, Table S2), based on
273 pool-seq data. The mutation I260T was only detected in the northern group and R119L was
274 mostly detected in the southern group. The mutation H146Q was found in four adjacent
275 populations (ZJHZ1, ZJHZ2, GXNN and BJCP4). This geographical heterogeneity in the
276 distribution of resistance mutations suggests multiple mechanisms of resistance evolution
277 underly these patterns, involving some spread via migration between populations (e.g. H146Q)
278 as well as possible independent origins of the same mutation (e.g. I260V).

279 We explored these ideas further using haplotype network analysis. When resistance
280 spreads via migration, these mutations should have identical haplotypes, unless recombination
281 has taken place which will introduce a second haplotype that segregates locally alongside the
282 initial haplotype. We constructed the haplotype network for the partial *sdhB* (216 of 1001 bp)
283 (Figure 4b) and *sdhD* (155 of 1,008 bp) (Figure 4c) genes determined from Sanger sequencing.
284 Since haplotypes could not be determined when two loci were heterozygous, we detected

285 haplotypes from sequencing data with at most one heterozygous locus. In total 844 and 696
286 individuals were used to detect haplotypes of *sdhB* and *sdhD*, respectively. The genes showed
287 high genetic diversity, with 8 SNPs (*sdhB*) and 11 SNPs (*sdhD*) detected, which generated 11
288 (*sdhB*) and 24 (*sdhD*) haplotypes, respectively. We assumed that common haplotypes located at
289 the center of the network are the ancestral haplotypes (H1 and H4 in the case of *sdhB*; H1, H2,
290 H3, and H11 in the case of *sdhD*). We assumed all other haplotypes were derived. Each derived
291 haplotype was only one nucleotide different from its nearest ancestral haplotype. Five of the
292 nine derived *sdhB* haplotypes carried a putative resistance mutation, as did 13 of the 22 derived
293 *sdhD* haplotypes. Combinations of these mutations between the two subunits were present.
294 However, there was no instance of two or more resistance mutations occurring within the same
295 haplotype.

296 Of the 10 putative resistance mutations detected through Sanger sequencing, all but four
297 (H258Y, D116N, R119G, and P120L) were found as multiple haplotypes (Figs. 4b and 4c). To test
298 whether these haplotypes were produced by multiple origins or by recombination, we inferred
299 the possible means by which recombination could produce each haplotype, and investigated
300 the geographical distributions of the requisite ancestral and derived haplotypes. For mutations
301 in *sdhB*, we inferred that recombination would have to involve the H4 ancestral haplotype as
302 donor, while the H11 ancestral haplotype was required for *sdhD* (Figure S3). Thus,
303 recombination could have produced the *sdhB* haplotypes H5 (H4 x H6) and H8 (H4 x H11), and
304 the *sdhD* haplotypes H17 (H11 x H10), H16 (H11 x H13) and H21 (H11 x H20). However, only
305 three populations (GXNN, YNKM3, and YNYX) had both of the two recombining haplotypes
306 required and one derived haplotype (Table S5). Additionally, the proximity of the mutations
307 was such that multiple recombination events would be required within the same 5 – 60 bp
308 regions, which seems unlikely even with a high recombination rate of (say) 10 cM/Mb where
309 two mutations might only be expected to occur once in 2.5×10^8 effective individuals across 60
310 bp; this assumes a population recombination rate $\rho = 2cN_er$, where r refers to recombination
311 rate per base-pair per generation, N_e refers to effective population size, and c refers to ploidy of

312 the genome (Li and Stephens, 2003). These results do not indicate an obvious role for
313 recombination in generating the derived haplotypes.

314 All haplotypes carrying putative resistance mutations in the *sdhB* gene arose in 2020-2021,
315 along with all haplotypes of the *sdhD* gene apart from H6, H7, H16 and H23 (Figure 4d, Table
316 S5). By 2024, the *sdhB* H6 haplotype became the most prevalent haplotype, while the non-
317 resistant *sdhB* H3 haplotype was almost lost despite it being prevalent between 2013 and 2021
318 (Figure 4d, Table S5). Some individual populations had multiple haplotypes of the same putative
319 resistance mutation (Figure 4d), and different haplotypes of the same mutation were often
320 found in adjacent populations (Figure 4a, Figure 4d, Table S5). In the ZJXS1 population used to
321 produce the LabR and LabS strains, four haplotypes of the *sdhB* gene (H1, H3, H4 and H7) and
322 six haplotypes of the *sdhD* gene (H1-H4, H11 and H24) were found, none of which had putative
323 resistance mutations. After 66 generations of cyetpyrafen selection, two resistant haplotypes
324 were fixed in the LabR population: H6 of *sdhB* and H10 of *sdhD*, which were likely derived from
325 H1 of *sdhB* and H2 of *sdhD*, respectively (Table S5). These results illustrate the rapid and
326 geographically widespread emergence of resistant alleles in these populations, linked to several
327 mutations found in a range of different haplotypes.

328 ***2.5 Single mutations have a large effect on resistance***

329 We next tested resistance of homozygous individuals carrying mutations singly or in
330 combination with other mutations. We first tested the population-level association between
331 allele frequencies of the mutations and levels of resistance, using the five mutations present in
332 at least ten populations (I260T, I260V, D116G, R119C, and R119L) and resistance indicated by
333 survival at 1000 mg/L cyetpyrafen. When each mutation was analyzed separately, a weak
334 association was found between survival and the frequency of the mutant allele (Table S6, linear
335 regression R^2 = 0.091-0.303). The multivariate regression analysis revealed that frequency of
336 three mutations, I260T (P = 0.00128), I260V (P = 0.00423) and D116G (P = 0.00058), were
337 significantly correlated with resistance of the field populations. When we considered the
338 frequency of individuals having any of the five mutations, we found a strong positive

339 association between survival and the frequency of the predominant allele (R^2 = 0.707), the
340 frequency of individuals carrying at least one resistant allele (heterozygous and homozygous
341 mutant genotypes) (R^2 = 0.747), and the frequency of individuals with homozygous mutant
342 genotypes (R^2 = 0.606) (Figure 5a-5c). These results showed that single mutations explained a
343 low proportion of resistance variation across populations. But when considering all five
344 mutations together, a substantial proportion of resistance variation was explained.

345 Homozygous lines at the mutation site(s) were created and tested for susceptibility to
346 cyetpyrafen (Figure 5d). These lines carried homozygous I260V, D116G, R119E, R119L, and
347 I260V+R119C mutations. When treated with 1000 mg/L cyetpyrafen, mortality of the
348 susceptible line was 100%, but was lower than 25.2% in all other lines, indicating that each
349 mutation and a combination of two mutations conferred high resistance to cyetpyrafen. These
350 results demonstrate that cyetpyrafen resistance is conferred by multiple large-effect mutations
351 in the *sdhB* and *sdhD* genes, although further functional validations are needed.

352 **3. Discussion**

353 Pesticide resistance can evolve rapidly, in contrast to traditional views of adaptation which
354 often emphasize a gradual process (Fisher, 1930). Due to the time taken for resistance to be
355 recognized, evolutionary studies of pesticide resistance are often *post hoc*. Here, we conducted
356 field monitoring of resistance to a novel acaricide, cyetpyrafen, following its 2017 release, with
357 the opportunity to track resistance as it develops. We provided a longitudinal picture of
358 resistance development in the field involving multiple and recurrently evolved large-effect
359 mutations. Cross-resistance was found between cyetpyrafen and two other SDHi acaricides—
360 cyflumetofen and cyenopyrafen—which were released in China before and after cyetpyrafen,
361 respectively. Although we could not clearly identify the contributions of these acaricides to
362 resistance evolution, they have only held a very small market share compared to cyetpyrafen
363 which is expected to be the main contributor to rapid resistance evolution.

364 Currently available pesticides mainly act by direct binding to receptors and enzymes,
365 resulting in the inhibition of physiological functions associated with the nervous system, energy

366 production system, growth and development, and midgut absorption (Sparks et al., 2021).
367 Mutations that alter the binding kinetics of pesticides on these proteins and thus potentially
368 confer pesticide resistance have been widely identified in agricultural pests and disease vectors
369 (De Rouck et al., 2023, Acford-Palmer et al., 2023). Resistant mutations of some target genes
370 are highly conserved both among populations and among species, perhaps due to the
371 functional constraints of these targets (Ffrench-Constant et al., 1993a, Hawkins et al., 2019).
372 However, multiple mutations on single pesticide targets have also been identified in the same
373 pest species and among different species. These cases often involve the voltage gated sodium
374 channel (*VGSC*) gene, where at least 5 and 10 mutations have been cumulatively reported
375 across studies on TSSM and the ectoparasite *Dermanyssus gallinae* respectively (De Rouck et al.,
376 2023). Similarly, the evolution of resistance to acetolactate synthase (ALS)-inhibiting herbicides
377 exhibits high repeatability across weed species, primarily driven by numerous SNPs within the
378 ALS target gene (Gaines et al., 2020). To date, 29 distinct target-site mutations have been
379 identified on eight amino acid residues across 67 weed species (Tranel et al., 2025). Notably,
380 even within a single weed species, up to eight different mutations can occur on three amino
381 acid residues (Tranel et al., 2025).

382 Here we found 15 mutations on eight amino acid residues in TSSM that all arose within a
383 few years following the introduction of cyetpyrafen. Notably, five of the substitutions involve
384 R119 of the *sdhD* gene. As far as we know, this is the highest number of both mutations and
385 altered residues ever found in a single pesticide target arthropod pests , all emerging in a short
386 period following initial detection of resistance. These mutations were located at the Q-site of
387 SDH targeted by acaricidal inhibitors of SDH. Among these mutations, I260T was previously
388 found in a cyflumetofen-resistant population (Sugimoto et al., 2020), while I260V (with S56L in
389 subunit C) was found in a pyflubumide and cyenopyrafen-resistant population (Maeoka and
390 Osakabe, 2021). R119L has previously been reported in cyflumetofen resistant and susceptible
391 strains of TSSM but had not yet been validated as having a role in resistance (Sugimoto et al.,
392 2020). These results provide information on the nature of novel mutations in a pesticide target

393 and the high level of resistance to cyetpyrafen that they can confer. It would be interesting to
394 further validate the impacts of the mutations across chemicals to establish patterns of cross
395 resistance given that the same mutation may confer a different level of resistance to different
396 chemicals (Maeoka and Osakabe, 2021).

