Co-occurrence of subunit B and C mutations in respiratory complex II confers high resistance levels to pyflubumide and cyenopyrafen in the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae)

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ABSTRACT

BACKGROUND: Pyflubumide and cyenopyrafen are respiratory complex II (complex II) inhibitors. Previous quantitative trait locus analyses suggested associations of I260V and S56L in complex II subunit B (B-I260V) and subunit C (C-S56L) with pyflubumide and cyenopyrafen resistance, respectively, in *Tetranychus urticae*. However, although resistant strains had been selected separately by these acaricides, all strains were homozygous for both *B-I260V* and *C-S56L*. Hence, the effects of each mutation on resistance development remain unclear.

RESULTS: We established strains homozygous for *B-I260V* with *C-S56* (*B-I260V_I260V/C-S56_S56*) and for *C-S56L* with *B-I260* (*B-I260_I260/C-S56L_S56L*). High resistance levels (LC_{50} > 1000 mg L^{-1}) to pyflubumide and cyenopyrafen was not conferred by *B-I260V* or *C-S56L* alone. Next, we prepared intermixed strains by crossing *B-I260V_I260V/C-S56_S56* and *B-I260_I260/C-S56L_S56L*. Selection of the intermixed strains by either acaricide caused very high resistance levels (LC_{50} ≥ 10,000 mg L^{-1}) to both acaricides and fixed both mutations. Allele-selected recoupling of the mutations without acaricide selection also conferred very high resistance levels to both acaricides in the intermixed strains. Unlike these, *B-I260V* or *C-S56L* alone conferred very high and high resistance levels to cyflumetofen, respectively.

CONCLUSION: We conclude that the effect of individual mutations characteristically varies among complex II inhibitors. Moreover, very high resistance levels to pyflubumide and cyenopyrafen is conferred by the co-occurrence of *B-I260V* and *C-S56L* mutations, which alone have limited effects on resistance level.

Keywords: mitochondrial electron transport chain, complex II, target site mutation, acaricide resistance, *Tetranychus urticae*

1 INTRODUCTION

Acaricide resistance is often caused by mutation of the target site or metabolic degradation of the pesticide by detoxification enzymes.[1] As an example, the amino acid substitution I1017F in chitin synthase I confers a very high level of resistance to etoxazole, clofentezine, and hexythiazox in the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae).[2–5] However, target site mutation alone does not confer high resistance levels to abamectin, milbemectin, or complex I inhibitors in *T. urticae*.[4–7] Additional significant effects of detoxification enzymes on resistance levels have been reported for those acaricides.[8–9] For complex III inhibitors such as bifenazate and acequinocyl, a specific combination of target site mutations (e.g., G126S with I136T or S141F in cytochrome *b*) confers high resistance levels in *T. urticae*, but a single mutation of G126S does not.[10–13] These findings indicate frequent involvement of multiple resistance factors in acquisition of resistance against acaricides, especially very high resistance levels such that the LC_{50} value exceeds 10,000 mg L^{-1}.

Cyflumetofen, cyenopyrafen and pyflubumide are pro-acaricides, as their metabolites inhibit the electron-transport function of respiratory complex II (i.e., succinate dehydrogenase, hereafter, complex II) in spider mites.[14–18] Those metabolites likely bind to the quinone binding pocket, consisting of complex II subunit B (iron-sulfur subunit; SdhB), subunit C (cytochrome b560 subunit; SdhC), and subunit D (cytochrome b small subunit),[14,17] in the same manner as fungicidal complex II inhibitors such as carboxin.[19–21] Many authors reported involvement of detoxification

60 systems in the development of resistance to acaricidal complex II inhibitors, and several
61 candidate genes have been identified in *T. urticae* [9,22–26] and also *Tetranychus*
62 *cinnabarinus* (Boisduval).[27,28] However, the resistance levels of mote strains used in
63 these studies were sometimes not high but rather moderate.
64 Using quantitative trait locus (QTL) analysis of microsatellite linkage maps,
65 Sugimoto et al.[29] identified I260T in SdhB (B-I260T), I260V in SdhB (B-I260V), and
66 S56L in SdhC (C-S56L) as candidate target site mutations responsible for very high
67 resistance levels to cyflumetofen, pyflubumide, and cyenopyrafen, respectively, in
68 selected *T. urticae* strains. The *SdhB* and *SdhC* loci are present on different
69 chromosomes.[29,30] Of these mutations, I260T (without C-S56L) also confers the very
70 high resistance levels to cyenopyrafen (LC_{50} > 10,000 mg L^{-1}, but relatively high
71 mortality also occurred at lower concentrations) but did not confer resistance to
72 pyflubumide.[29] A pyflubumide-resistant strain showed very high cross-resistance levels
73 to cyflumetofen and cyenopyrafen, and a cyenopyrafen-resistant strain also showed very
74 high cross-resistance levels to cyflumetofen (cross-resistance from cyenopyrafen to
75 pyflubumide was not tested).[29] Although QTL analyses identified distinctive target site
76 mutations associated with pyflubumide and cyenopyrafen resistance, strains resistant to
77 these two acaricides were selected from an identical field population, and consequently,
78 all strains possessed both *B-I260V* and *C-S56L*.[29] Hence, the individual and combination
79 effects of these mutations on resistance development have not yet been elucidated.
80 Our aims in this study were to elucidate the manner in which *SdhB* and *SdhC*
81 mutations are involved in very high resistance levels to pyflubumide and cyenopyrafen,
82 and whether those mutations are related to cyflumetofen resistance in *T. urticae*. For this
83 purpose, we uncoupled *B-I260V* and *C-S56L* and evaluated the individual effects of

these mutations on resistance levels. Then, we tested whether acaricidal selection leads to recoupling of these mutations and whether recoupling via genotype selection (allele-selected recoupling) confers very high resistance levels in mites.

