Unprecedented Number of Recurrent Mutations Drive the Rapid Evolution of Pesticide Resistance in a Notorious Pest

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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 drive the rapid evolution of the notorious pest, the two-spotted spider mite, to a recently commercialized acaricide, cyetpyrafen. We first observed high levels of resistance that appeared and became widespread within a few years. Genome scans revealed genetic heterogeneity of resistance among populations and identified 15 target mutations on eight amino acid residues of the pesticide target, with as many as five substitutions on one residue. No mutations were present in screened historical specimens, suggesting that mutations arose rapidly through *de novo* substitutions or from very rare segregating mutations. Identical mutations could recurrently appear in different genetic backgrounds, increasing the likelihood of resistance evolution. Additionally, we showed that single mutations conferred high levels of resistance, acting as large-effect alleles. This study demonstrates the high number of mutational options available for the evolution of target site resistance in this pest that challenges resistance management practices.

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

1. Introduction

The development of pesticide resistance in invertebrate pests represents rapid contemporary evolution 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 was documented in 1914, cases have exponentially increased to over 17870, involving more than 625 pest species and 360 compounds, and posing threats to agricultural production, human health, and the environment (Sparks et al., 2021, Melander, 1914, Gould et al., 2018, Fritz, 2022). 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., 2002b, 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 (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 set of resistant mutations in the field

71 (Legan et al., 2024). Such mismatches between laboratory-selected and field-evolved resistance
72 may lead to an inaccurate understanding of resistance evolution (Ffrench-Constant, 2013, Bras
73 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., 2022, 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 almost all chemical modes of action and is the most
92 resistant invertebrate pest worldwide (De Rouck et al., 2023, Van Leeuwen et al., 2010).
93 Succinate dehydrogenase inhibitor (SDHi) acaricides are the most recently developed mode of
94 action (IRAC group 25) for mite control, and include four chemicals: cyetpyrafen, cyenopyrafen,
95 cyflumetofen, and pyflubumide (Hayashi et al., 2013, Nakahira, 2011). Laboratory-selected
96 resistance has already been identified to these new acaricides, and is conferred by target-site
97 mutations of the SDH coding *sdhB* and *sdhD* genes, with mutations identified at three positions

98 (Njiru et al., 2022, Sugimoto et al., 2020). In China, three acaricidal SDH inhibitors have been
99 registered over the last decade: cyflumetofen (2013), cyetpyrafen (2017) and cyenopyrafen
100 (2019). Since its release in 2017, cyetpyrafen has become the most popular pesticide for
101 controlling spider mites, though low-level resistance was detected in the field only one year
102 after its release (Chen et al., 2019).

103 We have now tracked resistance through repeatedly monitoring field-evolved resistance of
104 TSSM to cyetpyrafen at a time when resistance has developed rapidly to high levels across
105 geographically distant populations. We investigated resistance using a combination of
106 population genomics, laboratory selection, bioassays, and studies on historically preserved
107 specimens before the introduction of cyetpyrafen. We find that multiple and recurrently
108 evolved mutations as large-effect alleles drive the rapid development of high-level resistance to
109 cyenopyrafen in field populations across a widespread geographical range. We also
110 demonstrate that these mutations likely evolved *de novo* after the introduction of cyetpyrafen,
111 though they may also have originated from extremely rare mutations segregating in
112 populations before cyetpyrafen selection. Two of the mutations from the field were also
113 identified from a laboratory selection experiment. Our results reveal diverse resistance
114 mechanisms encoded by major effect alleles underlying pesticide resistance in field populations.
115 We emphasize the benefits of integrating genomics with other approaches in longitudinal
116 studies to capture the dynamics of rapid evolutionary changes.

117 2. Results

118 *2.1 Strong resistance evolves rapidly in field and lab-selected populations*

119 To monitor the evolution and spread of cyetpyrafen resistance in the field, we conducted
120 bioassays on 46 mite population samples collected from various regions in China between 2020
121 and 2024 (Figure 1, Tables S1-S2). The susceptibility of these population samples to cyetpyrafen
122 was compared with previously reported data for populations collected between 2017 and 2018
123 during the early release stage of cyetpyrafen in China (Chen et al., 2019, Gong et al., 2017). In

124 2017-2018, all seven tested populations remained susceptible, with LC_{50} values ranging from
125 1.4 to 21.6 mg/L (Figure 1a). In 2020, one population from Shouguang City, Shandong Province
126 (SDSG2), showed resistance with an LC_{50} > 32,000 mg/L, while the other 13 populations
127 remained susceptible (Figure 1b). By 2021, high levels of resistance to cyetpyrafen quickly
128 became widespread in field populations: eight of 10 tested populations had an LC_{50} higher than
129 1606.2 mg/L (Figure 1c). In 2024, 11 of the 13 tested populations evolved resistance with an
130 LC_{50} higher than 1498.6 mg/L (Figure 1d). Overall, the susceptibility of all populations was
131 bimodal, with populations being highly susceptible (LC_{50} ranges from 0.61 to 30.1 mg/L, $\bar{x}$ = 6.72
132 mg/L) or highly resistant (LC_{50} > 1498.6 mg/L, $\bar{x}$ > 5892.5 mg/L) and an 877-fold difference
133 between mean LC_{50}s of the susceptible and resistant groups. While only a single sample was
134 resistant in 2020, by 2021 resistance was observed across the entire range of China (Figure 1c).
135 This pattern indicates that field populations of TSSMs have rapidly developed high levels of
136 resistance to cyetpyrafen across a wide geographical area.

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

149 Additional bioassays were used to test whether the selected LabR strain showed cross-
150 resistance to other pesticides that target proteins in the mitochondrial electron transport chain.

151 For the other two mitochondrial complex II inhibitors (IRAC group 25), the LC_{50} of cyenopyrafen
152 increased 21,254-fold (3.76 to 79918 mg/L), and the LC_{50} of cyflumetofen increased 69,861-fold
153 (6.47 to 452,003 mg/L). For the mitochondrial complex III inhibitors, the LC_{50} of bifenazate
154 (group 20D) increased 2-fold (9.87 to 18 mg/L), and the LC_{50} of pyridaben (group 21A) increased
155 3-fold (3,763.4 to 10,352 mg/L). The results indicate that cyetpyrafen selection led to high
156 cross-resistance to other mitochondrial complex II inhibitors but weak cross-resistance to the
157 mitochondrial complex III inhibitors (Table S3).

158 ***2.2 Selective sweep signals are clear but heterogeneous***

159 To identify genes potentially associated with cyetpyrafen resistance, we conducted pooled
160 whole-genome resequencing on 22 mite population samples, including historic samples of eight
161 geographical populations collected in 2017 before the first field releases of cyetpyrafen, 12
162 samples (10 resistant and two susceptible) collected afterwards, and two laboratory
163 populations (Tables S1, S4). To allow for variation in the genetic basis of resistance and genetic
164 backgrounds among populations, we chose the Population Branch Excess (PBE) analysis (Yassin
165 et al., 2016) method to scan selection signals across the genome; we used two susceptible
166 populations (NMHS2 and LabS) as nonfocal populations to correct for the varied genetic
167 backgrounds, and one resistant population as the focal population to identify population-
168 specific signals of pesticide selection. No clear and coincident selection signal was identified
169 among historical population samples (Figure 2a). The laboratory-selected resistant LabR
170 population had clear selection signals at two genes, *sdhB* and *sdhD,* that encode two subunits
171 of succinate dehydrogenase, the target of SDHi acaricides (Figure 2b). None of the 10 field-
172 collected resistant populations had selection signals at both of these genes, but four (BJDX6,
173 ZJHZ1, ZJHZ2, and LNDD) had selection signals at *sdhB*, and one (BJCP4) had a selection signal at
174 *sdhD*. Five of the resistant populations (QHHD, GXNN, SDSG3, SDQZ and YNYX) showed no
175 selection signal at either gene; this same pattern was observed in the one population sample
176 (HNCS2) that remained susceptible after cyetpyrafen was introduced. Additional PBE peaks
177 were observed at other genomic regions in historical, resistant, and susceptible populations;

178 these could reflect demographic processes or other selective forces not considered in this
179 study.