397 Our temporal collections of TSSM preceding cyetpyrafen release in the field indicated that
398 the resistant mutations were not at an appreciable frequency prior to pesticide selection.
399 Sequencing of 13 populations (involving a total of 2666 TSSMs) from 2013 to 2018 did not
400 recover any of the identified mutations. This points to a possible role of recurrent *de novo*
401 mutation, although resistant alleles could have been present at very low frequencies before
402 pesticide selection which can be difficult to detect (Hawkins et al., 2019). In laboratory
403 populations of TSSM, the H258Y mutation arose *de novo* and was subsequently favored by
404 continuous selection, resulting in resistance to pyflubumide and cyenopyrafen (Njiru et al.,
405 2023). Our selection experiments also led to new mutations (I260V, R119C) arising over 66
406 generations (2 years) of cyetpyrafen selection in the LabR population. Although it is hard to
407 completely exclude the possibility of contamination during laboratory selection, because TSSM
408 is tiny and can easily be dispersed passively, the haplotype analysis showed that the two
409 mutations in the lab-selected population likely arose *de novo*. In the same way, multiple *de*
410 *novo* mutations could have contributed to the rapid evolution of resistance in different
411 populations and regions as documented in our field surveys.

412 Although few studies have traced resistance evolution in the field, they suggest that both
413 *de novo* mutations and standing genetic variation can contribute to resistance, with their
414 relative importance depending on the specific pesticides and pests involved (Hawkins et al.,
415 2019). Resistant alleles can originate from a single event and spread to distant populations by
416 migration (Raymond et al., 1991, Daborn et al., 2002), but resistant mutations can also have
417 multiple origins (Guan et al., 2021, Ilias et al., 2014, Troczka et al., 2012, Kreiner et al., 2022b,
418 Schmidt et al., 2024). Direct evidence for multiple origins comes from the presence of the same
419 mutation on multiple haplotypes, assuming that recombination can be excluded as an

420 explanation because insufficient time has elapsed after a resistant mutation is introduced into a
421 new background (Semenov et al., 2019). In our study, the requisite recombinant haplotypes
422 were rarely found in the same population, and we therefore suspect that recombination is
423 unlikely to be the source of patterns of haplotype variation over short DNA sequences. We
424 suspect that populations of mites like TSSM have a strong evolutionary potential to evolve *in*
425 *situ* rather than relying on gene flow, and that there is a repeatability of evolutionary changes
426 when an organism is exposed to the same selection pressure, a long-standing issue in
427 evolutionary biology (Konecna et al., 2021). The recurrent appearance of the same mutation in
428 different genetic backgrounds suggests that the likelihood of resistance evolution is high in
429 TSSM and not dependent on gene flow, and the likelihood of this scenario is further increased
430 by the fact that resistance can develop through several mutational options. We observed 2.7
431 resistant mutations per population in samples collected in 2024, seven years after the release
432 of cyetpyrafen. The estimated mutation rate (Θ) is 0.0193, assuming 20 generations per year
433 for TSSM. An effective population size (N_e) of $2.29*10^6$ would be necessary to reach the number
434 of *de novo* mutation observed in this study, given $\Theta = 3N_e\mu$ (haplodiploid sex determination
435 of TSSM) and a mutation rate of $\mu = 2.8*10^{-9}$ per base pair per generation as estimated for
436 *Drosophila melanogaster* (Keightley et al., 2014). The high reproductive capacity (> 100 eggs per
437 female) and short generation time of TSSM make it possible to reach such a large population
438 size in the field.

439 Because field populations of TSSM can use multiple mutations to reach the same
440 phenotypic endpoint (resistance), it is unlikely that all resistant mutations will be identified
441 from laboratory selection studies particularly if these involve a single laboratory population.
442 Even in the case where multiple populations of TSSM have been selected for resistance (to
443 cyenopyrafen and the related chemicals cyflumetofen and pyflubumide), QTL mapping still only
444 identified a few of the relevant mutations providing resistance to each chemical (Sugimoto et
445 al., 2020). Additionally, mutations identified in laboratory selection may not be involved in
446 resistance in the field because of fitness costs. For instance, H258Y conferred resistance to

447 cyenopyrafen and pyflubumide in laboratory-selected populations is associated with a high
448 fitness cost in TSSM (Njiru et al., 2023), and also likely reinforces cyflumetofen binding and
449 toxicity (Njiru et al., 2022). Relying on a limited set of resistance alleles identified in the
450 laboratory for monitoring may bias inferences of resistance evolution in the field where many
451 alleles can produce resistance. Genetic and evolutionary studies on field populations are then
452 essential to develop a full picture of resistance evolution (Konecna et al., 2021, Lucas et al.,
453 2023, Schmidt et al., 2024, Kersten et al., 2023, Pezzini et al., 2024).

454 Theoretical and empirical studies have showed that field-evolved resistance often involves
455 large-effect alleles with a simple genetic basis, while laboratory selection may result in
456 polygenic genetic architectures, due to differences in the occurrence of rare alleles (more likely
457 in large field populations) and different selection pressures (more variable with intense periods
458 in the field on occasions) (Ffrench-Constant, 2013, Roush and McKenzie, 1987, Ffrench-
459 Constant et al., 1990, McKenzie and Batterham, 1994, Crow, 1954). This could potentially be
460 mitigated by establishing laboratory selection lines using large starting populations from the
461 field with pre-existing resistance alleles (Gould et al. 1997). Until recently, few studies have
462 directly compared the genetic basis of resistance in laboratory populations with field-evolved
463 resistance across a large geographical scale (McKenzie et al., 1992, Ffrench-Constant et al.,
464 1990, Crow, 1954, Legan et al., 2024). Here we identified large-effect alleles in both field- and
465 laboratory-selected resistant populations. High-level resistance was generated after lab
466 selection with cyetpyrafen for 33 of 65 generations, almost at the same rate as the field
467 populations where high-level resistance was observed three years after the commercial release
468 of cyetpyrafen. In field populations, it seems that multiple allelic options can favored by
469 selection due to parallel adaptation, and that the same allele may also end up residing in
470 different haplotypes in different areas. Through migration these alleles/backgrounds may
471 eventually end up in the same population to produce a polyallelic basis of adaptation where
472 particular alleles may then not reach high frequencies unless one haplotype is favored (Ralph
473 and Coop, 2010).

474 Pesticide resistance provides many fascinating examples of rapid and recent evolution
475 under high selection pressure, which can be leveraged to address fundamental issues about the
476 evolutionary basis of adaptations to new environments (Hawkins et al., 2019), and also to
477 examine the reliability of commonly used research methods. The development of high-
478 throughput sequencing and population genomics methodologies provides an opportunity to
479 detect selective signatures across the genome (Van Etten et al., 2020, Cohen et al., 2022,
480 Pélissié et al., 2022). Although pesticide resistance is a rapid evolutionary process under strong
481 selective pressures, identifying candidate genes directly from genomic signals remains difficult
482 when adaptation occurs via soft sweeps (Pennings and Hermisson, 2006, Schrider et al., 2015).
483 Soft sweeps may be common in adaptive evolution if the effective mutation rate ($4N_e\mu$) >>
484 0.01, e.g., through large population sizes (N_e), and/or high allelic mutation rates (Hermisson and
485 Pennings, 2005, Pennings and Hermisson, 2006). The multiple haplotypes and distinct
486 evolutionary history of SDH genes among resistant field populations suggest a prevalence of
487 soft selective sweeps through recurrent mutation as implicated in pesticide resistance in other
488 pest systems, such as *Drosophila* (Garud and Petrov, 2016), and the oomycete *Plasmopara*
489 *viticola* (Delmas et al., 2017). This is congruent with the 'messier' signals found in field
490 populations relative to the artificially-selected resistant laboratory population. Sweeps around
491 the SDH genes are incomplete, or other contributing loci are undergoing selective sweeps,
492 which may further reduce signal to detect adaptation (Pennings and Hermisson, 2006, Schrider
493 et al., 2015). Fewer mutation types and haplotypes along with higher mutation frequency are
494 more likely to be detected. Clearer signals of selective sweeps could emerge from haplotype-
495 based linkage-disequilibrium methods using individual-level genomic data (Hermisson and
496 Pennings, 2017).

497 In conclusion, we have captured a detailed picture of contemporary evolutionary
498 processes under pesticide selection in field populations of an invertebrate pest. We
499 demonstrate that the rapid evolution of pesticide resistance is driven by an unprecedented
500 number of population-specific mutations in target genes that arose recurrently. These findings

501 reveal complex origins and diverse genetic bases of pesticide resistance in the field. These
502 findings represent a challenge for SNP-specific molecular monitoring of resistance and for the
503 implementation of effective resistance management strategies. They also highlight the value of
504 amplicon and whole-genome sequencing to detect multiple target-site mutations. Finally, we
505 call for future studies on field-evolved resistance to provide theoretical and practical insights
506 into effective pest control, leveraging the genomics era.

507 **4. Materials and Methods**

508 ***4.1 Samples***

509 We used 72 field-collected and laboratory-selected populations of TSSM across China for
510 bioassays, whole-genome resequencing, Sanger sequencing of target genes, and establishment
511 of mutant strains (Table S1), including 15 populations collected before (2013 - 2017) and 55
512 populations collected after (2018 - 2024) the release of cyetpyrafen, as well as one laboratory-
513 selected resistant population, and one laboratory-maintained susceptible population. More
514 than 500 adults were collected from at least three points separated by 100 meters for each
515 population. Samples were kept in 100% alcohol or transported live to the laboratory for
516 chemical bioassays. Because TSSM has a haplodiploid sex-determination system, only female
517 adults were randomly selected for bioassays and genotyping. A initial colony of about 4,000
518 individuals was established from the field population ZJXS1 in 2018, which was subsequently
519 divided into two parallel cohorts under controlled laboratory conditions. The selection group
520 (LabR) was subjected to continuous selection pressure using cyetpyrafen, while the control
521 group (LabS) was maintained under identical laboratory conditions without exposure to
522 acaricidal agents. The TSSM strain was reared on French bean, *Pemphigus vulgaris* L., under
523 25±1 °C, 60±5% relative humidity and a photoperiod of L16h: D8h. We continuously selected
524 this population in the laboratory by killing about 60% to 80% of randomly selected individuals
525 every two generations, to obtain a laboratory-selected resistant strain. When the mortality was
526 lower than 60%, another round of spraying was applied by increasing the dosage of cyetpyrafen.