2 MATERIALS AND METHODS

2.1 Acaricides

Commercial formulations of cyenopyrafen (Starmite,® 30 SC; Nissan Chemical Corp., Tokyo, Japan), pyflubumide (Dani-kong,® 20 SC; Nihon Nohyaku Co., Ltd., Tokyo, Japan), and cyflumetofen (Danisaraba,® 20 SC; OAT Agrio Co., Ltd., Tokyo, Japan) were used for selection and the toxicological bioassay.

2.2 Mites

The pyflubumide-resistant strain SoKg1_PflR and susceptible strain Tsukuba_S used in this study were the same strains used by Sugimoto et al.[29] Briefly, SoKg1_PflR was selected by pyflubumide from a field population originally collected in 2012 from commercially cultivated strawberry greenhouses in Kakegawa (SoKg1), Shizuoka Prefecture, Japan.[29] Tsukuba_S is a laboratory strain transferred from the Central Region Agricultural Research Center, NARO (Tsukuba, Ibaraki, Japan) in December 2011, and it was maintained in a laboratory with no acaricide application for 18 years.[5] Mites were reared on kidney bean (*Phaseolus vulgaris* L.) leaves placed atop water-soaked cotton in Petri dishes (9 cm in diameter) in a laboratory at 25°C under a photoperiod of 16:8 h light/dark. DNA sequences of SoKg1 and Tsukuba_S are available from the DDBJ/EMBL/GenBank databases under accession numbers LC511606 and LC511608, respectively, for *SdhB* and LC509025 and LC509026, respectively, for *SdhC*.[29]

2.3 Sequencing analyses of *SdhB* and *SdhC*

Crude DNA samples were individually extracted from mites and prepared for PCR amplification following the method of Osakabe et al.[5] Adult female or male mites were individually homogenized using a plastic pestle (Pellet mixer; Toho, Tokyo, Japan) in 20 μL lysis buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% Igepal CA-630 (Sigma-Aldrich Co. LLC., Tokyo, Japan), 10 mM NaCl, and 1 mg mL^{-1} proteinase K (TaKaRa Bio Inc., Kusatsu, Japan) in 200 μL PCR tubes. The homogenate was incubated at 65°C for 20 min and 95°C for 10 min. The lysate was diluted with nuclease-free water at 1:80 and 1:40 for females and males, respectively, and stored in a freezer at −25°C until PCR amplification.

Genomic DNA regions of *T. urticae* including the whole coding sequences (CDSs) of *SdhB* and *SdhC* were separately amplified via PCR using 1.6 μL DNA template in a total reaction volume of 8 μL containing PCR buffer for KOD FX Neo (TOYOBO Co. Ltd., Osaka, Japan), 0.4 mM of each dNTP, 0.25 μM each of specific forward and reverse primers, and 0.4 U KOD FX Neo DNA polymerase (TOYOBO). The primer sets used for PCR amplification were those reported by Sugimoto et al.,[29] namely tuSdhB-gF, 5′-GAAGGACCTCACGTTTGAATCACAG-3′, and tuSdhB-gR, 5′-CAACGCCCGATTCTCTTGTTACC-3′, for *SdhB* (amplicon: 1942 bp) and tuSdhC-gF, 5′-TCTGGACTCACTGCGATCGAAAG-3′, and tuSdhC-gR, 5′-GGTGACTGGGATCAAGATTGAGTACC-3′, for *SdhC* (amplicon: 1284 bp). The PCR conditions were an initial 2 min at 96°C followed by 40 cycles of 10 s at 98°C, 10 s at 60°C, and 120 s and 77 s for *SdhB* and *SdhC*, respectively, at 68°C, and then a final 7 min at 68°C. After the remaining primers were removed by the polyethylene glycol precipitation method, cycle sequencing was performed using the BigDye ™ Terminator

v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) with the primers tuSdhB-900R, 5′-GTCCAGATATCTGAGGCTCACTCTTC-3′, and tuSdhC-gFseq, 5′-GCTATTCATGAATATGCTACAAAACCAC-3′, for *SdhB* and *SdhC*, respectively. The amplicons obtained using these primers included the 260th and 56th codons of *SdhB* and *SdhC*, respectively. The sequence of tuSdhC-gFseq was the same as that of the primer used by Sugimoto et al.,[29] and tuSdhB-900R was newly designed to target the CDS of *SdhB* (*tetur01g15710*) obtained from Online Resource for Community Annotation of Eukaryotes (https://bioinformatics.psb.ugent.be/orcae/) using GENETYX® ver. 14 software (GENETYX Corp., Tokyo, Japan). The DNA sequences were analyzed using the ABI 3130 Genetic Analyzer (Thermo Fisher Scientific).

2.4 *SdhB* and *SdhC* genotyping by high resolution melting analysis

The quantitative PCR (qPCR) solution consisted of a total volume of 8 µL containing 4 µL TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Inc.), 0.5 µM each of forward and reverse primers, and 1 µL of the crude DNA sample prepared as described in Section 2.3.