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

190 ***2.3 Multiple mutations have arisen in the field***

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

204 R119 on sdhD was a notable extreme case, involving five amino acid changes. The second
205 codon position of R119 (G) was substituted by the other three nucleotides, while the first codon
206 position (C) was substituted by two other nucleotides (T and G), indicating that the first two
207 codon positions of R119 are almost saturated in terms of possible nucleotide substitutions.

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 3c).

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 3d, 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. Seven mutations were first detected in 2020
230 (I260T, D116G, D116N, R119C, R119L, R119P, and R119H), three mutations were first detected

231 in 2021 (H146Q, S212I, and I260V), and the remaining four mutations were first detected in
232 2024 (H258Y, D116E, R119G, and P120L) (Figure 3d, Table S2). Among the target-site mutations,
233 I260V and R119C were the most common, found in 65.9% and 46.3% (respectively) of the
234 populations collected after the release of cyetpyrafen, with their frequency increasing annually
235 (Figure 3d). In the laboratory-selected population LabR, two mutations (I260V and R119C) were
236 fixed, but neither was detected in either the field population from which it was sourced (ZJXS1)
237 or the unselected susceptible laboratory population derived from ZJXS1 (LabS). These results
238 indicate that the putative resistance mutations were not likely to be present as standing genetic
239 variation unless they were segregating at very low frequencies, and they may have evolved
240 recently through *de novo* substitutions although this cannot be demonstrated definitively.

241 ***2.4 Recurrent evolution of identical mutations***

242 When a specific resistance mutation is found in multiple populations, this can be the result of
243 migration between populations or to multiple independent origins of the same mutation. We
244 evaluated these evolutionary hypotheses using two methods: TreeMix (Pickrell and Pritchard,
245 2012) and haplotype network analysis.

246 We used TreeMix to investigate whether populations with identical alleles were more
247 genetically similar or had recent histories of migration. TreeMix builds maximum likelihood
248 trees from population allele frequencies and then adds optional migration edges between
249 populations. We set the SCCD population from southwest China as the root population as it had
250 the highest genetic diversity, and we built trees that followed a strict drift model (i.e. no
251 migration) and trees with up to 10 migration edges. The strict drift model explained most of the
252 variance in relatedness among populations (mean over 1000 replicates of 0.916). The best
253 number of historical migration events was one. However, this migration edge was placed
254 between the laboratory-selected population and a field clade different to the one used for the
255 laboratory population (Figure 4a), an impossible evolutionary scenario, suggesting that
256 migration among populations is low. The consensus tree over the 1000 replicates assigned most

257 of the populations into northern and southern groups by geography, but not grouped by
258 resistant status (Figure 4a). We also observed several instances where geographically distant
259 populations grouped together. Some southern populations were placed with the northern
260 group (YNYX), and vice versa (BJDX6); these populations may have been recently established via
261 long-distance dispersal, possibly by the transport of seedlings.

262 The above-mentioned mutations in *sdhB* and *sdhD* genes were detected in six populations
263 from the southern group and seven populations from the northern group. The mutation I260V
264 was detected in every field population sampled after the release of cyetpyrafen (Figure 4a,
265 Table S2), using pool-seq data. I260T was only detected in the northern group and R119L was
266 mostly detected in the southern group. The mutation H146Q was found in four adjacent
267 populations (ZJHZ1, ZJHZ2, GXNN and BJCP4). This geographical heterogeneity in the
268 distribution of resistance mutations suggests multiple mechanisms of resistance evolution
269 underly these patterns, involving spread via migration between populations (e.g. H146Q) as
270 well as possible independent origins of the same mutation (e.g. I260V).

271 We explored these ideas further using haplotype network analysis. When resistance
272 spreads via migration, these mutations should have identical haplotypes, unless recombination
273 has taken place which will introduce a second haplotype that segregates locally alongside the
274 initial haplotype. We constructed the haplotype network for the partial *sdhB* (Figure 4b) and
275 *sdhD* (Figure 4c) genes determined from Sanger sequencing. Since haplotypes could not be
276 determined when two loci were heterozygous, we detected haplotypes from sequencing data
277 with at most one heterozygous locus. The genes showed high genetic diversity, with 11 (*sdhB*)
278 and 24 (*sdhD*) haplotypes detected. We assumed that common haplotypes located at the
279 centre of the network are the ancestral haplotypes (H1 and H4 in *sdhB*; H1, H2, H3, and H11 in
280 *sdhD*). We assumed all other haplotypes were derived. Each derived haplotype was only one
281 nucleotide different from the nearest ancestral haplotype. Five of the nine derived *sdhB*
282 haplotypes carried a putative resistance mutation, as did 13 of the 22 derived *sdhD* haplotypes.

283 Of the 10 putative resistance mutations detected through Sanger sequencing, all but four
284 (H258Y, D116N, R119G, and P120L) were found as multiple haplotypes (Figs. 4b and 4c). To test
285 whether these haplotypes were produced by multiple origins or by recombination, we inferred
286 the possible means by which recombination could produce each haplotype, and investigated
287 the geographical distributions of the requisite ancestral and derived haplotypes. For mutations
288 in *sdhB*, we inferred that recombination would have to involve the H4 ancestral haplotype as
289 donor, while the H11 ancestral haplotype was required for *sdhD* (Figure S3). Thus,
290 recombination could have produced the *sdhB* haplotypes H5 (H4 x H6) and H8 (H4 x H11), and
291 the *sdhD* haplotypes H17 (H11 x H10), H16 (H11 x H13) and H21 (H11 x H20). However, only
292 three populations (GXNN, YNKM3, and YNYX) had both of the two recombining haplotypes
293 required and one derived haplotype (Table S5). Additionally, the proximity of the mutations
294 was such that multiple recombination events would be required within the same 5 – 60 bp
295 regions, which seems unlikely even with a high recombination rate of (say) 10 cM/Mb where
296 two mutations might only be expected to occur once in 3.6×10^9 individuals across 60 bp. These
297 results do not indicate an obvious role for recombination in generating the derived haplotypes.