527 The LC_{50} values were tested at F_1, F_{32}, F_{54}, F_{60}, F_{62}, and F_{66} generations to quantify resistance
528 development trajectories.

529 ***4.2 Bioassay***

530 Bioassays were conducted to test the susceptibility of TSSM to acaricides using a Potter Spray
531 Tower (Burkard Scientific, London, UK). We used transparent plastic cups (6 cm in diameter and
532 4 cm in height) to keep the female adult mites. A layer of 0.2% agar was placed on the bottom
533 of the cups to avoid leaves drying out. Fresh leaves of *P. vulgaris* were cut into a circle with a
534 diameter of 6 cm to fit the cup and put onto the agar. The borders of the leaves were sealed
535 using 0.2% agar. Thirty female adults were carefully moved into a plastic cup by suction trap.
536 Two ml acaricide solution was applied through a Potter spray tower (Burkard Manufacturing
537 Co. Ltd., UK) at 68.9 kPa and a sedimentation time of 30 s. Each population was exposed to
538 between five and eight pre-estimated concentrations of acaricides, diluted with water
539 containing 0.1% Triton X-100 (Beijing Solar BioScience and Technology Limited Company,
540 China). The 0.1% Triton X-100 solution was used as a control treatment to correct the potential
541 influence of adjuvants in the acaricides. The treated mites were kept under 25 °C, 60-70%
542 relative humidity, and a photoperiod of 16 h:8 h (L:D). Mortality was assessed under a Stemi
543 305 stereomicroscope (Zeiss, Germany), 48 hours after spraying. The tested TSSMs were
544 considered dead if no movement of appendages was observed when they were prodded with a
545 fine brush at 24 hours and 48 hours after spraying. The 50% lethal concentration (LC_{50}) and its
546 95% confidence intervals were calculated using probit regressions implemented in the
547 statistical software DPS v12.01 (Tang and Zhang, 2013).

548 ***4.3 Pooled whole-genome resequencing and SNP calling***

549 Between 50 and 400 individuals per population were used for pooled genome resequencing to
550 obtain population genomic data for diversity estimates and demographic analyses. DNA
551 libraries with insert sizes of 400 bp were constructed and sequenced on the Illumina NovaSeq
552 6000 platform to obtain 150 bp paired-end reads. Raw reads were filtered using fastp v0.23.0

553 (Chen et al., 2018). The filtered reads were mapped to our recently assembled chromosome-
554 level genome (Cao et al., 2024) using bwa-mem2 2.2.1 with default parameters (Vasimuddin et
555 al., 2019). SAMtools v1.14 (Li et al., 2009) was used to sort the sequence data, removing
556 duplicated reads, removing reads with mapping quality < 20, and converting bam files to
557 mpileup files. Indels in mpileup files were filtered out (with a 5-bp margin on each side) to avoid
558 spurious SNP calling using scripts from PoPoolation (Kofler et al., 2011a). Then mpileup files
559 were converted using scripts from PoPoolation2 (Kofler et al., 2011b).

560 ***4.4 Population genetic diversity and demography analyses***

561 Genetic diversity in each population was quantified by estimating nucleotide diversity (π) and
562 Tajima's *D* across 5 kb non-overlapping windows, using PoPoolation (Kofler et al., 2011a). To
563 reduce the impact of read depth on estimates of nucleotide diversity, each alignment was
564 converted into a population-specific pileup file using SAMtools, and reads were subsampled
565 without replacement up to a depth of 50 (Hoban et al., 2016). The mean F_{ST} across all windows
566 for each pairwise population comparison was calculated using PoPoolation2 (Kofler et al.,
567 2011a) across 5 kb non-overlapping windows. To infer population relationships and historical
568 migration, a rooted maximum likelihood tree was constructed with a strict drift model (no
569 migration) using Treemix v1.13 (Pickrell and Pritchard, 2012). The SCCD population was set as
570 root and we tested migration events from 0 to 10 with at least 100 runs for each case. The best
571 number of migration events was determined by the second-order rate of change in likelihood
572 (Δm) across incremental migration events (m) using the Evanno method (Evanno et al., 2005)
573 embedded in R package 'optM' (Fitak, 2021). For the best m, we performed 1000 runs to
574 generate 1000 maximum likelihood trees and obtained a consensus tree using the program
575 SumTrees version 4.10 (Sukumaran and Holder, 2010).

576 ***4.5 Genome scan of outlier SNPs***

577 We investigated the genomic signals of selection around the four SDH genes (± 1 Mb) and
578 across the whole genome. The main statistic used to identify outlier regions was the population

579 branch excess (PBE) statistic (Yassin et al., 2016). The PBE statistic is based on comparisons of
580 F_{ST} values among three populations, comprising a focal population and two nonfocal
581 populations (Pool et al., 2017). In the analysis, each resistant population was set as the focal
582 population, and two susceptible populations (LabS and NMHH2) were set as the nonfocal
583 populations. PBE values were calculated for 5 kb non-overlapping sliding windows. Windows
584 with top 5% PBE value were considered outliers. Patterns of nucleotide diversity (π) estimates
585 and Tajima's D values around the SDH genes were also examined. We expected resistant
586 populations to have lower nucleotide diversity and Tajima's D values near the SDH genes due to
587 directional selection favoring resistant haplotypes. The coding effects of SNPs within the SDH
588 genes were obtained using SnpEff v4.3 (Cingolani et al., 2012).

589 ***4.6 SDH homology modeling***

590 The three-dimensional (3D) structures of the wild and mutational SDH from TSSM were
591 modeled with SWISS-MODEL (Waterhouse et al., 2018) using 8GS8.1 (Du et al., 2023) as a
592 template. To examine the conservation of *SdhB* and *SdhD* genes, we collected the protein
593 sequences of two genes of different animals in the protein database of NCBI
594 (https://www.ncbi.nlm.nih.gov/protein). Then, MUSCLE in MEGA v11 (Tamura et al., 2021)
595 were used to perform sequence alignment and trim protein sequences around mutation points.
596 After removing identical sequences of the same species, the multiple sequence alignment
597 sequences were used to determine conservation and visualized with WebLogo 3 (Crooks et al.,
598 2004).

599 ***4.7 PCR amplification and sequencing of target genes***

600 The target genes sequences of 16 - 48 individuals from each population were used to
601 characterize the spatiotemporal variation of these target-site mutations in these populations
602 before and after the release of cyetpyrafen (Table S1). PCR primers were designed for
603 sequences of the *sdhB* gene (sdhb_F: GAGTCTAGCCGCAGTCTGAT, sdhb_R:
604 ACTTGGGACCTGCTGTACTC) and the *sdhD* gene (sdhd_F: GCGATGGATAAGGCATAGACG, sdhd_R:

605 TCTGGTCAGTGTGGTTCCTC) to capture the candidate mutation sites. Polymerase chain reaction
606 (PCR) was conducted in the Mastercycler pro system (Eppendorf, Germany) under an annealing
607 temperature of 54 °C for 1 min using Promega Go Taq™ Master Mixes (Madison, Wisconsin,
608 USA). Amplified products were purified and sequenced directly from one strand using an ABI
609 3730xl DNA Analyzer by Sangon Biotech Co. Ltd. (Shanghai, China).

610 ***4.8 Haplotype analysis***

611 Haplotypes of each gene were identified from Sanger sequencing. We constructed haplotype
612 networks for *sdhB* and *sdhD* from individuals that contained homozygous resistant genotypes.
613 Haplotype networks were constructed using the R package *pegas* (Paradis, 2010), and were
614 visualized with the R package *ggplot2*.

615 ***4.9 Association analysis between mutation frequency and resistance level***

616 Regressions were run to assess the strength of association between target-site mutation
617 frequencies and mean population-level resistance phenotypes. Since LC_{50} of some populations
618 could not be calculated or were inaccurate due to high resistance levels, the survival rate after
619 spraying with 1000 mg/L cyetpyrafen, which is about 6.7 to 10-fold of field-recommended dose,
620 was used to quantify the resistance level of TSSM to cyetpyrafen. Because multiple target-site
621 mutations existed, we evaluated the associations using different metrics of target-site mutation
622 frequencies: (i) the frequency of the predominant mutation in each population, (ii) the
623 frequency of individuals carrying any mutation in each population, and (iii) the frequency of
624 individuals with homozygous mutations in each population. Regressions were fitted as linear
625 models using the *lm* function from the R package *stats* and visualized with *ggplot2*. We did not
626 adjust associations for relatedness among populations because the evolutionary events leading
627 to resistance appear to be largely independent. A logistic regression model was used to track
628 trajectories of allele frequencies. The selection coefficient of each allele (s) and their joint effect
629 ($\tilde{s}$) were estimated (Kreiner et al., 2022a). We calculated F_{ST} using the frequency of resistance
630 alleles following Hartl et al. (1997) and explored isolation by distance (IBD) with Mantel tests in
631 the R package *vegan*.

632 ***4.10 Establishment of homozygous lines***

633 Single immature TSSM females were picked from resistant populations and reared individually
634 in a petri dish. When unmated, females will only produce haploid male eggs. To obtain the
635 genotype of males for the *sdh* genes, the DNA of each female was extracted and genotyped as
636 mentioned above after it had laid more than 10 eggs, while the eggs (> 10) were allowed to
637 develop into male adults (F_1 male adults). To obtain the genotype of virgin females, the first six
638 eggs were genotyped while the females were kept live (F_0 females). Then, each pair of F_0 female
639 and F_1 male adults with the same homozygous genotypes for the six mutation sites was placed
640 into the same petri dish and left to mate and produce male and female progeny, with females
641 from the line generated defined as a line homozygous for the target resistance mutation(s). The
642 mated male and female pairs were from the same population to reduce differences in their
643 genetic backgrounds. The homozygous lines at the mutation sites were tested for susceptibility
644 to cyetpyrafen with the bioassay method described above.

645 **Author contributions:** S.J.W. designed research; J.C.C., L.J.C., L.X.Z., Y.Y, M.C.Y., J.Y.Z., X.Y.Z.,
646 and Y.J.G., performed research; L.J.C., S.J.W., J.A.T, T.L.S, Q.Y., and A.A.H analyzed data; S.J.W.,
647 A.A.H., L.J.C., J.A.T., T.L.S., H.L., R.F.C., and X.X.C. wrote the paper.