High-resolution melting (HRM) analysis was performed to determine the genotypes of *SdhB* and *SdhC* using a real-time PCR instrument (LightCycler 96 System; Roche Diagnostics, Tokyo, Japan). The PCR conditions consisted of preheating for 5 s at 95°C, followed by 40 cycles of 5 s at 95°C, 30 s at 60°C, and 30 s at 72°C and one cycle of 60 s at 95°C, 60 s at 40°C, and 1 s at 65°C. HRM was then performed by heating to 97°C with 15 readings per 1°C. The primer pairs used for the HRM analysis to obtain PCR products including the mutation sites (B-I260 and C-S56) were tuSdhB-736F, 5′-GATCAATTGAGGGATCCCTTCTCAC-3′, and tuSdhB-826R, 5′-

154 CTCGACCAGGATTCAAGTTCTTAGG-3′, for *SdhB* (amplicon: 91 bp) and tuSdhC-
155 96F, 5′- AAGCTCATCGTCGCCATCAC-3′, and tuSdhC-211R, 5′-
156 TTGATAGGACCGAGGTTAATTGAGG-3′, for *SdhC* (amplicon: 116 bp). These
157 primer pairs were designed from the CDSs of *SdhB* and *SdhC* (*tetur30g00210*;
158 https://bioinformatics.psb.ugent.be/orcae/) using GENETYX® ver. 14 (Supplementary
159 Data 1 and Data 2). The genotypes of *SdhB* for I260 and I260V and *SdhC* for S56 and
160 S56L were determined from the shape of the melting curve (Supplementary Fig. S1).

161 ## 2.5 Establishment of homozygous strains of *SdhB* and *SdhC*

162 Strains homozygous for *B-I260V* and *C-S56L* (mutant-type strain) and for *B-I260* and
163 *C-S56* (wild-type strain) were established by intra-strain crosses of SoKg1_PflR and
164 Tsukuba_S, respectively. Eight pairs of females and males from each strain were
165 separately mated on kidney bean leaf disks (2 × 2 cm). Offspring from three mating
166 pairs homozygous for the mutation or wild-type gene at both *SdhB* and *SdhC* loci were
167 pooled and used for production of the next generations, thereby establishing the mutant-
168 type (SoKg1_PflR_MT) and wild-type (TsukubaS_WT) strains. The genotypes of all
169 pairs used in the crosses were determined by sequencing analysis (Data was not shown).

170 ## 2.6 Toxicological bioassay

171 A kidney bean leaf disk (2 × 2 cm) containing 10–14 adult females was immersed in
172 acaricide solution at the assigned concentration for 10 s and then dried on filter paper at
173 room temperature. The leaf disk was placed atop water-soaked cotton in a Petri dish,
174 and mortality was determined after 72 h, as pyflubumide and cyenopyrafen act slowly.
175 Any individual that could walk was counted as alive. Individuals that escaped from the
176 leaf disks and drowned were excluded from computation of the LC_{50} values. Corrected

mortality (M_C) was calculated using Abbott's formula:[31]

$$M_C = \frac{B-A}{B},$$

where A and B are the survival rates on leaves immersed in acaricide solution (test leaves) and in water without acaricide (control), respectively. The LC_{50} value and its 95% fiducial limit were computed using a program designed to determine the 50% effective dose (http://aoki2.si.gunma-u.ac.jp/R/ed50.html)[32] with some modifications made by Sugimoto et al.[29] in R software.[33]

We used several leaf disks for each assigned concentration and combined the data from those leaf disks to compute a LC_{50} value. The average number of females used for the analysis per concentration in each experiment was shown in Table 1, 2, and 4.

2.7 Evaluation of the independent effects of *B-I260V* and *C-S56L*

2.7.1 Decoupling of SdhB and SdhC mutations

To construct strains homozygous for *B-I260V* and *C-S56* and homozygous for *B-I260* and *C-S56L*, four SoKg1_PflR_MT males were individually crossed with TsukubaS_WT females (mating lines: ML1, 2, 3 and 4 [1–4]; Supplementary Fig. S2). Eight F1 females (heterozygous for both *SdhB* and *SdhC*) obtained from each parental pair were individually mated with TsukubaS_WT males (*B-I260*/*C-S56*). Then, mating of unmated F2 females (44 ♀♀, 16 ♀♀, 62 ♀♀, and 38 ♀♀ for ML1, ML2, ML3, and ML4, respectively) with their own sons (F3 males) was performed. For this cross experiment, after oviposition of the unfertilized (haploid) male eggs for 2–4 days at 25°C, the F2 females were kept at 10°C until the F3 males reached adulthood. After mother (F2)–son (F3) mating, each pair was reared for a week at 25°C until a sufficient number of eggs was laid for subsequent cross experiments. Genotypes of *SdhB* and

200 *SdhC* in the F2 females and F3 males (48 pairs) were confirmed by HRM analysis
201 (Table S1). As a result, the offspring (F3 females) from four mating pairs (sublines
202 ML1-2-6, ML3-1-1, ML1-4-4, and ML1-4-5; branch numbers show identification of
203 parent-F1-F2 females) were selected for use in subsequent mother–son mating
204 experiments. Genotypes of *SdhB* and *SdhC* in the four mating pairs were confirmed by
205 sequencing analysis, and no discrepancy were found (Data was not shown). In sublines
206 ML1-2-6 and ML3-1-1, *SdhB* and *SdhC* of the F2 females were heterozygous and fixed
207 for wild-type, respectively (*B-I260_I260V*/*C-S56_S56*), and F3 males had *B-I260V* and
208 *C-S56* (*B-I260V*/*C-S56*). In the sublines ML1-4-4 and ML1-4-5, the genotype of F2
209 females was *B-I260_I260*/*C-S56_S56L*, and that of F3 males was *B-I260*/*C-S56L*. Four
210 F3 females produced from each mother (F2)–son (F3) mating were used for the next
211 mother (F3)–son (F4) mating experiment, and the offspring of F3 females of *B-*
212 *I260V_I260V*/*C-S56_S56* and *B-I260_I260*/*C-S56L_S56L* were established as the *B-*
213 *I260V* fixed strain and *C-S56L* fixed strain, respectively (Table S2). Consequently, three
214 *B-I260V* fixed strains (strains I260V-1, 2 and 3 [1–3]) were obtained from subline ML1-
215 2-6, and three *C-S56L* fixed strains (strains S56L-1, 2 and 3 [1–3]) were obtained from
216 sublines ML1-4-4 and ML1-4-5. These six fixed strains were all derived from a single
217 parental mating pair (mating line: ML1). Genotypes of *SdhB* and *SdhC* in the decoupled
218 strains were confirmed by sequencing analysis (Supplementary Data 1 and Data 2).