298 All haplotypes carrying putative resistance mutations in the *sdhB* gene arose in 2020-2021,
299 along with all haplotypes of the *sdhD* gene apart from H6, H7, H16 and H23 (Figure 4d, Table
300 S5). By 2024, the *sdhB* H6 haplotype became the most prevalent haplotype, while the non-
301 resistant *sdhB* H3 haplotype was almost lost despite it being prevalent between 2013 and 2021
302 (Figure 4d, Table S5). Some individual populations had multiple haplotypes of the same putative
303 resistance mutation (Figure 4d), and different haplotypes of the same mutation were often
304 found in adjacent populations (Figure 4a, Figure 4d, Table S5). In the ZJXS1 population used to
305 produce the LabR and LabS strains, four haplotypes of the *sdhB* gene (H1, H3, H4 and H7) and
306 six haplotypes of the *sdhD* gene (H1-H4, H11 and H24) were found, none of which had putative
307 resistance mutations. After 66 generations of cyetpyrafen selection, two resistant haplotypes
308 were fixed in the LabR population: H6 of *sdhB* and H10 of *sdhD*, which were likely derived from
309 H1 of *sdhB* and H2 of *sdhD*, respectively (Table S5). These results illustrate the rapid and

310 geographically widespread emergence of resistant alleles in these populations, linked to several
311 mutations found in a range of different haplotypes.

312 ***2.5 Single mutations have a large effect on resistance***

313 We next characterized the function of each mutation by itself and in combination with other
314 mutations. We first tested the population-level association between allele frequencies of the
315 mutations and levels of resistance, using the five mutations present in at least ten populations
316 (I260T, I260V, D116G, R119C, and R119L) and resistance indicated by survival at 1000 mg/L
317 cyetpyrafen. When each mutation was analyzed separately, a weak association was found
318 between survival and the frequency of the mutant allele (Table S6, linear regression R^2 = 0.091-
319 0.303). When we considered the frequency of individuals having any of the five mutations, we
320 found a strong positive association between survival and the frequency of predominant allele
321 (R^2 = 0.707), the frequency of individuals carrying at least one resistant allele (heterozygous and
322 homozygous mutant genotypes) (R^2 = 0.747), and the frequency of individuals with
323 homozygous mutant genotypes (R^2 = 0.606) (Figure 5a-5c). These results showed that single
324 mutations explained a low proportion of resistance variation across populations. But when
325 considering all five mutations together, a substantial proportion of resistance variation was
326 explained.

327 Near iso-female lines were created and tested for susceptibility to cyetpyrafen (Figure 5d).
328 These carried I260V, D116G, R119E, R119L, and one selected line carried both I260V and R119C.
329 When treated with 1000 mg/L cyetpyrafen, mortality of the susceptible line was 100%, but was
330 lower than 25.2% in all other lines, indicating that each mutation and a combination of two
331 mutations conferred high resistance to cyetpyrafen. These results demonstrate that
332 cyetpyrafen resistance is often conferred by different large-effect mutations in the *sdhB* and
333 *sdhD* genes.

334 **3. Discussion**

335 Pesticide resistance can evolve rapidly, in contrast to traditional views of adaptation which
336 often emphasize a gradual process (Fisher, 1930). Due to the time taken for resistance to be
337 recognized, evolutionary studies of pesticide resistance are often *post hoc*. Here, we conducted
338 field monitoring of resistance to a novel acaricide, cyetpyrafen, following its 2017 release, with
339 the opportunity to track resistance as it develops. We demonstrated a longitudinal picture of
340 resistance development in the field involving multiple and recurrently evolved large-effect
341 mutations.

342 Currently available pesticides mainly act by direct binding to receptors and enzymes,
343 resulting in the inhibition of physiological functions associated with the nervous system, energy
344 production system, growth and development, and midgut absorption (Sparks et al., 2021).
345 Mutations that alter the binding kinetics of pesticides on these proteins and thus potentially
346 confer pesticide resistance have been widely identified in agricultural pests and disease vectors
347 (De Rouck et al., 2023, Acford-Palmer et al., 2023). Resistant mutations of some target genes
348 are highly conserved both among populations and among species, perhaps due to the
349 functional constraints of these targets (Ffrench-Constant et al., 1993a, Hawkins et al., 2018).
350 However, multiple mutations on single pesticide targets have been identified in the same pest
351 species and among different species. These cases often involve the voltage gated sodium
352 channel (*VGSC*) gene, where at least 5 and 10 mutations have been cumulatively reported
353 across studies on TSSM and the ectoparasite *Dermanyssus gallinae* respectively (De Rouck et al.,
354 2023).

355 Here we found 15 mutations on eight amino acid residues in TSSM that all arose within a
356 few years following the introduction of cyetpyrafen. Notably, five of the substitutions involve
357 R119 of the *sdhD* gene. As far as we know, this is the highest number of both mutations and
358 altered residues ever found in a single pesticide target from the same pest species, all emerging
359 in a short period following initial detection of resistance. These mutations were located at the
360 Q-site of SDH targeted by acaricidal inhibitors of SDH. Among these mutations, I260T was
361 previously found in a cyflumetofen-resistant population (Sugimoto et al., 2020), while I260V

362 (with S56L in subunit C) was found in a pyflubumide and cyenopyrafen-resistant population
363 (Maeoka and Osakabe, 2021). R119L has previously been reported in cyflumetofen resistant
364 and susceptible strains of TSSM but had not yet been validated as having a role in resistance
365 (Sugimoto et al., 2020). These results provide information on the nature of novel mutations in a
366 pesticide target and the high level of resistance to cyetpyrafen that they can confer. It would be
367 interesting to further validate the impacts of the mutations across chemicals to establish
368 patterns of cross resistance given that the same mutation may confer a different level of
369 resistance to different chemicals (Maeoka and Osakabe, 2021).

370 Our temporal collections of TSSM preceding cyetpyrafen release in the field indicated that
371 the resistant mutations were not at an appreciable frequency prior to pesticide selection.
372 Sequencing of 13 populations (involving a total of 2666 TSSMs) from 2013 to 2018 did not
373 recover any of the identified mutations. This points to a possible role of recurrent *de novo*
374 mutation, although resistant alleles could have been present at very low frequencies before
375 pesticide selection which can be difficult to detect (Hawkins et al., 2018). In laboratory
376 populations of TSSM, continuous selection pressure resulted in the appearance of the *de novo*
377 mutation H258Y, which confers resistance to pyflubumide and cyenopyrafen (Njiru et al., 2023).
378 Our selection experiments also led to new mutations (I260V, R119C) arising over 66 generations
379 (2 years) of cyetpyrafen selection in the LabR population. Although it is hard to completely
380 exclude the possibility of contamination during laboratory selection, because TSSM is tiny and
381 can easily be dispersed passively, the haplotype analysis showed that the two mutations in the
382 lab-selected population likely arose *de novo*. In the same way, multiple *de novo* mutations
383 could have contributed to the rapid evolution of resistance in different populations and regions
384 as documented in our field surveys.

385 Although few studies have traced resistance evolution in the field, they suggest that both
386 *de novo* mutations and standing genetic variation can contribute to resistance, with their
387 relative importance depending on the specific pesticides and pests involved (Hawkins et al.,
388 2018). Resistant alleles can originate from a single event and spread to distant populations by

389 migration (Raymond et al., 1991, Daborn et al., 2002a), but resistant mutations can also have
390 multiple origins (Guan et al., 2021, Ilias et al., 2014, Troczka et al., 2012, Kreiner et al., 2022,
391 Schmidt et al., 2024). Direct evidence for multiple origins comes from the presence of the same
392 mutation on multiple haplotypes, assuming that recombination can be excluded as an
393 explanation because insufficient time has elapsed after a resistant mutation is introduced into a
394 new background (Semenov et al., 2019). In our study, the requisite recombinant haplotypes
395 were rarely found in the same population, and we therefore suspect that recombination is
396 unlikely to be the source of patterns of haplotype variation over short DNA sequences. We
397 suspect that populations of mites like TSSM have a strong evolutionary potential to evolve *in*
398 *situ* rather than relying on gene flow, and that there is a repeatability of evolutionary changes
399 when an organism is exposed to the same selection pressure, a long-standing issue in
400 evolutionary biology (Konecna et al., 2021). The recurrent appearance of the same mutation in
401 different genetic backgrounds suggests that the likelihood of resistance evolution is high in
402 TSSM and not dependent on gene flow, and this is further increased by the fact that resistance
403 can develop through several mutational options.