648 **Acknowledgments:** We thank Ming-Liang Li, Hong-Ping Tian, Liang-Bin Zeng, for their assistance
649 with sample collections. This study is supported by National Key R&D Program of China
650 (2023YFD1401200) and Program of Beijing Academy of Agriculture and Forestry Sciences
651 (QNJJ202006, JKZX202208). Ary Hoffmann and Joshua Thia are supported by the Grains
652 Research and Development Corporation and Horticulture Innovation (Australia).

653 **Data availability statement:** Raw reads of whole genome resequencing data were submitted to
654 NCBI Sequence Read Archive, with accession numbers SRR30666541 - SRR30666561 and
655 SRR28000465. All scripts and data used in the study were deposited to GitHub:
656 https://github.com/caolijun/TU_pool-seq and Figshare: 10.6084/m9.figshare.27231003 (Wei
657 and Cao, 2025).

658 **Conflict of interest:** No competing interests.

659 **References**

660 ACFORD-PALMER, H., CAMPOS, M., BANDIBABONE, J., N'DO, S., BANTUZEKO, C., ZAWADI, B., WALKER, T.,
661 PHELAN, J. E., MESSENGER, L. A., CLARK, T. G. & CAMPINO, S. 2023. Detection of insecticide
662 resistance markers in *Anopheles funestus* from the Democratic Republic of the Congo using a
663 targeted amplicon sequencing panel. *Scientific Reports,* 13**,** 17363.

664 ADESANYA, A. W., LAVINE, M. D., MOURAL, T. W., LAVINE, L. C., ZHU, F. & WALSH, D. B. 2021.
665 Mechanisms and management of acaricide resistance for *Tetranychus urticae* in agroecosystems.
666 *Journal of Pest Science,* 94**,** 639-663.

667 ANDREEV, D., KREITMAN, M., PHILLIPS, T. W. & BEEMAN, R. W. 1999. Multiple origins of cyclodiene
668 insecticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Journal of Molecular*
669 *Evolution,* 48**,** 615-624.

670 BRAS, A., ROY, A., HECKEL, D. G., ANDERSON, P. & KARLSSON GREEN, K. 2022. Pesticide resistance in
671 arthropods: Ecology matters too. *Ecology Letters,* 25**,** 1746-1759.

672 CAO, L. J., GUAN, T. B., CHEN, J. C., YANG, F. Y., LIU, J. X., JIN, F. L. & WEI, S. J. 2024. Chromosome-level
673 genome assembly of the two-spotted spider mite *Tetranychus urticae*. *Scientific Data,* 11**,** 798.

674 CHEN, J. C., GONG, Y. J., SHI, P., WANG, Z. H., CAO, L.-J., WANG, P. & WEI, S. J. 2019. Field-evolved
675 resistance and cross-resistance of the two-spotted spider mite, *Tetranychus urticae*, to
676 bifenazate, cyenopyrafen and SYP-9625. *Experimental and Applied Acarology,* 77**,** 545-554.

677 CHEN, S., ZHOU, Y., CHEN, Y. & GU, J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor.
678 *Bioinformatics,* 34**,** i884-i890.

679 CHEN, Y. H., COHEN, Z. P., BUENO, E. M., CHRISTENSEN, B. M. & SCHOVILLE, S. D. 2023. Rapid evolution
680 of insecticide resistance in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Current*
681 *Opinion in Insect Science,* 55**,** 101000.

682 CINGOLANI, P., PLATTS, A., WANG LE, L., COON, M., NGUYEN, T., WANG, L., LAND, S. J., LU, X. & RUDEN, D.
683 M. 2012. A program for annotating and predicting the effects of single nucleotide
684 polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-
685 3. *Fly (Austin),* 6**,** 80-92.

686 COHEN, Z. P., CHEN, Y. H., GROVES, R. & SCHOVILLE, S. D. 2022. Evidence of hard-selective sweeps
687 suggests independent adaptation to insecticides in Colorado potato beetle (Coleoptera:
688 Chrysomelidae) populations. *Evolutionary Applications,* 15**,** 1691-1705.

689 CROOKS, G. E., HON, G., CHANDONIA, J.-M. & BRENNER, S. E. 2004. WebLogo: A squence logo generator.
690 *Genome Research,* 14**,** 1188-1190.

691 CROW, J. F. 1954. Analysis of a DDT-resistant strain of *Drosophila*. *Journal of Economic Entomology,* 47**,**
692 393–398.

693 D'COSTA, V. M., KING, C. E., KALAN, L., MORAR, M., SUNG, W. W., SCHWARZ, C., FROESE, D., ZAZULA, G.,
694 CALMELS, F. & DEBRUYNE, R. J. N. 2011. Antibiotic resistance is ancient. *Nature,* 477**,** 457-461.

695 DABORN, P. J., YEN, J. L., BOGWITZ, M. R., LE GOFF, G., FEIL, E., JEFFERS, S., TIJET, N., PERRY, T., HECKEL, D.,

696 BATTERHAM, P., FEYEREISEN, R., WILSON, T. G. & FFRENCH-CONSTANT, R. H. 2002. A single p450
697 allele associated with insecticide resistance in *Drosophila*. *Science,* 297**,** 2253-2256.

698 DE ROUCK, S., İNAK, E., DERMAUW, W. & VAN LEEUWEN, T. 2023. A review of the molecular mechanisms
699 of acaricide resistance in mites and ticks. *Insect Biochemistry and Molecular Biology,* 159**,**
700 103981.

701 DELMAS, C. E., DUSSERT, Y., DELIèRE, L., COUTURE, C., MAZET, I. D., RICHART CERVERA, S. & DELMOTTE, F.
702 2017. Soft selective sweeps in fungicide resistance evolution: recurrent mutations without
703 fitness costs in grapevine downy mildew. *Molecular Ecology,* 26**,** 1936-1951.

704 DU, Z., ZHOU, X., LAI, Y., XU, J., ZHANG, Y., ZHOU, S., FENG, Z., YU, L., TANG, Y., WANG, W., YU, L., TIAN, C.,
705 RAN, T., CHEN, H., GUDDAT, L. W., LIU, F., GAO, Y., RAO, Z. & GONG, H. 2023. Structure of the
706 human respiratory complex II. *Proceedings of the National Academy of Sciences of the United*
707 *States of America,* 120**,** e2216713120.

708 EVANNO, G., REGNAUT, S. & GOUDET, J. 2005. Detecting the number of clusters of individuals using the
709 software STRUCTURE: a simulation study. *Molecular Ecology,* 14**,** 2611-20.

710 FFRENCH-CONSTANT, R., ROUSH, R. T., MORTLOCK, D. & DIVELY, G. 1990. Isolation of dieldrin resistance
711 from field populations of *Drosophila melanogaster* (Diptera: Drosophilidae). *Journal of Economic*
712 *Entomology,* 83**,** 1733-1737.

713 FFRENCH-CONSTANT, R., STEICHEN, J., ROCHELEAU, T., ARONSTEIN, K. & ROUSH, R. 1993a. A single-
714 amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated
715 with cyclodiene insecticide resistance in Drosophila populations. *Proceedings of the National*
716 *Academy of Sciences of the United States of America,* 90**,** 1957-1961.

717 FFRENCH-CONSTANT, R. H. 2007. Which came first: insecticides or resistance? *Trends in Genetics,* 23**,** 1-4.

718 FFRENCH-CONSTANT, R. H. 2013. The molecular genetics of insecticide resistance. *Genetics,* 194**,** 807-815.

719 FFRENCH-CONSTANT, R. H., DABORN, P. J. & LE GOFF, G. 2004. The genetics and genomics of insecticide
720 resistance. *Trends in Genetics,* 20**,** 163-170.

721 FFRENCH-CONSTANT, R. H., ROCHELEAU, T. A., STEICHEN, J. C. & CHALMERS, A. E. 1993b. A point
722 mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature,* 363**,** 449-451.

723 FISHER, R. A. 1930. *The genetical theory of natural selection*, Oxford University Press.

724 FITAK, R. R. 2021. OptM: estimating the optimal number of migration edges on population trees using
725 Treemix. *Biology Methods and Protocols,* 6**,** bpab017.

726 FRITZ, M. L. 2022. Utility and challenges of using whole-genome resequencing to detect emerging insect
727 and mite resistance in agroecosystems. *Evolutionary Applications,* 15**,** 1505-1520.

728 GAINES, T. A., DUKE, S. O., MORRAN, S., RIGON, C. A. G., TRANEL, P. J., KüPPER, A. & DAYAN, F. E. 2020.
729 Mechanisms of evolved herbicide resistance. *Journal of Biological Chemistry,* 295**,** 10307-10330.

730 GARUD, N. R. & PETROV, D. A. 2016. Elevated linkage disequilibrium and signatures of soft sweeps are
731 common in *Drosophila melanogaster*. *Genetics,* 203**,** 863-880.

732 GONG, Y.-J., CHEN, J.-C., JIANG, J.-Y., WANG, Z.-H., CAO, L.-J. & WEI, S.-J. 2017. Toxicity and field control
733 efficacy of the new acaricide SYP-9625 to the two-spotted spider mite (*Tetranychus urticae* koch).
734 *Agrochemicals,* 56**,** 561-563.

735 GOULD, F., BROWN, Z. S. & KUZMA, J. 2018. Wicked evolution: Can we address the sociobiological
736 dilemma of pesticide resistance? *Science,* 360**,** 728-732.

737 GROETERS, F. R. & TABASHNIK, B. E. 2000. Roles of selection intensity, major genes, and minor genes in
738 evolution of insecticide resistance. *Journal of Economic Entomology,* 93**,** 1580-1587.

739 GUAN, F., HOU, B., DAI, X., LIU, S., LIU, J., GU, Y., JIN, L., YANG, Y., FABRICK, J. A. & WU, Y. 2021. Multiple
740 origins of a single point mutation in the cotton bollworm tetraspanin gene confers dominant
741 resistance to Bt cotton. *Pest Management Science,* 77**,** 1169-1177.

742 HARTL, D. L., CLARK, A. G. & CLARK, A. G. 1997. *Principles of population genetics*, Sinauer associates
743 Sunderland, MA.