219 ### *2.7.2 Effects of decoupled mutations on resistance level*

220 The resistance levels of the *B-I260V* fixed strains (I260V-1–3) and *C-S56L* fixed strains
221 (S56L-1–3) were tested against pyflubumide, cyenopyrafen and cyflumetofen by
222 toxicological bioassays. The LC_{50} values were compared with those of

SoKg1_PflR_MT and TsukubaS_WT, which were tested simultaneously.

2.8 Evaluation of co-occurrence effects of *B-I260V* and *C-S56L*

2.8.1 Intermixing of the B-I260V *and* C-S56L *fixed strains*

We intermixed *B-I260V* fixed strains I260V-1–3 with *C-S56L* fixed strains S56L-1–3. Three combinations of intermixed strains (PflMix-1, 2 and 3 [1–3]) were selected based on the LC_{50} values for pyflubumide presented in Section *2.7.2* (Table S3), although the order of I260V-1 and I260V-2 strains with a slight difference in LC_{50} values (see Table 1) had been reversed. Three further combinations (CyeMix-1, 2 and 3 [1–3]) were similarly constructed using the LC_{50} values for cyenopyrafen (Table S3). For each combination, four pairs of forward and reverse mating events were separately performed using kidney bean leaf disks (2 × 2 cm) placed on water-soaked cotton in a Petri dish. After mating, females from the reciprocal crosses were moved to a newly prepared kidney bean leaf (~25 cm^2) placed atop water-soaked cotton in a Petri dish. After 20 days, the intermixed strains were used for toxicological testing of pyflubumide and cyenopyrafen. To confirm co-occurrence of the fixed mutations in each Sdh gene in intermixed strains, we preliminarily analyzed *SdhB* and *SdhC* genotype frequencies in PflMix-1–3 by sequencing analysis (Table S4).

2.8.2 Effects of acaricide selection on genotype frequencies

PflMix-1–3 and CyeMix-1–3 were selected using pyflubumide and cyenopyrafen, respectively, five times at increasing concentrations of 100, 200, 400, 1000, and 2000 mg L^{-1}. For selection, a small piece of kidney bean leaf containing >100 individuals was immersed in acaricide solution for 10 s. After drying on filter paper at room temperature, a small piece of the leaf was placed on water-soaked cotton in a Petri dish

246 for 24 h and then moved onto a newly prepared kidney bean leaf (~25 cm^2).

247 After acaricide selection, the resistance levels of pyflubumide-selected and
248 cyenopyrafen-selected intermixed strains (PflMixR-1–3 and CyeMixR-1–3,
249 respectively) against pyflubumide, cyenopyrafen, and cyflumetofen were evaluated by
250 toxicological bioassays. Genotypes of *SdhB* and *SdhC* were analyzed by DNA
251 sequencing of 12 females randomly chosen from each the selected intermixed strain.

252 ### *2.8.3 Effects of allele-selected recoupling of* B-I260V *and* C-S56L *without*
253 ### *acaricide selection on resistance*

254 Eight mating groups consisting of one male and six unmated females from a single
255 intermixed strain (PflMix-1–3 and CyeMix-1–3; 48 mating groups in total) were
256 introduced to kidney bean leaf disks (2 × 2 cm) and allowed to mate for 24 h. Females
257 that mated with males of the *B-I260V*/*C-S56L* genotype, as determined using the HRM
258 method, were then individually reared on kidney bean leaf disks and allowed to lay eggs
259 for 1 week. Next, the genotypes of the females were determined using the HRM
260 method. F1 offspring of females that were heterozygous or homozygous for both
261 mutations were used for subsequent mother–son mating experiments. After mother–son
262 mating, the *SdhB* and *SdhC* genotypes of the females were determined using the HRM
263 method. The offspring of *B-I260V_I260V*/*C-S56L_S56L* females were established as the
264 recoupled strains. Consequently, two recoupled strains, PflMixMT-1 and PflMixMT-2,
265 were obtained from PflMix-1 and PflMix-2, respectively, and another two strains,
266 CyeMixMT-2 and CyeMixMT-3, were obtained from CyeMix-2 and CyeMix-3,
267 respectively (Table S5). Genotypes of *SdhB* and *SdhC* in the recoupled strains were
268 confirmed by sequencing analysis (Supplementary Data 1 and Data 2). The resistance

levels of the recoupled strains against pyflubumide and cyenopyrafen were evaluated by toxicological bioassays.

3 RESULTS

3.1 Effects of decoupled mutations on resistance level

SoKg1_PflR_MT exhibited a very high resistance level to pyflubumide, cyenopyrafen, and cyflumetofen. The LC_{50} of this strain for pyflubumide was 10,900 mg L^{-1} (Fig. 1a, Table 1). As mortality rates in the presence of cyenopyrafen (21.2%) and cyflumetofen (34.6%) remained low at a concentration of 20,000 mg L^{-1} (Fig. 1b and Fig. 1c), we described the LC_{50} values for these acaricides as > 10,000 mg L^{-1} (Table 1). In contrast, TsukubaS_WT was susceptible to all three complex II inhibitors, although the slope of the regression line for cyflumetofen (0.49) was small, and the associated LC_{50} of 23.7 mg L^{-1} was 7–10 times higher than those reported by Sugimoto et al.[29] for other susceptible strains (2.40 for Nara_S and 3.39 mg L^{-1} for NS_CflS) (Fig. 1, Table 1).