404 Because field populations of TSSM can use multiple mutations to reach the same
405 phenotypic endpoint (resistance), it is unlikely that all resistant mutations will be identified
406 from laboratory selection studies particularly if these involve a single laboratory population.
407 Even in the case where multiple populations of TSSM have been selected for resistance (to
408 cyenopyrafen and the related chemicals cyflumetofen and pyflubumide), QTL mapping still only
409 identified a few of the relevant mutations providing resistance to each chemical (Sugimoto et
410 al., 2020). Additionally, mutations identified in laboratory selection may not be involved in
411 resistance in the field because of fitness costs. For instance, H258Y conferred resistance to
412 cyenopyrafen and pyflubumide in laboratory-selected populations, but likely reinforced
413 cyflumetofen binding and toxicity (Njiru et al., 2022) to produce a fitness cost (Njiru et al.,
414 2023). Relying on a limited set of resistance alleles identified in the laboratory for monitoring
415 may bias inferences of resistance evolution in the field when many alleles can produce

416 resistance. Genetic and evolutionary studies on field populations are then essential to develop
417 a full picture of resistance evolution (Konecna et al., 2021, Lucas et al., 2023, Schmidt et al.,
418 2024, Kersten et al., 2023, Pezzini et al., 2024).

419 Theoretical and empirical studies have showed that field-evolved resistance often involves
420 large-effect alleles with a simple genetic basis, while laboratory selection may result in
421 polygenic genetic architectures, due to differences in the occurrence of rare alleles (more likely
422 in large field populations) and different selection pressures (more variable with intense periods
423 in the field on occasions) (Ffrench-Constant, 2013, Roush and McKenzie, 1987, Ffrench-
424 Constant et al., 1990, McKenzie and Batterham, 1994, Crow, 1954). This could potentially be
425 mitigated by establishing laboratory selection lines using large starting populations from the
426 field with pre-existing resistance alleles (Gould et al. 1997). Until recently, few studies have
427 directly compared the genetic basis of resistance in laboratory populations with field-evolved
428 resistance across a large geographical scale (McKenzie et al., 1992, Ffrench-Constant et al.,
429 1990, Crow, 1954, Legan et al., 2024). Here we identified large-effect alleles in both field- and
430 laboratory-selected resistant populations. High-level resistance was generated after lab
431 selection with cyetpyrafen for 33 of 65 generations, almost at the same rate as the field
432 populations where high-level resistance was observed three years after the commercial release
433 of cyetpyrafen. In field populations, it seems that multiple allelic options can favored by
434 selection due to parallel adaptation, and that the same allele may also end up residing in
435 different haplotypes in different areas. Through migration these alleles/backgrounds may
436 eventually end up in the same population to produce a polyallelic basis of adaptation where
437 particular alleles may then not reach high frequencies unless one haplotype is favored (Ralph
438 and Coop, 2010).

439 Pesticide resistance provides many fascinating examples of rapid and recent evolution
440 under high selection pressure, which can be leveraged to address fundamental issues about the
441 evolutionary basis of adaptations to new environments (Hawkins et al., 2019), and also examine
442 the reliability of commonly used research methods. The development of high-throughput

443 sequencing and population genomics methodologies provides an opportunity to detect
444 selective signatures across the genome (Van Etten et al., 2020, Cohen et al., 2022, Pélissié et al.,
445 2022). Although pesticide resistance is a rapid evolutionary process under strong selective
446 pressures, identifying candidate genes directly from genomic signals remains difficult when
447 adaptation occurs via soft sweeps (Pennings and Hermisson, 2006, Schrider et al., 2015). Soft
448 sweeps may be common in adaptive evolution if the effective mutation rate ($2N_e\mu$) >> 0.01,
449 e.g., through large population sizes (N_e), and/or high allelic mutation rates (Hermisson and
450 Pennings, 2005, Pennings and Hermisson, 2006). The multiple haplotypes and distinct evolution
451 history of SDH genes among resistant field populations suggest a prevalence of soft selective
452 sweeps through recurrent mutation as implicated in pesticide resistance in other pest systems,
453 such as *Drosophila* (Garud and Petrov, 2016), and the oomycete *Plasmopara viticola* (Delmas et
454 al., 2017). This is congruent with the 'messier' signals found in field populations relative to the
455 artificially selected laboratory resistant population. Sweeps around the SDH genes are
456 incomplete, or other contributing loci are undergoing selective sweeps, which may further
457 reduce signal to detect adaptation (Pennings and Hermisson, 2006, Schrider et al., 2015). Fewer
458 mutation types and haplotypes along with higher mutation frequency are more likely to be
459 detected. Clearer signals of selective sweeps could emerge from haplotype-based linkage-
460 disequilibrium methods using individual-level genomic data (Hermisson and Pennings, 2017).

461 In conclusion, we have captured a detailed picture of contemporary evolutionary
462 processes under pesticide selection in field populations of an invertebrate pest. We
463 demonstrate that the rapid evolution of pesticide resistance is driven by an unprecedented
464 number of population-specific mutations on target genes that arose recurrently. These findings
465 reveal complex origins and diverse genetic bases of pesticide resistance in the fields,
466 challenging the molecular monitoring of resistance and the implementation of effective
467 resistance management strategies. Finally, we call for future studies on field-evolved resistance
468 to provide theoretical and practical insights into effective pest control, leveraging the genomics
469 era.

470 **4. Materials and Methods**

471 ***4.1 Samples***

472 We used 72 field-collected and laboratory-selected populations of TSSM for bioassays, whole-
473 genome resequencing, Sanger sequencing of target genes, and establishment of mutant strains
474 (Table S1). Samples of TSSM were collected across China from 2013 to 2017 and from 2018 to
475 2024 before and after the release of cyetpyrafen. More than 500 adults were collected from at
476 least three points separated by 100 meters for each population. Samples were kept in 100%
477 alcohol or transported live to the laboratory for chemical bioassays. Because TSSM has a
478 haplodiploid sex-determination system, only female adults were used for bioassays and
479 genotyping. A field population, ZJXS1, was collected in 2018 and selected for 60 generations by
480 spraying cyetpyrafen. The TSSM strain were reared on French bean, *Pemphigus vulgaris* L.
481 under 25±1 °C, 60±5% relative humidity and photoperiod of L16h: D8h. We continuously
482 selected this population in the laboratory for another 40 generations by killing 60% of
483 individuals every two generations, to obtain a laboratory-selected resistant strain.