744 HARTLEY, C., NEWCOMB, R., RUSSELL, R., YONG, C., STEVENS, J., YEATES, D., LA SALLE, J. & OAKESHOTT, J.
745 2006. Amplification of DNA from preserved specimens shows blowflies were preadapted for the
746 rapid evolution of insecticide resistance. *Proceedings of the National Academy of Sciences of the*
747 *United States of America,* 103**,** 8757-8762.

748 HAWKINS, N. J., BASS, C., DIXON, A. & NEVE, P. 2019. The evolutionary origins of pesticide resistance.
749 *Biological Reviews,* 94**,** 135-155.

750 HAYASHI, N., SASAMA, Y., TAKAHASHI, N. & IKEMI, N. 2013. Cyflumetofen, a novel acaricide–its mode of
751 action and selectivity. *Pest Management Science,* 69**,** 1080-1084.

752 HERMISSON, J. & PENNINGS, P. S. 2005. Soft sweeps: molecular population genetics of adaptation from
753 standing genetic variation. *Genetics,* 169**,** 2335-52.

754 HERMISSON, J. & PENNINGS, P. S. 2017. Soft sweeps and beyond: understanding the patterns and
755 probabilities of selection footprints under rapid adaptation. *Methods in Ecology and Evolution,* 8**,**
756 700-716.

757 HOBAN, S., KELLEY, J. L., LOTTERHOS, K. E., ANTOLIN, M. F., BRADBURD, G., LOWRY, D. B., POSS, M. L.,
758 REED, L. K., STORFER, A. & WHITLOCK, M. C. 2016. Finding the genomic basis of local adaptation:
759 pitfalls, practical solutions, and future directions. *American Naturalist,* 188**,** 379-397.

760 ILIAS, A., VONTAS, J. & TSAGKARAKOU, A. 2014. Global distribution and origin of target site insecticide
761 resistance mutations in *Tetranychus urticae*. *Insect Biochemistry and Molecular Biology,* 48**,** 17-
762 28.

763 KEIGHTLEY, P. D., NESS, R. W., HALLIGAN, D. L. & HADDRILL, P. R. 2014. Estimation of the spontaneous
764 mutation rate per nucleotide site in a *Drosophila melanogaster* full-sib family. *Genetics,* 196**,**
765 313-20.

766 KERSTEN, S., CHANG, J., HUBER, C. D., VOICHEK, Y., LANZ, C., HAGMAIER, T., LANG, P., LUTZ, U.,
767 HIRSCHBERG, I., LERCHL, J., PORRI, A., PEER, Y. V. D., SCHMID, K., WEIGEL, D. & RABANAL, F. A.
768 2023. Standing genetic variation fuels rapid evolution of herbicide resistance in blackgrass.
769 *Proceedings of the National Academy of Sciences of the United States of America,* 120**,**
770 e2206808120.

771 KOFLER, R., OROZCO-TERWENGEL, P., DE MAIO, N., PANDEY, R. V., NOLTE, V., FUTSCHIK, A., KOSIOL, C. &
772 SCHLOTTERER, C. 2011a. PoPoolation: a toolbox for population genetic analysis of next
773 generation sequencing data from pooled individuals. *PLoS ONE,* 6**,** e15925.

774 KOFLER, R., PANDEY, R. V. & SCHLOTTERER, C. 2011b. PoPoolation2: identifying differentiation between

775 populations using sequencing of pooled DNA samples (Pool-Seq). *Bioinformatics,* 27**,** 3435-3436.

776 KONECNA, V., BRAY, S., VLCEK, J., BOHUTINSKA, M., POZAROVA, D., CHOUDHURY, R. R., BOLLMANN-
777 GIOLAI, A., FLIS, P., SALT, D. E., PARISOD, C., YANT, L. & KOLAR, F. 2021. Parallel adaptation in
778 autopolyploid *Arabidopsis arenosa* is dominated by repeated recruitment of shared alleles.
779 *Nature Communications,* 12**,** 4979.

780 KREINER, J. M., GIACOMINI, D. A., BEMM, F., WAITHAKA, B., REGALADO, J., LANZ, C., HILDEBRANDT, J.,
781 SIKKEMA, P. H., TRANEL, P. J., WEIGEL, D., STINCHCOMBE, J. R. & WRIGHT, S. I. 2019. Multiple
782 modes of convergent adaptation in the spread of glyphosate-resistant *Amaranthus tuberculatus*.
783 *Proceedings of the National Academy of Sciences of the United States of America,* 116**,** 21076-
784 21084.

785 KREINER, J. M., LATORRE, S. M., BURBANO, H. A., STINCHCOMBE, J. R., OTTO, S. P., WEIGEL, D. & WRIGHT,
786 S. I. 2022a. Rapid weed adaptation and range expansion in response to agriculture over the past
787 two centuries. *Science,* 378**,** 1079-1085.

788 KREINER, J. M., SANDLER, G., STERN, A. J., TRANEL, P. J., WEIGEL, D., STINCHCOMBE, J. R. & WRIGHT, S. I.
789 2022b. Repeated origins, widespread gene flow, and allelic interactions of target-site herbicide
790 resistance mutations. *elife,* 11**,** 70242.

791 LEGAN, A. W., ALLAN, C. W., JENSEN, Z. N., DEGAIN, B. A., YANG, F., KERNS, D. L., BENOWITZ, K. M.,
792 FABRICK, J. A., LI, X., CARRIèRE, Y., MATZKIN, L. M. & TABASHNIK, B. E. 2024. Mismatch between
793 lab-generated and field-evolved resistance to transgenic Bt crops in Helicoverpa zea. *Proceedings*
794 *of the National Academy of Sciences of the United States of America,* 121**,** e2416091121.

795 LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G.,
796 DURBIN, R. & GENOME PROJECT DATA PROCESSING, S. 2009. The sequence alignment/map
797 format and SAMtools. *Bioinformatics,* 25**,** 2078-2079.

798 LI, N. & STEPHENS, M. 2003. Modeling linkage disequilibrium and identifying recombination hotspots
799 using single-nucleotide polymorphism data. *Genetics,* 165**,** 2213-2233.

800 LUCAS, E. R., NAGI, S. C., EGYIR-YAWSON, A., ESSANDOH, J., DADZIE, S., CHABI, J., DJOGBENOU, L. S.,
801 MEDJIGBODO, A. A., EDI, C. V., KETOH, G. K., KOUDOU, B. G., VAN'T HOF, A. E., RIPPON, E. J.,
802 PIPINI, D., HARDING, N. J., DYER, N. A., CERDEIRA, L. T., CLARKSON, C. S., KWIATKOWSKI, D. P.,
803 MILES, A., DONNELLY, M. J. & WEETMAN, D. 2023. Genome-wide association studies reveal novel
804 loci associated with pyrethroid and organophosphate resistance in *Anopheles gambiae* and
805 *Anopheles coluzzii*. *Nature Communications,* 14**,** 4946.

806 MAEOKA, A. & OSAKABE, M. 2021. Co-occurrence of subunit B and C mutations in respiratory complex II
807 confers high resistance levels to pyflubumide and cyenopyrafen in the two-spotted spider mite
808 *Tetranychus urticae* (Acari: Tetranychidae). *Pest Management Science,* 77**,** 5149-5157.

809 MCKENZIE, J. A. & BATTERHAM, P. 1994. The genetic, molecular and phenotypic consequences of
810 selection for insecticide resistance. *Trends in Ecology and Evolution,* 9**,** 166-169.

811 MCKENZIE, J. A., PARKER, A. & YEN, J. 1992. Polygenic and single gene responses to selection for
812 resistance to diazinon in *Lucilia cuprina*. *Genetics,* 130**,** 613-620.

813 MELANDER, A. 1914. Can insects become resistant to sprays? *Journal of Economic Entomology,* 7**,** 167.

814 NAKAHIRA, K. 2011. Strategy for discovery of a novel miticide Cyenopyrafen which is one of electron

815 transport chain inhibitors. *Journal of Pesticide Science,* 36, 511-515.

816 NJIRU, C., SAALWAECHTER, C., GUTBROD, O., GEIBEL, S., WYBOUW, N. & VAN LEEUWEN, T. 2022. A
817 H258Y mutation in subunit B of the succinate dehydrogenase complex of the spider mite
818 *Tetranychus urticae* confers resistance to cyenopyrafen and pyflubumide, but likely reinforces
819 cyflumetofen binding and toxicity. *Insect Biochemistry and Molecular Biology,* 144, 103761.

820 NJIRU, C., SAALWAECHTER, C., MAVRIDIS, K., VONTAS, J., GEIBEL, S., WYBOUW, N. & VAN LEEUWEN, T.
821 2023. The complex II resistance mutation H258Y in succinate dehydrogenase subunit B causes
822 fitness penalties associated with mitochondrial respiratory deficiency. *Pest Management Science,*
823 79, 4403-4413.

824 PARADIS, E. 2010. pegas: an R package for population genetics with an integrated-modular approach.
825 *Bioinformatics,* 26, 419-20.

826 PéLISSIé, B., CHEN, Y. H., COHEN, Z. P., CROSSLEY, M. S., HAWTHORNE, D. J., IZZO, V. & SCHOVILLE, S. D.
827 2022. Genome resequencing reveals rapid, repeated evolution in the Colorado potato beetle.
828 *Molecular Biology and Evolution,* 39, msac016.

829 PENNINGS, P. S. & HERMISSON, J. 2006. Soft sweeps II—molecular population genetics of adaptation
830 from recurrent mutation or migration. *Molecular Biology and Evolution,* 23, 1076-1084.

831 PETERSON, M. A., COLLAVO, A., OVEJERO, R., SHIVRAIN, V. & WALSH, M. J. 2018. The challenge of
832 herbicide resistance around the world: a current summary. *Pest Management Science,* 74, 2246-
833 2259.

834 PEZZINI, D., TAYLOR, K. L., REISIG, D. D. & FRITZ, M. L. 2024. Cross-pollination in seed-blended refuge and
835 selection for Vip3A resistance in a lepidopteran pest as detected by genomic monitoring.
836 *Proceedings of the National Academy of Sciences of the United States of America,* 121,
837 e2319838121.

838 PICKRELL, J. K. & PRITCHARD, J. K. 2012. Inference of population splits and mixtures from genome-wide
839 allele frequency data. *PLoS Genetics,* 8, e1002967.