The effect of decoupling of *SdhB* and *SdhC* mutations on susceptibility varied among complex II inhibitors and mutations. I260V-1–3 had a higher LC_{50} value for pyflubumide (2.93–8.83 mg L^{-1}) compared with TsukubaS_WT (0.093 mg L^{-1}) but remained susceptible because the LC_{50} values were much lower than the field concentration (100 mg L^{-1}; Table 1, Fig. 1a). S56L-1–3 showed pyflubumide resistance, with a LC_{50} value of 375–892 mg L^{-1} higher than field concentrations. For cyenopyrafen, I260V-1–3 and S56L-1–3 showed moderate resistance, with LC_{50} values of 107–199 mg L^{-1} and 50.2–150 mg L^{-1}, respectively, which were similar to the field concentration (150 mg L^{-1}; Table 1, Fig. 1b). I260V-1–3 showed very high resistance levels to cyflumetofen (LC_{50} > 10,000 mg L^{-1}), equivalent to the resistance exhibited by

292 SoKg1_PflR_MT (Table 1, Fig. 1c). S56L-1–3 also showed high-revel resistance to
293 cyflumetofen, with LC_{50} values exceeding 3000 mg L^{-1}, which were markedly higher
294 than the field concentration (200 mg L^{-1}).

295 ## 3.2 Co-occurence effects of *B-I260V* and *C-S56L*

296 ### *3.2.1 Effects of acaricide selection on genotype frequencies*

297 Prior to acaricide selection, the LC_{50} values of the intermixed strains PflMix-1–3 (4.05–
298 101 mg L^{-1}) (Table 2, Fig. 2a) for pyflubumide were somewhat increased relative to
299 those of I260V-1–3 (2.93–8.83 mg L^{-1}; Table 1) but decreased relative to those of S56L-
300 1–3 (375–892 mg L^{-1}; Table 1). The slope of the regression line was reduced to 0.37–
301 0.72 in PflMix-1–3, suggesting heterogeneous resistance. After pyflubumide selection,
302 the LC_{50} of PflMixR-1–3 increased markedly to > 10,000 mg L^{-1}, and the slope of the
303 regression line increased to 1.88–2.76, suggesting homogenous resistance (Table 2, Fig.
304 2a). The intermixed strains CyeMix-1–3 showed moderate resistance (LC_{50} = 20.3–219
305 mg L^{-1}) to cyenopyrafen, and the slope of the regression line was small (0.42–0.89)
306 (Table 2, Fig. 2b). Cyenopyrafen selection markedly increased the LC_{50} to 9975–19,225
307 mg L^{-1} and the regression line slope to 1.73–2.58 (Table 2, Fig. 2b). The mortality rate
308 of the acaricide-selected intermixed strains caused by cyflumetofen at concentrations of
309 1000–10,000 mg L^{-1} was low, at 0–28.5% (Fig. 2c), suggesting very high cross-
310 resistance (Table 2).

311 After acaricide selection, most individuals were homozygous for mutant *SdhB* and
312 *SdhC* (*B-I260V_I260V*/*C-S56L_S56L*). The percentages of individuals with *B-*
313 *I260V_I260V*/*C-S56L_S56L* were 83.3–100% and 83.3–91.7% in PflMixR-1–3 and
314 CyeMix-1–3, respectively (Table 3). The genotypes of all other individuals were *B-*

315 *I260_I260V/C-S56L_S56L*. This suggests that selection using these acaricides promoted
316 homozygous mutant alleles at both the *SdhB* and *SdhC* loci.

317 ### *3.2.2 Effects of allele-selected recoupling of B-I260V and C-S56L without*
318 ### *acaricide selection on resistance levels*

319 Strains homozygous for both *B-I260V* and *C-S56L* mutations established without
320 acaricide selection showed low mortality rates in the presence of pyflubumide
321 (PflMixMT-1–2, 35–47%) and cyenopyrafen (CyeMixMT-2–3, 32–41%), respectively,
322 even at a concentration of 20,000 mg L^{-1} (Fig. 3), indicating very high resistance levels
323 to these acaricides. However, substantial mortality was observed at concentrations of
324 100–1000 mg L^{-1} (15.6–31.3% [number of females tested: 98 ± 1 (SD)] and 5.5–23.1%
325 [99 ± 5] for pyflubumide and cyenopyrafen, respectively; Fig. 3). This result might
326 indicate the presence of unknown resistance factors, which were selected with the
327 application of acaricides as described in Section *3.2.1* but not by genotype selection,
328 leading to unrealistic LC_{50} values (0.8–20 kg L^{-1}). Therefore, we excluded the data
329 obtained using concentrations of 100–1000 mg L^{-1} and determined approximate LC_{50}
330 values above 10,000 mg L^{-1} for both pyflubumide (PflMixMT-1–2) and cyenopyrafen
331 (CyeMixMT-1–2) (Table 4, Fig. 3).

332 ## 4 DISCUSSION

333 The mutations of *B-I260V* and *C-S56L* have been suggested to drive pyflubumide and
334 cyenopyrafen resistance, respectively, based on the QTL analyses of Sugimoto et al.[29]
335 Decoupled strains homozygous for either *B-I260V* (I260V-1–3) or *C-S56L* (C56L-1–3)
336 showed intermediate susceptibilities to both pyflubumide and cyenopyrafen between the
337 very high-resistance-level strain (SoKg1_PflR_MT) and the susceptible strain

(TsukubaS_WT). Consequently, neither *B-I260V* nor *C-S56L* alone conferred very high resistance levels to these acaricides. Moreover, the LC_{50} value for pyflubumide was unexpectedly higher in the decoupled strain with *C-S56L* mutation than in that with *B-I260V*, while the LC_{50} values for cyenopyrafen did not differ greatly between the two decoupled strains. The reason for this discrepancy between the decoupling experiment and the QTL analysis that suggested *B-I260V* and *C-S56L* as factors of pyflubumide and cyenopyrafen resistance, respectively,[29] cannot be explained. The detailed molecular mechanisms underlying resistance to these acaricides should be elucidated in future research.