484 ***4.2 Bioassay***

485 Bioassays were conducted to test the susceptibility of TSSM to acaricides using a Potter Spray
486 Tower (Burkard Scientific, London, UK). We used transparent plastic cups (6 cm in diameter and
487 4 cm in height) to keep the female adult mites. A layer of 0.2% agar was placed on the bottom
488 of the cups to avoid leaves drying out. Fresh leaves of *P. vulgaris* were cut into a circle with a
489 diameter of 6 cm to fit the cup and put onto the agar. The borders of the leaves were sealed
490 using 0.2% agar. Thirty female adults were carefully moved into a plastic cup by suction trap.
491 Two ml acaricide solution was applied through a Potter spray tower (Burkard Manufacturing
492 Co. Ltd., UK) at 68.9 kPa and a sedimentation time of 30 s. Each population was exposed to
493 between five and eight pre-estimated concentrations of acaricides, diluted with water
494 containing 0.1% Triton X-100 (Beijing Solar BioScience and Technology Limited Company,
495 China). The 0.1% Triton X-100 solution was used as a control treatment to correct the potential
496 influence of adjuvants in the acaricides. The treated mites were kept under 25 °C, 60-70%

497 relative humidity, and a photoperiod of 16 h:8 h (L:D). Mortality was assessed under a Stemi
498 305 stereomicroscope (Zeiss, Germany) 48 hours after spraying. The testing TSSMs were
499 considered dead if no movement of appendages was observed when they were prodded with a
500 fine brush at 24 hours and 48 hours after spraying. The 50% lethal concentration (LC_{50}) and its
501 95% confidence intervals were calculated using probit regressions implemented in the
502 statistical software DPS v12.01 (Tang and Zhang, 2013).

503 ***4.3 Pooled whole-genome resequencing and SNP calling***

504 Between 50 and 400 individuals per population were used for pooled genome resequencing to
505 obtain population genomic data for diversity estimates and demographic analyses. DNA
506 libraries with insert sizes of 400 bp were constructed and sequenced on the Illumina NovaSeq
507 6000 platform to obtain 150 bp paired-end reads. Raw reads were filtered using fastp v0.23.0
508 (Chen et al., 2018). The filtered reads were mapped to our recently assembled chromosome-
509 level genome (Cao et al., 2024) using bwa-mem2 2.2.1 with default parameters (Vasimuddin et
510 al., 2019). SAMtools v1.14 (Li et al., 2009) was used to sort, remove duplication reads, remove
511 reads with mapping quality < 20, and convert bam files to mpileup files. Indels in mpileup files
512 were filtered out (with a 5-bp margin on each side) to avoid spurious SNP calling using scripts
513 from PoPoolation (Kofler et al., 2011a). Then mpileup files were converted using scripts of
514 PoPoolation2 (Kofler et al., 2011b).

515 ***4.4 Population genetic diversity and demography analyses***

516 Genetic diversity in each population was quantified by estimating nucleotide diversity (π) and
517 Tajima's *D* across 5 kb non-overlapping windows, using PoPoolation (Kofler et al., 2011a). To
518 reduce the impact of read depth on estimates of nucleotide diversity, each alignment was
519 converted into a population-specific pileup file using SAMtools, and reads were subsampled
520 without replacement up to a depth of 50 (Hoban et al., 2016). The mean F_{ST} across all windows
521 for each pairwise population comparison was calculated using PoPoolation2 (Kofler et al.,
522 2011a) across 5 kb non-overlapping windows. To infer population relationships and historical

523 migration, a rooted maximum likelihood tree was constructed with a strict drift model (no
524 migration) using Treemix v1.13 (Pickrell and Pritchard, 2012). The SCCD population was set as
525 root and we tested migration events from 0 to 10 with at least 100 runs for each case. The best
526 number of migration events was determined by the second-order rate of change in likelihood
527 (Δm) across incremental migration events (m) using the Evanno method (Evanno et al., 2005)
528 embedded in R package 'optM' (Fitak, 2021). For the best m, we performed 1000 runs to
529 generate 1000 maximum likelihood trees and obtained a consensus tree using the program
530 SumTrees version 4.10 (Sukumaran and Holder, 2010).

531 ***4.5 Genome scan of outlier SNPs***

532 We investigated the genomic signals of selection around the four SDH genes (± 1 Mb) and
533 across the whole genome. The main statistic used to identify outlier regions was the population
534 branch excess (PBE) statistic (Yassin et al., 2016). The PBE statistic is based on comparisons of
535 F_{ST} values among three populations, comprising a focal population and two nonfocal
536 populations (Pool et al., 2017). In the analysis, each resistant population was set as the focal
537 population, and two susceptible populations (LabS and NMHH2) were set as the nonfocal
538 populations. PBE values were calculated for 5 kb non-overlapping sliding windows. Windows
539 with top 5% PBE value were considered outliers. Patterns of nucleotide diversity (π) estimates
540 and Tajima's D values around the SDH genes were also examined. We expected resistant
541 populations to have lower nucleotide diversity and Tajima's D values near the SDH genes due to
542 directional selection favoring resistant haplotypes. The coding effects of SNPs within the SDH
543 genes were obtained using SnpEff v4.3 (Cingolani et al., 2012).

544 ***4.6 SDH homology modeling***

545 The three-dimensional (3D) structures of the wild and mutational SDH from TSSM were
546 modeled with SWISS-MODEL (Waterhouse et al., 2018) using 8GS8.1 (Du et al., 2023) as a
547 template. To examine the conservation of *SdhB* and *SdhD* genes, we collected the protein
548 sequences of two genes of different animals in the protein database of NCBI

549 (https://www.ncbi.nlm.nih.gov/protein). Then, MUSCLE in MEGA v11 (Tamura et al., 2021)
550 were used to perform sequence alignment and trim protein sequences around mutation points.
551 After removing identical sequences of the same species, the multiple sequence alignment
552 sequences were used to determine conservation and visualized with WebLogo 3 (Crooks et al.,
553 2004).

554 ***4.7 PCR amplification and sequencing of target genes***

555 Sanger sequencing was used to characterize the spatiotemporal variation of these target-site
556 mutations in populations before and after the release of cyetpyrafen (Table S1). PCR primers
557 were designed for sequences of the *sdhB* gene (sdhb_F: GAGTCTAGCCGCAGTCTGAT, sdhb_R:
558 ACTTGGGACCTGCTGTACTC) and the *sdhD* gene (sdhd_F: GCGATGGATAAGGCATAGACG, sdhd_R:
559 TCTGGTCAGTGTGGTTCCTC) to capture the candidate mutation sites. Polymerase chain reaction
560 (PCR) was conducted in the Mastercycler pro system (Eppendorf, Germany) under an annealing
561 temperature of 54 °C for 1 min using Promega Go Taq™ Master Mixes (Madison, Wisconsin,
562 USA). Amplified products were purified and sequenced directly from one strand using an ABI
563 3730xl DNA Analyzer by Sangon Biotech Co. Ltd. (Shanghai, China).

564 ***4.8 Haplotype analysis***

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

569 ***4.9 Association analysis between mutation frequency and resistance level***

570 Regressions were run to assess the strength of association between target-site mutation
571 frequencies and mean population-level resistance phenotypes. Since LC_{50} of some populations
572 could not be calculated or were inaccurate due to high resistance levels, the survival rate after
573 spraying with 1000 mg/L cyetpyrafen, which is about 6.7 to 10-fold of field-recommended dose,
574 was used to quantify the resistance level of TSSM to cyetpyrafen. Because multiple target-site

575 mutations existed, we evaluated the associations using different metrics of target-site mutation
576 frequencies: (i) the frequency of the predominant mutation in each population, (ii) the
577 frequency of individuals carrying any mutation in each population, and (iii) the frequency of
578 individuals with homozygous mutations in each population. Regressions were fitted as linear
579 models using the *lm* function from the R package *stats* and visualized with *ggplot2*. We did not
580 adjust associations for relatedness among populations because the evolutionary events leading
581 to resistance appear to be largely independent.