840 POOL, J. E., BRAUN, D. T. & LACK, J. B. 2017. Parallel evolution of cold tolerance within *Drosophila*
841 *melanogaster*. *Molecular Biology and Evolution,* 34, 349-360.

842 RALPH, P. & COOP, G. 2010. Parallel adaptation: one or many waves of advance of an advantageous allele?
843 *Genetics,* 186, 647-668.

844 RAYMOND, M., CALLAGHAN, A., FORT, P. & PASTEUR, N. 1991. Worldwide migration of amplified
845 insecticide resistance genes in mosquitoes. *Nature,* 350, 151.

846 ROUSH, R. T. & MCKENZIE, J. A. 1987. Ecological genetics of insecticide and acaricide resistance. *Annual*
847 *Review of Entomology,* 32, 361-380.

848 SCHMIDT, J. M., GOOD, R. T., APPLETON, B., SHERRARD, J., RAYMANT, G. C., BOGWITZ, M. R., MARTIN, J.,
849 DABORN, P. J., GODDARD, M. E. & BATTERHAM, P. 2010. Copy number variation and
850 transposable elements feature in recent, ongoing adaptation at the *Cyp6g1* locus. *PLoS Genetics,*
851 6, e1000998.

852 SCHMIDT, T. L., ENDERSBY-HARSHMAN, N. M., VAN ROOYEN, A. R. J., KATUSELE, M., VINIT, R., ROBINSON,
853 L. J., LAMAN, M., KARL, S. & HOFFMANN, A. A. 2024. Global, asynchronous partial sweeps at
854 multiple insecticide resistance genes in *Aedes* mosquitoes. *Nature Communications,* 15, 6251.

855 SCHRIDER, D. R., MENDES, F. K., HAHN, M. W. & KERN, A. D. 2015. Soft shoulders ahead: spurious
856 signatures of soft and partial selective sweeps result from linked hard sweeps. *Genetics,* 200**,**
857 267-284.

858 SEMENOV, G. A., SAFRAN, R. J., SMITH, C. C. R., TURBEK, S. P., MULLEN, S. P. & FLAXMAN, S. M. 2019.
859 Unifying theoretical and empirical perspectives on genomic differentiation. *Trends in Ecology*
860 *and Evolution,* 34**,** 987-995.

861 SPARKS, T. C., STORER, N., PORTER, A., SLATER, R. & NAUEN, R. 2021. Insecticide resistance management
862 and industry: the origins and evolution of the Insecticide Resistance Action Committee (IRAC)
863 and the mode of action classification scheme. *Pest Management Science,* 77**,** 2609-2619.

864 SUGIMOTO, N., TAKAHASHI, A., IHARA, R., ITOH, Y., JOURAKU, A., VAN LEEUWEN, T. & OSAKABE, M. 2020.
865 QTL mapping using microsatellite linkage reveals target-site mutations associated with high
866 levels of resistance against three mitochondrial complex II inhibitors in *Tetranychus urticae*.
867 *Insect Biochemistry and Molecular Biology,* 123**,** 103410.

868 SUKUMARAN, J. & HOLDER, M. T. 2010. DendroPy: a Python library for phylogenetic computing.
869 *Bioinformatics,* 26**,** 1569-1571.

870 TABASHNIK, B. E., BRéVAULT, T. & CARRIèRE, Y. 2013. Insect resistance to Bt crops: lessons from the first
871 billion acres. *Nature Biotechnology,* 31**,** 510-521.

872 TAMURA, K., STECHER, G. & KUMAR, S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version
873 11. *Molecular Biology and Evolution,* 38**,** 3022-3027.

874 TANG, Q. Y. & ZHANG, C. X. 2013. Data Processing System (DPS) software with experimental design,
875 statistical analysis and data mining developed for use in entomological research. *Insect science,*
876 20**,** 254-260.

877 TAYLOR, K. L., HAMBY, K. A., DEYONKE, A. M., GOULD, F. & FRITZ, M. L. 2021. Genome evolution in an
878 agricultural pest following adoption of transgenic crops. *Proceedings of the National Academy of*
879 *Sciences of the United States of America,* 118**,** e2020853118.

880 TRANEL, P. J., WRIGHT, T. R. & HEAP, I. M. 2025. *Mutations in herbicide-resistant weeds to Inhibition of*
881 *Acetolactate Synthase* [Online]. Available: http://www.weedscience.org [Accessed 4/24 2025].

882 TROCZKA, B., ZIMMER, C. T., ELIAS, J., SCHORN, C., BASS, C., DAVIES, T. E., FIELD, L. M., WILLIAMSON, M.
883 S., SLATER, R. & NAUEN, R. 2012. Resistance to diamide insecticides in diamondback moth,
884 *Plutella xylostella* (Lepidoptera: Plutellidae) is associated with a mutation in the membrane-
885 spanning domain of the ryanodine receptor. *Insect Biochemistry and Molecular Biology,* 42**,** 873-
886 880.

887 VAN ETTEN, M., LEE, K. M., CHANG, S.-M. & BAUCOM, R. S. 2020. Parallel and nonparallel genomic
888 responses contribute to herbicide resistance in *Ipomoea purpurea*, a common agricultural weed.
889 *PLoS Genetics,* 16**,** e1008593.

890 VAN LEEUWEN, T., DEMAEGHT, P., OSBORNE, E. J., DERMAUW, W., GOHLKE, S., NAUEN, R., GRBIC, M.,
891 TIRRY, L., MERZENDORFER, H. & CLARK, R. M. 2012. Population bulk segregant mapping uncovers
892 resistance mutations and the mode of action of a chitin synthesis inhibitor in arthropods.
893 *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 4407-
894 4412.

895 VAN LEEUWEN, T., VONTAS, J., TSAGKARAKOU, A., DERMAUW, W. & TIRRY, L. 2010. Acaricide resistance
896 mechanisms in the two-spotted spider mite Tetranychus urticae and other important Acari: a
897 review. *Insect Biochemistry and Molecular Biology,* 40**,** 563-572.

898 VASIMUDDIN, M., MISRA, S., LI, H. & ALURU, S. Efficient Architecture-Aware Acceleration of BWA-MEM
899 for Multicore Systems. 2019 IEEE International Parallel and Distributed Processing Symposium
900 (IPDPS), 20-24 May 2019 2019. 314-324.

901 WALSH, T., HECKEL, D. G., WU, Y., DOWNES, S., GORDON, K. & OAKESHOTT, J. 2022. Determinants of
902 insecticide resistance evolution: comparative analysis among *Heliothines*. *Annual Review of*
903 *Entomology,* 67**,** 387-406.

904 WATERHOUSE, A., BERTONI, M., BIENERT, S., STUDER, G., TAURIELLO, G., GUMIENNY, R., HEER, F. T.,
905 DE BEER, T. A P., REMPFER, C., BORDOLI, L., LEPORE, R. & SCHWEDE, T. 2018. SWISS-MODEL:
906 homology modelling of protein structures and complexes. *Nucleic Acids Research,* 46**,** W296-
907 W303.

908 WEI, S.-J. & CAO, L.-J. 2025. Unprecedented number of recurrent mutations drive the rapid evolution of
909 pesticide resistance in a notorious pest.

910 YASSIN, A., DEBAT, V., BASTIDE, H., GIDASZEWSKI, N., DAVID, J. R. & POOL, J. E. 2016. Recurrent
911 specialization on a toxic fruit in an island *Drosophila* population. *Proceedings of the National*
912 *Academy of Sciences of the United States of America,* 113**,** 4771-4776.

913

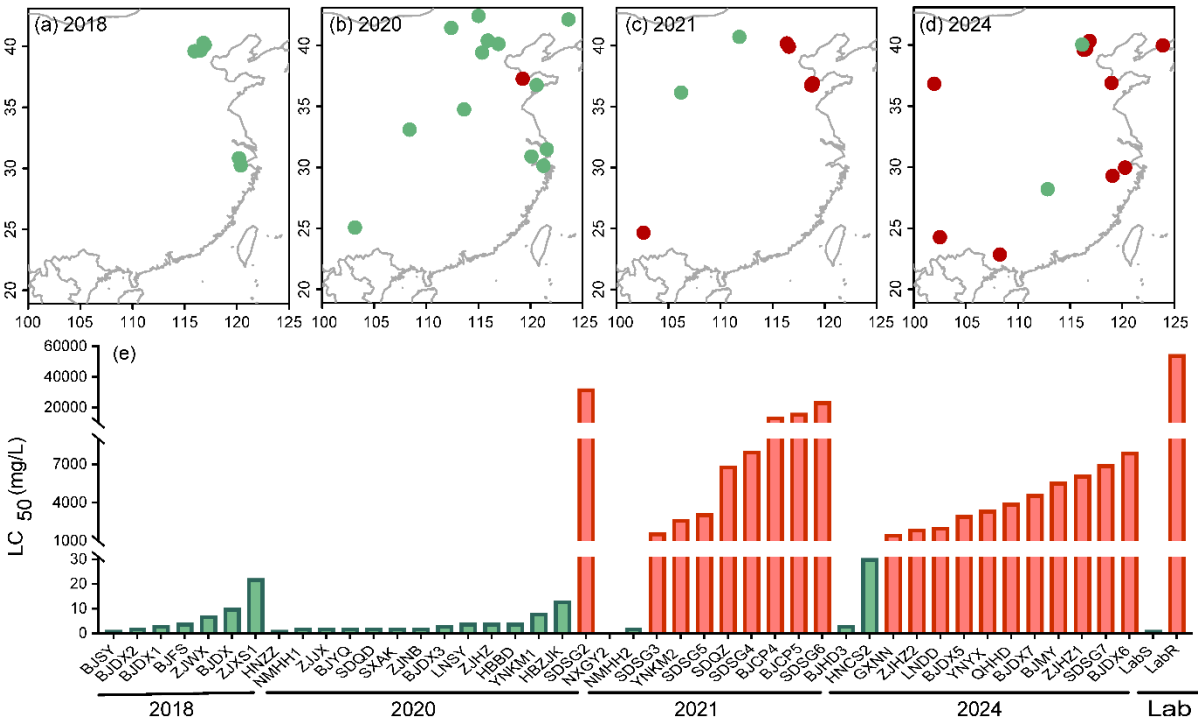


Figure 1 Susceptibility of the two-spotted spider mite *Tetranychus urticae* to cyetpyrafen. (a-d) Collection maps of populations used for bioassay in different years. Red and green points represent resistant and susceptible populations, respectively. (e) LC_{50} values of each population collected in 2018 (7 populations) (Chen et al., 2019), 2020 (14), 2021 (10), 2024 (13), and a laboratory-selected resistant population (LabR) and a parallel reared susceptible population (LabS) to cyetpyrafen. The green bars show the susceptible populations, while the red bars show the resistant populations.