In contrast, *B-I260V* alone conferred very high resistance levels to cyflumetofen, as did *B-I260T* in Sugimoto et al.,[29] and *C-S56L* alone conferred high resistance levels to cyflumetofen. Although no detailed studies have been conducted on the binding site of cyflumetofen to complex II, I260 in *SdhB* of *T. urticae* is located two residues downstream of the ubiquinone-binding residue (H207 in *Escherichia coli*, corresponding to H258 in *T. urticae*) in the Q_2 site[19] within cysteine-rich cluster III,[34] and corresponds to I269V in *Zymoseptoria tritici* (synonym: *Mycosphaerella graminicola*), which confers high resistance levels to the fungicidal complex II inhibitor carboxin.[35] The potential ubiquinone-binding histidine (H267) in *Z. tritici* SdhB appears to bind to the "core" moiety of carboxamides via hydrogen bonding at the bottom of the cavity.[21] B-I260V in *T. urticae* may alter the binding of the cyflumetofen core to SdhB and weaken the interactions of SdhB with pyflubumide and cyenopyrafen, or their active metabolites.[14,17]

Acaricide selection for the mixtures of decoupled strains (intermixed strains) led to the development of very high resistance levels to both pyflubumide and cyenopyrafen,

362 and selection with either pyflubumide or cyenopyrafen alone enhanced fixation of both
363 the *SdhB* and *SdhC* loci to the *B-I260V* and *C-S56L* alleles, respectively. Conversely,
364 allele-selected homogenization of the intermixed strains for both the *SdhB* and *SdhC*
365 loci to the mutant alleles without acaricide selection increased the LC_{50} values for
366 pyflubumide and cyenopyrafen to achieve very high resistance levels. Therefore, the
367 occurrence of *B-I260V* and *C-S56L* mutations is likely essential for very high resistance
368 levels to pyflubumide and cyenopyrafen to occur (Table 5). This finding suggests the
369 possibility of very high cross-resistance levels between pyflubumide and cyenopyrafen,
370 as well as between these acaricides and cyflumetofen. In contrast, development of very
371 high resistance levels to cyflumetofen with *B-I260V* or *B-I260T*[29] alone does not cause
372 cross-resistance to pyflubumide, although these mutations cause moderate cross-
373 resistance and very high cross-resistance levels to cyenopyrafen, respectively (Table 5).
374 Substantial mortality due to pyflubumide and cyenopyrafen at relatively low
375 concentrations (100–1000 mg L^{-1}) was observed in the intermixed strains after allele-
376 selected homogenization, despite low mortality at the higher concentration of 20,000
377 mg L^{-1}. When the intermixed strains were selected by these acaricides (PflMixR-1–3
378 and CyeMixR-1–3), slopes of the concentration–mortality regression lines clearly
379 increased, and the substantial mortality at lower concentrations above described was not
380 observed. TsukubaS_WT possibly included genetic variance other than SdhB and SdhC.
381 Moreover, in our experimental design, decoupled strains (I260V-1–3 and S56L-1–3) had
382 25% of SoKg1_PflR_MT-derived genes theoretically. Therefore, to explain the
383 difference in the response to lower concentrations between acaricide-selected and allele-
384 selected intermixed strains, we inferred from previous studies that additional factors
385 such as detoxification enzymes contributed to general resistance to these complex II

386 inhibitors.

387 Previous synergism experiments have suggested that detoxification enzymes are
388 involved in cyenopyrafen,[9,22,23] pyflubumide,[24] and cyflumetofen[22,23] resistance. The
389 most promising upregulated candidate genes are *CYP392A11* (*tetur20g01390*) and
390 *CYP392A12* (*tetur06g02130*) for cyenopyrafen,[23] *CYP392A16* (*tetur06g04520*) for
391 pyflubumide,[25] and *TuGSTd05* (*tetur01g02510*) for cyflumetofen.[26] Fotoukkiaii et al.[25]
392 suggested the involvement of cis-regulatory variation of *CYP392A16* in pyflubumide
393 resistance. Associations of variations in the expression regulatory systems of certain
394 glutathione *S*-transferases have been reported in strains with low and moderate
395 cyflumetofen resistance in *T. cinnabarinus*.[27,28] Besides increasing expression of
396 detoxification enzymes, suppressed expression of enzymes that metabolize and activate
397 the complex II inhibitors[14–18] also possibly contributes resistance development.
398 Although the synergistic effects by esterase inhibitors on susceptibility to the complex II
399 inhibitors varies among mite strains and acaricides.[9,22–24] Sugimoto et al.[29] pointed out
400 the down regulation of two esterase genes, *TuCCE04* (*tetur01g10750*) and *TuCCE09*
401 (*tetur01g10830*), in Japanese and European cyflumetofen resistant strains of *T. urticae*.
402 These findings support our idea that additional factors are involved in the resistance to
403 complex II inhibitors.

404 **5 CONCLUSION**

405 The effect of each individual mutation varied among complex II inhibitors. *B-I260V* and
406 *C-S56L* alone conferred very high and high resistance levels to cyflumetofen,
407 respectively, but not to pyflubumide or cyenopyrafen, in *T. urticae*. Very high resistance
408 levels to pyflubumide and cyenopyrafen was induced by co-occurrence of the *B-I260V*

409 and *C-S56L* mutations. Such synergistic (or additive) effects of mutations in two
410 separate target site genes provide a new aspect of polygenic resistance. Moreover, even
411 if the LC_{50} values are low, populations having both mutations possibly achieve the very
412 high cross-resistance levels through coupling of the mutations accelerated by the
413 selection with pyflubumide or cyenopyrafen. Therefore, the synergistic effects should
414 take into consideration in a resistance management program, for which development of
415 a simple and efficient monitoring technique for resistance alleles[5,36–38] is desired.