582 ***4.10 Establishment of near isofemale lines***

583 Single immature TSSM females were picked from resistant populations and reared individually
584 in a petri dish. When unmated, females will only produce haploid male eggs. To obtain the
585 genotype of males for the *sdh* genes, the DNA of each female was extracted and genotyped as
586 mentioned above after it had laid more than 10 eggs, while the eggs (> 10) were allowed to
587 develop into male adults (F_1 male adults). To obtain the genotype of virgin female, the first six
588 eggs were genotyped, while the female were kept (F_0 female). Then, a pair of F_0 female and F_1
589 male adults with the same homozygous genotypes for the three mutation sites were placed
590 into the same petri dish to allow them to mate and produce male and female progeny, with the
591 subsequent line defined as a near isofemale line. The mated male and female pairs were from
592 the same population to reduce differences in their genetic backgrounds. The near isofemale
593 lines with homozygous genotypes at the mutation sites were tested for susceptibility to
594 cyetpyrafen using the bioassay method.

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

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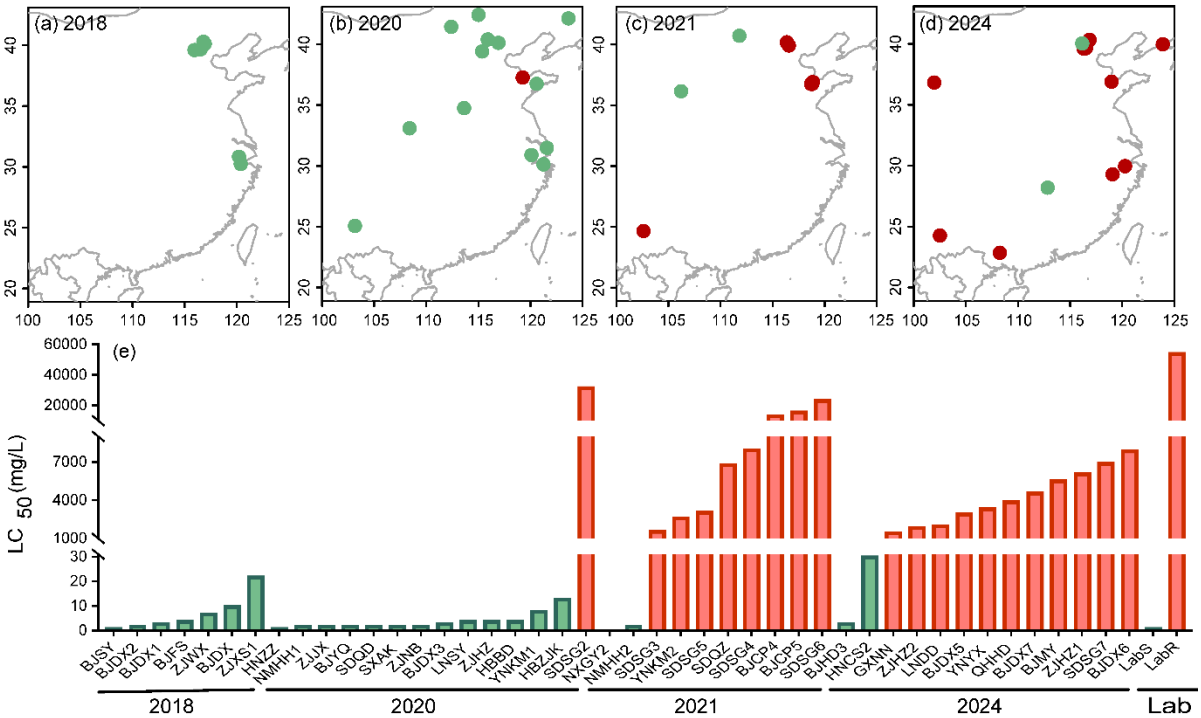

Figure 1 Susceptibility of the two-spotted spider mite *Tetranychus urticae* to cyetpyrafen. (a-d) Collection maps of populations used for bioassay in different years. Red and green points represent resistant and susceptible populations, respectively. (e) LC_{50} values of each population collected in 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.

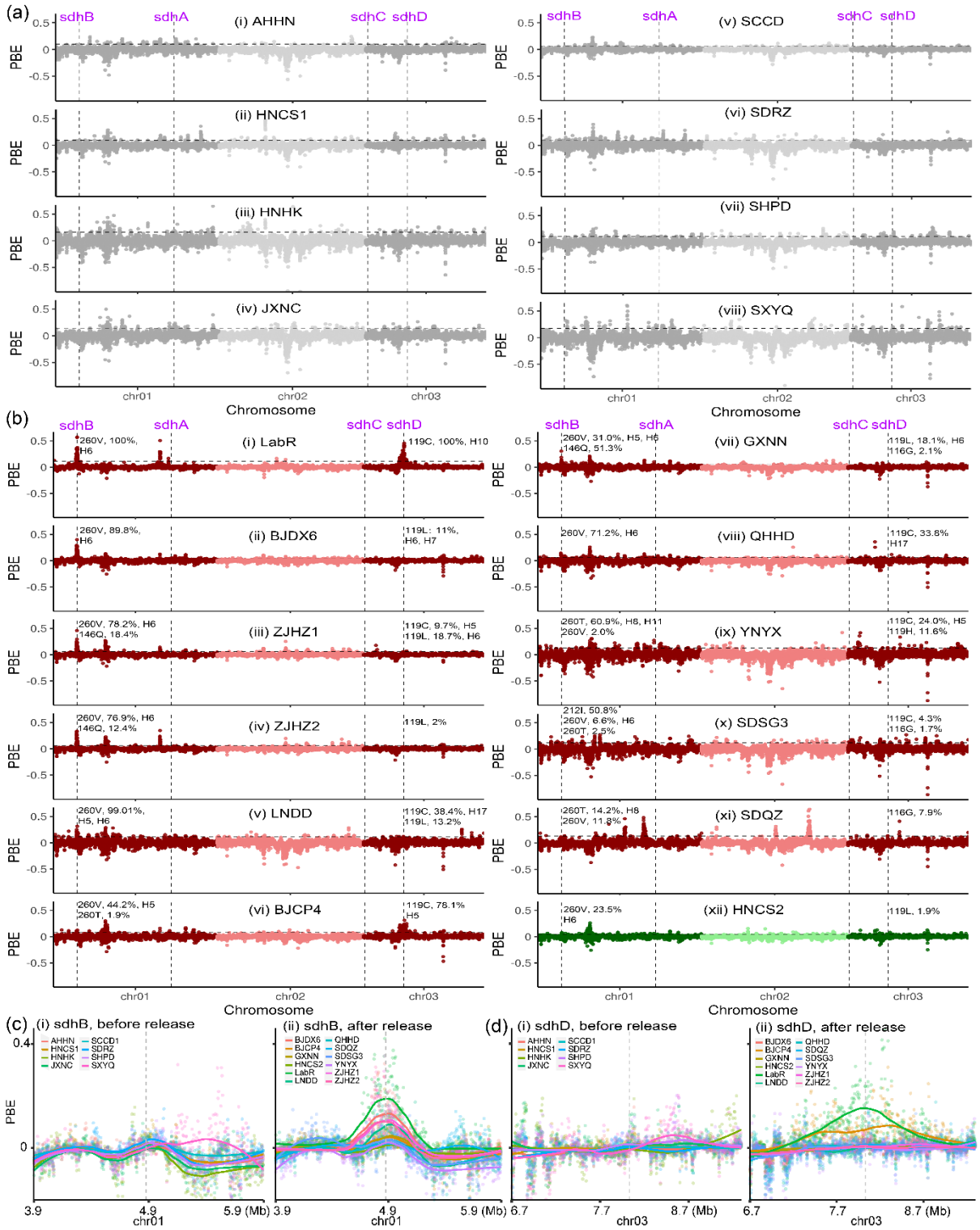

Figure 2 Signals of selective sweep across genomes of the two-spotted spider mite *Tetranychus urticae*. (a) PBE values across the genome for eight historic populations collected