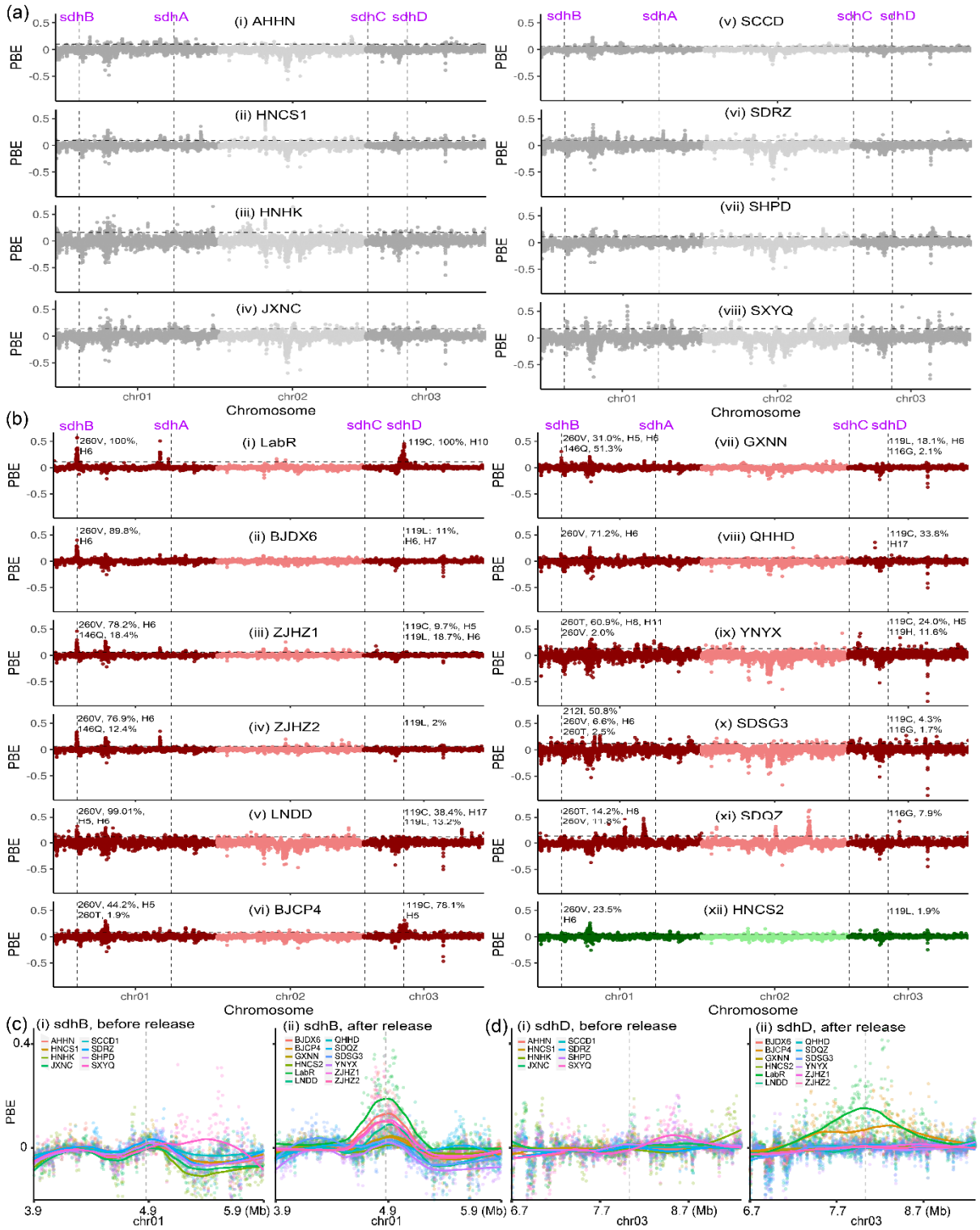


Figure 2 Signals of selective sweep across genomes of the two-spotted spider mite *Tetranychus urticae*. (a) PBE values across the genome for eight historic populations collected

926 in 2017 before the commercial release of cyetpyrafen. No clear and consistent selection signal
927 was identified among these samples. Chromosomes are colored alternately dark grey and light
928 grey. (b) PBE values for one lab-selected resistant population (LabR), 10 resistant field
929 populations and one susceptible population (HNCS) collected in 2021 and 2024 after the release
930 of cyetpyrafen. The laboratory-selected resistant LabR population had clear selection signals at
931 two genes, *sdhB* and *sdhD*. Four (BJDX6, ZJHZ1, ZJHZ2, and LNDD) field-collected resistant
932 populations had selection signals at *sdhB*, and one (BJCP4) had a selection signal at *sdhD*.
933 Chromosomes are colored alternately dark red and light red for resistant populations, dark
934 green and light green for susceptible population HNCS. Allele frequency (pool-seq data) and
935 haplotypes of mutations are indicated near the corresponding *sdhB* and *sdhD* genes of each
936 population in (a) and (b). (c) PBE values for genomic regions around the *sdhB* gene, comparing
937 values between the historic (left) and recently collected (right) populations. (d) As above but for
938 the *sdhD* gene. Each point represents a genomic window of 5-kp wide when compared to
939 reference genomes involving the susceptible laboratory line (LabS) and a susceptible field
940 population (NMHH2). The vertical lines indicate the position of *sdh* genes, while the horizontal
941 lines represent the 1% threshold of PBE values.

942

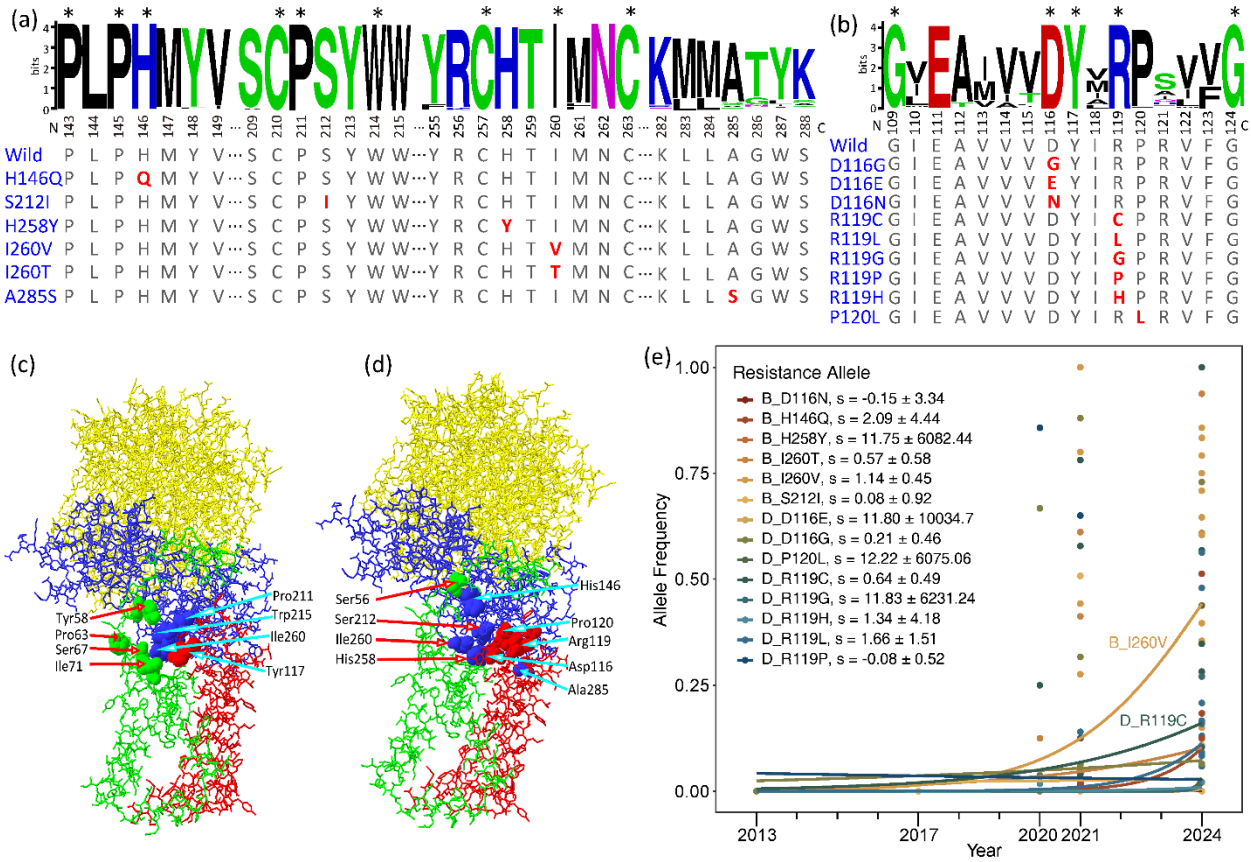
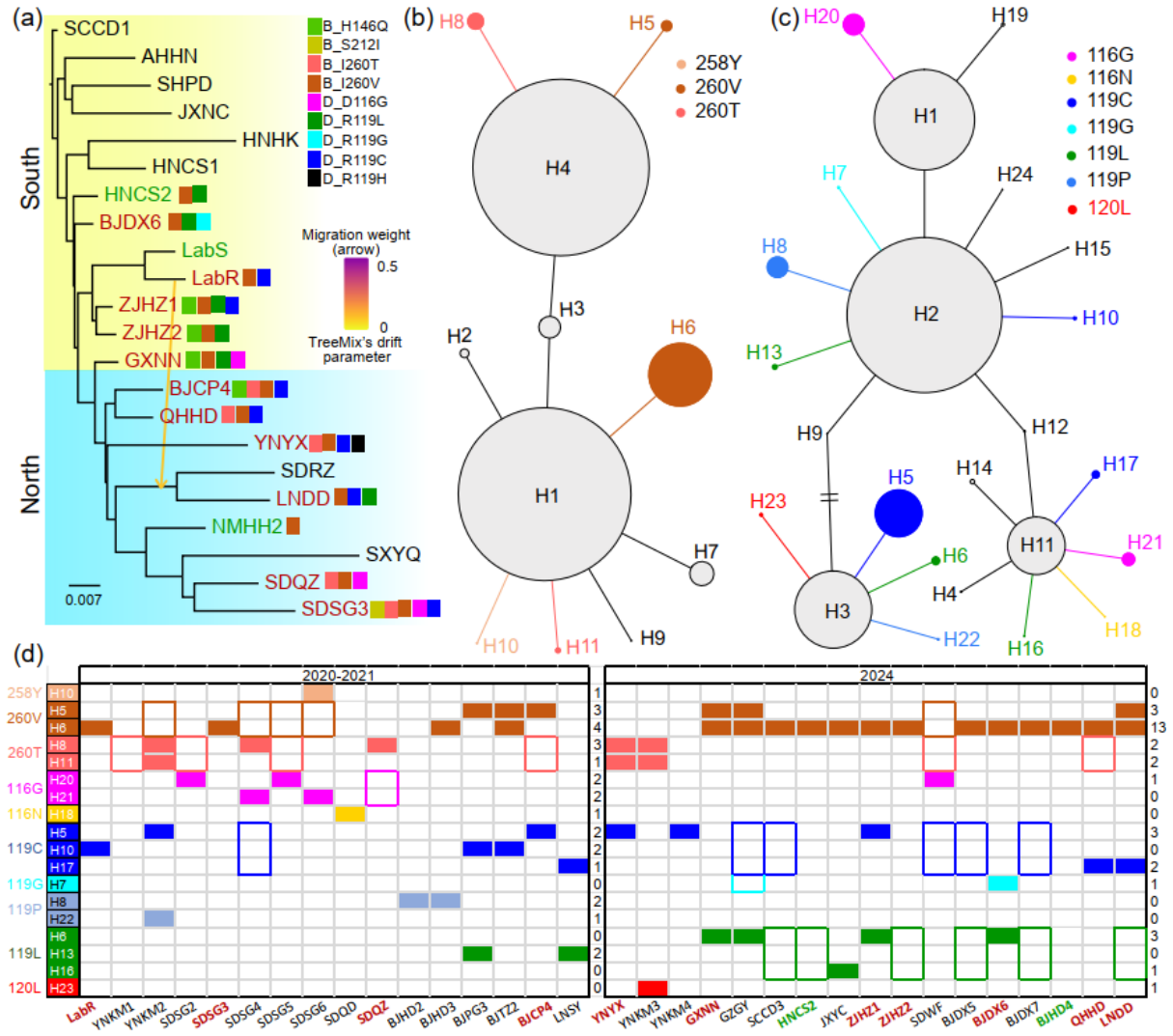