416 ## SUPPORTING INFORMATION

417 Supporting information may be found in the online version of this article.

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548

Table 1. Resistance levels against complex II inhibitors in each strain including the strains with single mutation in *SdhB* (*B-I260V*) or *SdhC* (*C-S56L*)

Acaricides	Strains	LC_{50} values (mg L^{-1}) [a]	95% confidence intervals	Regression lines	RF [b]	n ± SD [c]
Pyflubumide	SoKg1_PflR_MT	10,900	8905–13764	$Y = 1.32\,X - 0.33$	117,204	96 ± 4
	TsukubaS_WT	0.093	0.0248–0.196	$Y = 1.07\,X + 6.10$	1	58 ± 25
	I260V-1	7.53	5.62–9.97	$Y = 1.18\,X + 3.96$	81.0	92 ± 25
	I260V-2	8.83	5.47–14.9	$Y = 0.64\,X + 4.40$	94.9	87 ± 24
	I260V-3	2.93	2.04–4.13	$Y = 1.00\,X + 4.53$	31.5	85 ± 27
	S56L-1	892	796–1002	$Y = 2.41\,X - 2.10$	9591	97 ± 15
	S56L-2	375	297–452	$Y = 1.62\,X + 0.83$	4032	91 ± 14
	S56L-3	641	530–764	$Y = 1.59\,X + 0.55$	6893	84 ± 17
Cyenopyrafen	SoKg1_PflR_MT	> 10,000	—	—	> 7407	93 ± 33
	TsukubaS_WT	1.35	1.07–1.70	$Y = 1.45\,X + 4.81$	1	98 ± 20
	I260V-1	107	92.1–122	$Y = 2.01\,X + 0.91$	79.3	99 ± 18
	I260V-2	194	173–219	$Y = 2.42\,X - 0.528$	143	93 ± 16
	I260V-3	199	179–223	$Y = 2.47\,X - 0.671$	147	105 ± 23
	S56L-1	50.2	42.5–59.1	$Y = 1.66\,X + 2.17$	37.2	94 ± 20
	S56L-2	77.2	68.0–88.3	$Y = 2.19\,X + 0.86$	57.2	98 ± 18
	S56L-3	150	134–170	$Y = 2.78\,X - 1.04$	111	97 ± 32
Cyflumetofen	SoKg1_PflR_MT	> 10,000	—	—	> 422	98 ± 3
	TsukubaS_WT	23.7	14.7–46.8	$Y = 0.49\,X + 4.33$	1	95 ± 3
	I260V-1	15,034	8214–48,749	$Y = 0.72\,X + 2.00$	634	102 ± 15
	I260V-2	> 10,000	—	—	> 422	102 ± 9
	I260V-3	13,831	8437–32,344	$Y = 0.88\,X + 1.37$	584	104 ± 11
	S56L-1	4566	3830–5597	$Y = 1.83\,X - 1.68$	193	97 ± 9
	S56L-2	3650	3135–4306	$Y = 2.09\,X - 2.46$	154	99 ± 14
	S56L-3	4665	3851–5837	$Y = 1.69\,X - 1.19$	197	92 ± 13

[a] If concentration–mortality curves did not rise within the range of the tested concentration due to the extremely high resistance levels, the LC_{50} values were indicated as > 10,000 mg L^{-1}.

[b] Resistance factors (RF) were calculated as a ratio of LC_{50} to TsukubaS_WT.

[c] The average numbers of females used for the analysis per concentration ± standard deviation

Table 2. Resistance levels against complex II inhibitors in the intermixed strains before (PfMix-1–3 and CyeMix-1–3) and after selection with pyflubumide (PfMixR-1–3) and cyenopyrafen (CyeMixR-1–3)

Acaricides	Strains [a]	LC_{50} values (mg L^{-1}) [b]	95% confidence intervals	Regression lines	RF [c]	n ± SD [d]
Pyflubumide	PflMix-1	101	37.7–221	$Y = 0.55\,X + 3.90$	1086	50 ± 2
	PflMix-2	45.2	19.2–87.5	$Y = 0.72\,X + 3.81$	486	50 ± 1
	PflMix-3	4.05	0.0717–20.7	$Y = 0.37\,X + 4.77$	43.5	49 ± 2
	PflMixR-1	10,225	8273–13171	$Y = 2.19\,X - 3.76$	109,946	49 ± 2
	PflMixR-2	11,413	9525–14105	$Y = 2.76\,X - 6.20$	122,720	47 ± 4
	PflMixR-3	10,853	8545–14687	$Y = 1.88\,X - 2.59$	116,699	48 ± 3
Cyenopyrafen	CyeMix-1	20.3	0.0200–122	$Y = 0.44\,X + 4.43$	15.0	44 ± 3
	CyeMix-2	219	90.7–399	$Y = 0.89\,X + 2.92$	162	47 ± 4
	CyeMix-3	75.9	0.938–296	$Y = 0.42\,X + 4.21$	56.2	46 ± 1
	CyeMixR-1	13,818	11327–17902	$Y = 2.58\,X - 5.68$	10,236	47 ± 2
	CyeMixR-2	9975	7282–14022	$Y = 1.73\,X - 1.93$	7389	46 ± 2
	CyeMixR-3	19,225	14075–32610	$Y = 1.87\,X - 3.02$	14,241	47 ± 1
Cyflumetofen [e]	PflMixR-1	> 10,000	—	—	> 422	46 ± 6
	PflMixR-2	> 10,000	—	—	> 422	44 ± 4
	PflMixR-3	> 10,000	—	—	> 422	48 ± 3
	CyeMixR-1	> 10,000	—	—	> 422	48 ± 2
	CyeMixR-2	> 10,000	—	—	> 422	46 ± 3
	CyeMixR-3	> 10,000	—	—	> 422	49 ± 2

[a] PflMix-1–3 and CyeMix-1–3 were intermixed strains established by cross of I260V-1–3 and S56L-1–3. PflMixR-1–3 and CyeMixR-1–3 were established by selection of PflMix-1–3 and CyeMix-1–3 with pyflubumide and cyenopyrafen, respectively.