865 in 2017 before the commercial release of cyetpyrafen. Chromosomes are colored alternately
866 dark grey and light grey. (b) PBE values for one lab-selected resistant population, 10 resistant
867 field populations and one susceptible population collected in 2021 and 2024 after the release of
868 cyetpyrafen. Chromosomes are colored alternately dark red and light red for resistant
869 populations, dark green and light green for susceptible populations. Allele frequency (pool-seq
870 data) and haplotypes of mutations are indicated near the corresponding *sdhB* and *sdhD* genes
871 of each population in (a) and (b). (c) PBE values for genomic regions around the *sdhB* gene,
872 comparing values between the historic (left) and recently collected (right) populations. (d) As
873 above but for the *sdhD* gene. Each point represents a genomic window of 5-kp wide when
874 compared to reference genomes involving the susceptible laboratory line (LabS) and a
875 susceptible field population (NMHH2). The vertical lines indicate the position of *sdh* genes,
876 while the horizontal lines represent the 1% threshold of PBE values.

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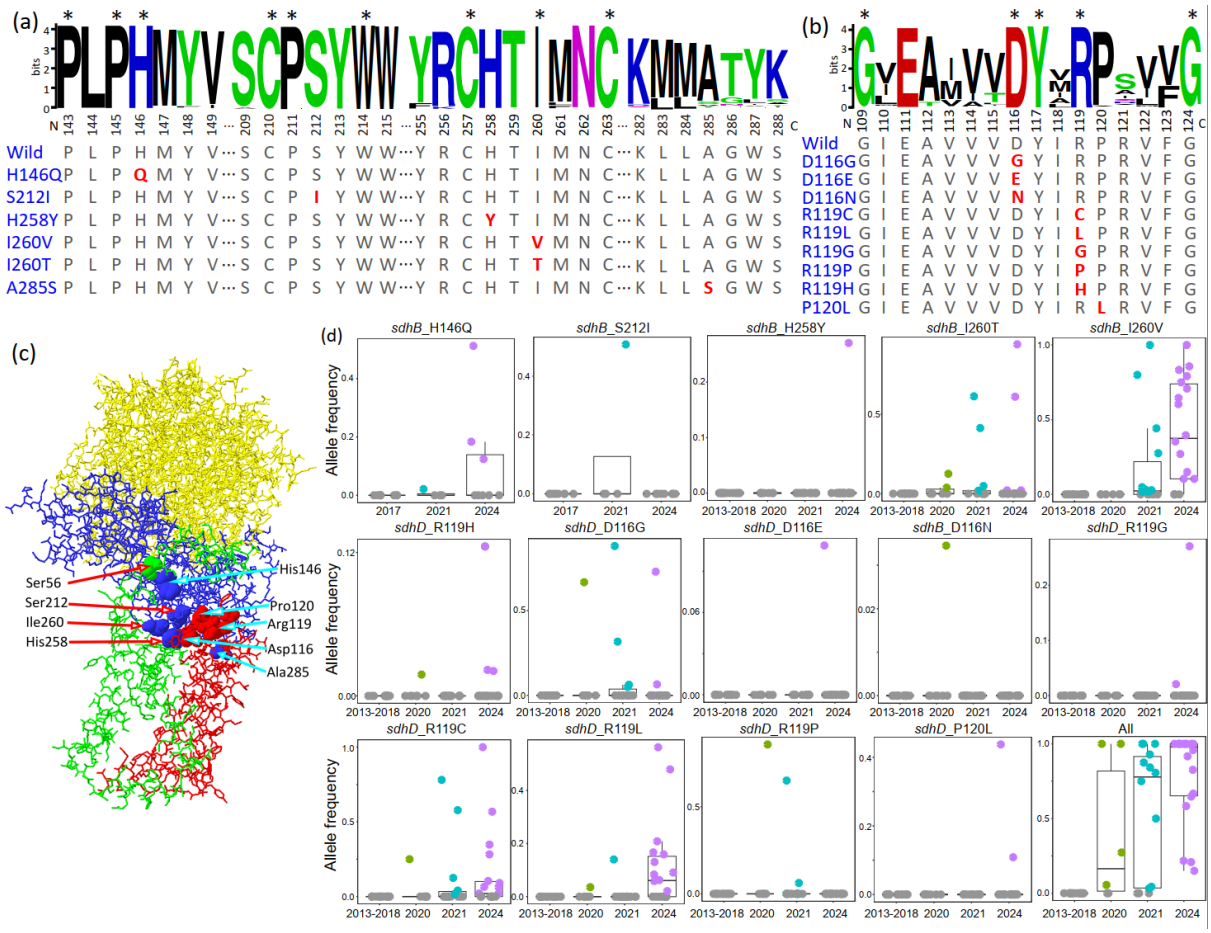
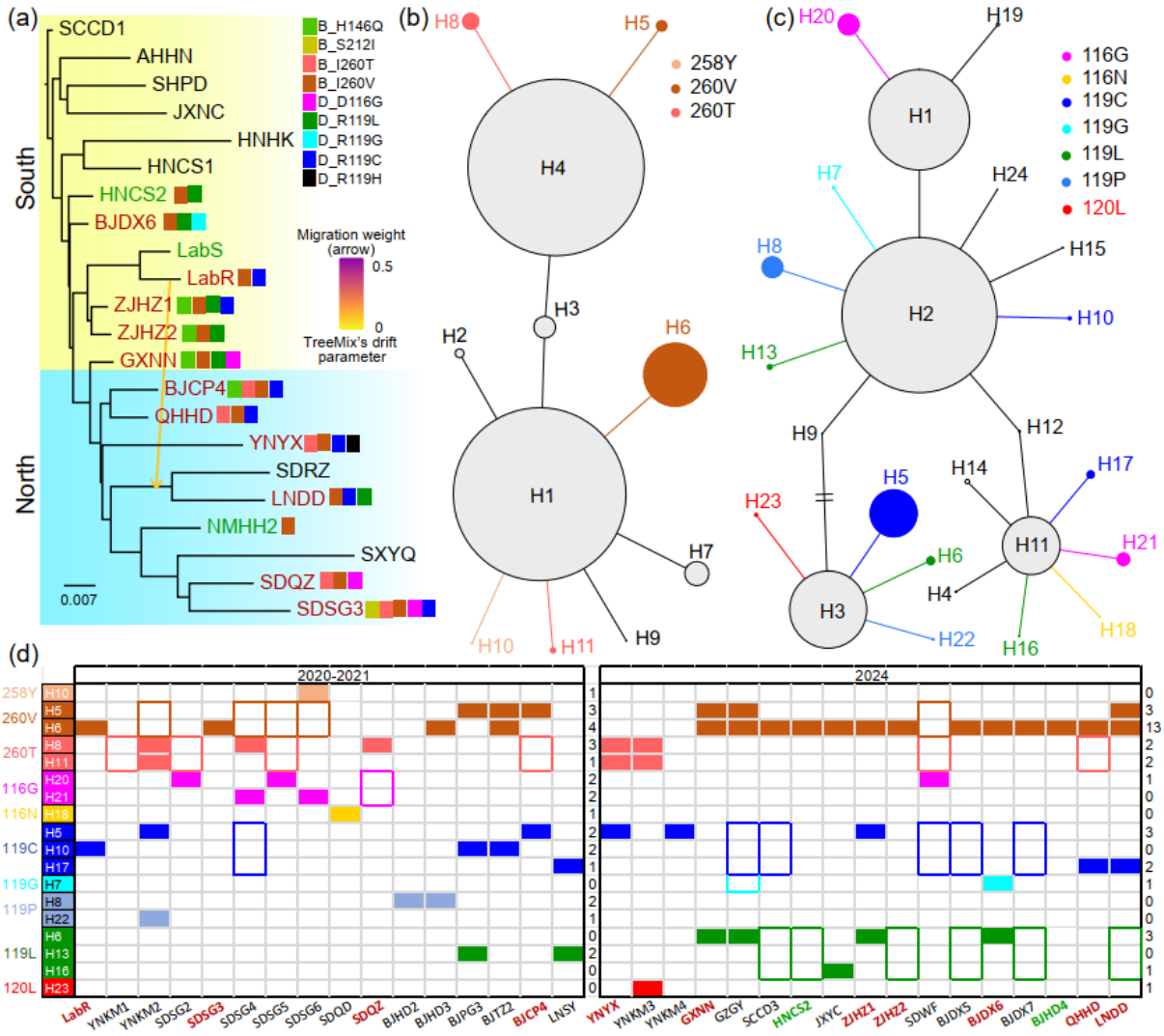