Figure 3 Amino acid mutations on *sdhB* and *sdhD* genes in populations of the two-spotted spider mite *Tetranychus urticae*. (a, b) Sequence logos at mutations related to cyetpyrafen resistance in *sdhB* (a, 757 sequences) and *sdhD* (b, 158 sequences). The numbers under logos indicate the position of amino acid residues on protein chains. Excluding *T. urticae*, conserved amino acids without any alternative are labeled with asterisks. Amino acids of *T. urticae* are shown below. Amino acids in red indicate mutations. (c) 3D structure of the wild-type succinate dehydrogenase of *T. urticae*. Solid 3D shapes show eight amino acid residues at the Q-site, which is the binding site of cyetpyrafen. The sdhA, sdhB, sdhC and sdhD chains are shown in yellow, blue, green, and red, respectively. (d) Amino acid mutation sites on a 3D structure of the wild-type succinate dehydrogenase of *T. urticae*. Solid 3D shapes indicate mutations on five residues identified in this study (His258, Ile260, Asp116, Arg119, and Pro120) and one residue (Ser56) reported by previous studies (Njiru et al., 2022, Sugimoto et al., 2020) that confer resistance to SDH inhibitors. (d) The trajectory of allele frequencies of resistance mutations through time fitted by logistic regression. Each dot represents one population. A258S was omitted as it was only present in one population (SDRZ) sampled in 2017.



959

960 **Figure 4 Genetic structure and haplotype networks of *sdhB* and *sdhD* genes of the two-**
961 **spotted spider mite *Tetranychus urticae*.** (a) Treemix result showing genetic structure and gene
962 flow among populations. The mutations detected by pool-seq data were labeled near the
963 corresponding population. (b) Haplotype network of *sdhB* gene inferred from homozygous
964 individuals. In figures (a) and (d), the red population codes show resistant populations, the
965 green ones show susceptible populations, while the black ones show populations of unknown
966 resistance status. (c) Haplotype network of *sdhD* gene inferred from homozygous individuals.
967 The colored haplotypes are those carrying the resistant mutation. (d) Presence of each
968 haplotype carrying the target mutations in two periods. In the early resistance development
969 stage of 2020-2021, each haplotype was mainly distributed across a limited range, while in
970 2024, the same haplotype was distributed across a wide geographical range. The filled

971 rectangle indicates that the entire haplotype is present in the population, while a colored
972 border suggests that only the mutations were detected, but the haplotype was not assigned.
973 The number of populations carrying the corresponding mutation are listed in the right column
974 for the two time periods.

975

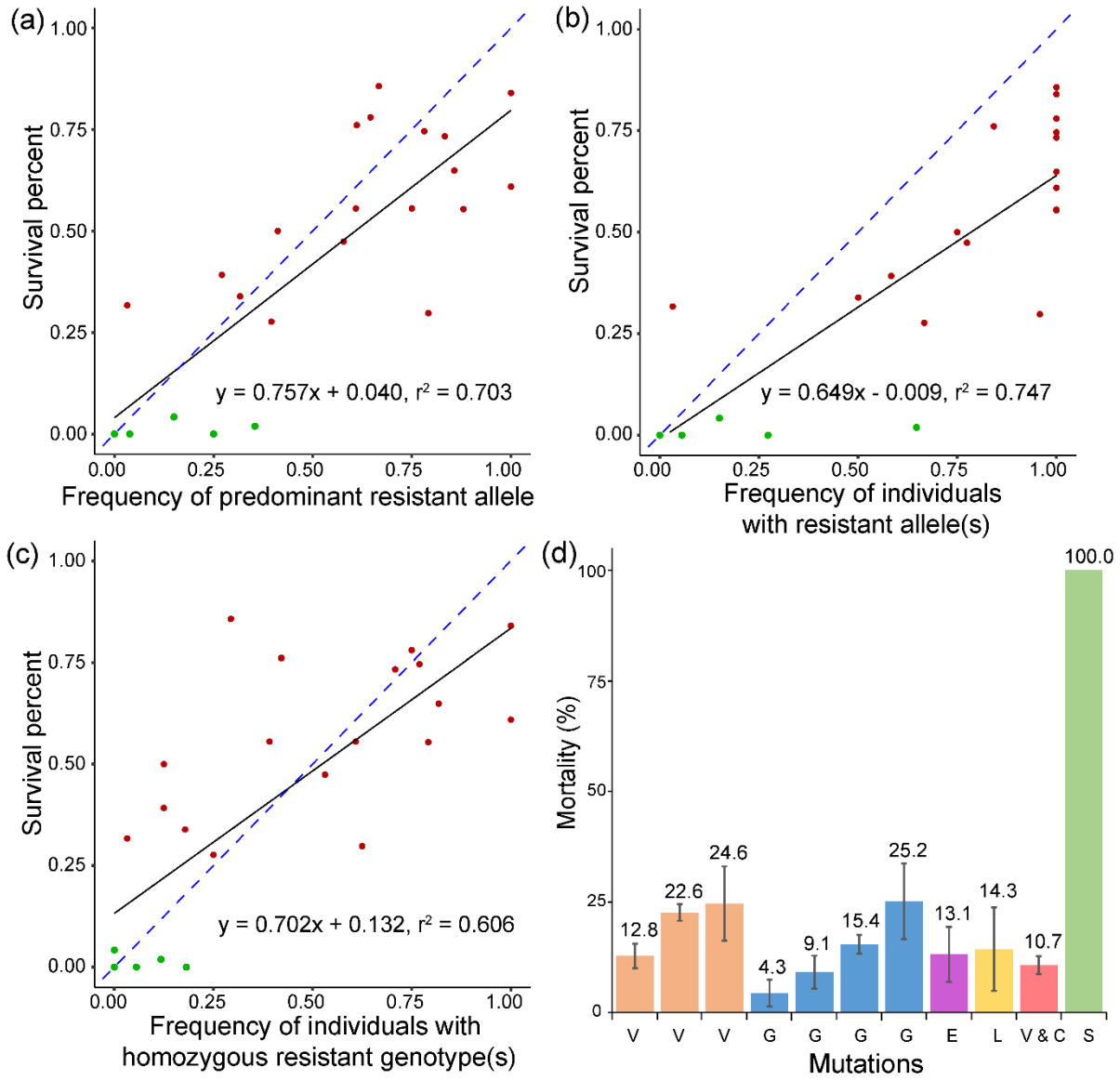


Figure 5 Function of target-site mutations in the *sdhB* and *sdhD* genes and survival of two-spotted spider mite *Tetranychus urticae* in cyetpyrafen resistance. (a-c) Association between frequency of target-site mutations in the *sdhB* and *sdhD* genes and survival of two-spotted spider mite *T. urticae* exposed to 1000 mg/L of cyetpyrafen. (a) Allele frequencies are measured as the predominant mutation in the population at either the *sdhB* or *sdhD* genes. (b) Allele frequency is the proportion of individuals carrying a resistant allele at either the *sdhB* or *sdhD* genes. (c) Allele frequency is the proportion of individuals carrying a homozygous genotype for either the *sdhB* or *sdhD* genes. Red and dark green points represent resistant and susceptible populations, respectively. Blue dashed lines indicate the expected association between two

986 variables, i.e. $y = x$. (d) Mortality of mite lines carrying one of the homozygous mutations or a
987 combination of two mutations exposed to 1000 mg/L of cyetpyrafen. V, I260V; G, D116G; E,
988 R119E; L, R119L; V and C, I260V and R119C; S, susceptible populations LabS. Populations with
989 the same homozygous mutations are shown in the same color. Error bars show standard error
990 of the mean.