Figure 3 Amino acid mutations on *sdhB* and *sdhD* genes in populations of the two-spotted spider mite *Tetranychus urticae*. (a, b) Sequence logos at mutations related to cyetpyrafen resistance in *sdhB* (a, 757 sequences) and *sdhD* (b, 158 sequences). The numbers under logos indicate the position 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) Amino acid mutation sites on a 3D structure of the wild-type succinate dehydrogenase of *T. urticae*. The sdhA, sdhB, sdhC and sdhD chains are shown in yellow, blue, green, and red, respectively. 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) Temporal variation in allele frequencies of resistance mutations in populations of *T. urticae* determined through Sanger sequencing of target genes. Each dot represents one population. Allele frequencies of populations collected from 2013-2018, 2020, 2021, and 2024 are shown as different colors. A258S was omitted as it was only present in one population (SDRZ) sampled in 2017.



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

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

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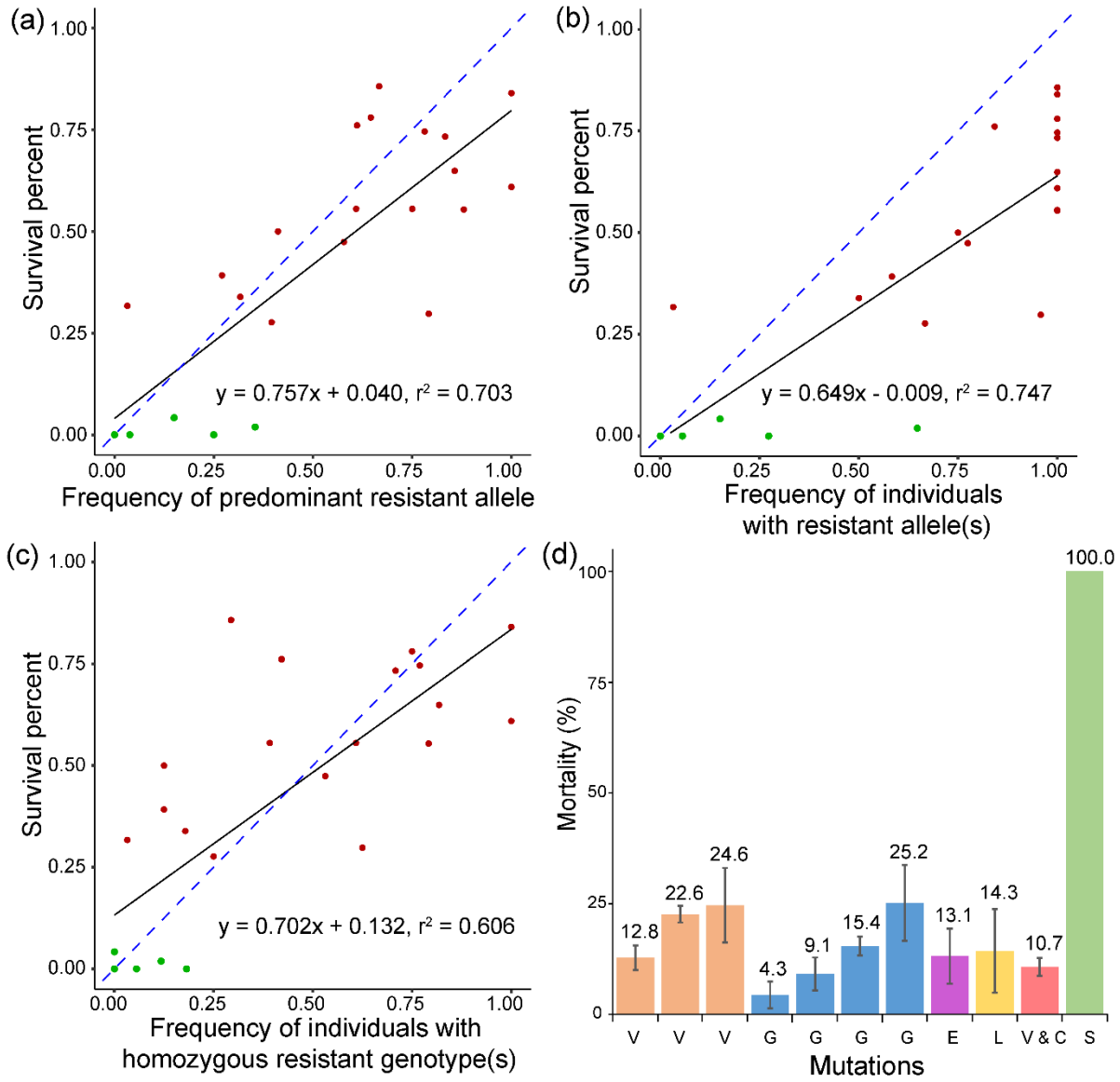

Figure 5 Function of target-site mutations in the *sdhB* and *sdhD* genes and survival of two-spotted spider mite *Tetranychus urticae* in cyetpyrafen resistance. (a-c) Association between frequency of target-site mutations in the *sdhB* and *sdhD* genes and survival of two-spotted spider mite *T. urticae* 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

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