[b] See Table 1.

[c] Resistance factors (RF) were calculated as a ratio of LC_{50} to TsukubaS_WT in Table 1.

[d] The average numbers of females used for the analysis per concentration ± standard deviation

[e] Because *B-I260V* alone caused very high resistant levels to cyflumetofen (see Table 1), we did not test the resistance levels of the non-selected intermixed strains against cyflumetofen.

Table 3. *SdhB* and *SdhC* genotype frequencies in the intermixed strains after the selection with pyflubumide (PfMixR-1–3) and cyenopyrafen (CyeMixR-1–3)

Genotypes [a]	Genotype frequencies (%)					
	PflMixR-1	PflMixR-2	PflMixR-3	CyeMixR-1	CyeMixR-2	CyeMixR-3
WbWb/WcWc	0.0	0.0	0.0	0.0	0.0	0.0
WbWb/WcMc	0.0	0.0	0.0	0.0	0.0	0.0
WbWb/McMc	0.0	0.0	0.0	0.0	0.0	0.0
WbMb/WcWc	0.0	0.0	0.0	0.0	0.0	0.0
WbMb/WcMc	0.0	0.0	0.0	0.0	0.0	0.0
WbMb/McMc	16.7	8.3	0.0	8.3	8.3	16.7
MbMb/WcWc	0.0	0.0	0.0	0.0	0.0	0.0
MbMb/WcMc	0.0	0.0	0.0	0.0	0.0	0.0
MbMb/McMc	83.3	91.7	100	91.7	91.7	83.3

[a] Wild type *B-I260* and mutant type *B-I260V* of *SdhB* are represented as Wb and Mb, respectively, and wild type *C-S56* and mutant type *C-S56L* of *SdhC* are shown as Wc, and Mc, respectively. The number of females analyzed was 12 per strain.

551

Table 4. Resistance levels of the strains where *B-I260V* and *C-S56L* were recoupled without acaricide selection

Acaricides	Strains [a]	LC_{50} (mg L^{-1})	95% confidence intervals	Regression lines	RF [b]	n ± SD [c]
Pyflubumide	PflMixMT-1	49,559	25,122–1,199,230	$Y = 0.93\ X + 0.65$	532,892	102 ± 5
	PflMixMT-2	19,417	14,307–37,869	$Y = 1.27\ X - 0.43$	208,785	99 ± 3
Cyenopyrafen	CyeMixMT-2	49,382	28,747–240,004	$Y = 1.42\ X - 1.64$	36,579	97 ± 5
	CyeMixMT-3	29,404	22,572–48,297	$Y = 2.28\ X - 5.19$	21,781	100 ± 6

[a] PflMixMT-1, PflMixMT-2, CyeMixMT-2, and CyeMixMT-3 were selected from PflMix-1, PflMix-2, CyeMix-2, and CyeMix-3, respectively, based on genotypes of *SdhB* and *SdhC* without acaricide selection and homozygous for *B-I260V* and *C-S56L*.

[b] Resistance factors (RF) were calculated as a ratio of LC_{50} to TsukubaS_WT in Table 1.

[c] The average numbers of females used for the analysis per concentration ± standard deviation over the concentrations of 5000, 10000, and 20000 mg L^{-1} that were used to compute the LC_{50} values (see Fig. 3).

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Table 5. Summary of mutation in complex II subunits and resistance levels to the three complex II inhibitors

Mutant alleles	Resistance levels [a]		
	Pyflubumide	Cyenopyrafen	Cyflumetofen
B-I260T	S [b]	VHR [b, c]	VHR [b]
B-I260V	S	MR	VHR
C-S56L	R	MR	HR
B-I260V + *C-S56L*	VHR [c]	VHR [c]	VHR [b]

[a] VHR: very high resistance levels (LC_{50} > 10,000 mg L^{-1}), HR: high resistance levels (1000–10,000 mg L^{-1}), R: resistance (200–1000 mg L^{-1}), MR: moderate resistance (20–200 mg L^{-1}), S: susceptible (< 20 mg L^{-1})

[b] Data from Sugimoto et al.[22]

[c] Substantial mortalities were detected at relatively low concentrations (100–1000 mg L^{-1} or less).

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Figure legends

Figure 1. Concentration–mortality plots of strains in which *SdhB* and *SdhC* mutations were decoupled (I260V-1–3 and S56L-1–3, respectively) and strains fixed to the wild-type (TsukubaS_WT) or mutant-type (SoKg1_PflR_MT) of both genes. (a) Pyflubumide, (b) cyenopyrafen, and (c) cyflumetofen. Gray square: I260V-1; gray circle: I260V-2; gray triangle: I260V-3; open square: S56L-1; open circle: S56L-2; open triangle: S56L-3; cross: TsukubaS_WT; plus: SoKg1_PflR_MT.

Figure 2. Concentration–mortality plots of intermixed strains derived from *B-I260V* fixed strains (I260V-1–3) and *C-S56L* fixed strains (S56L-1–3) before (PyflMix-1–3 and CyeMix-1–3) and after selection with pyflubumide (PyflMixR-1–3) or cyenopyrafen (CyeMixR-1–3), tested using pyflubumide (a), cyenopyrafen (b), and cyflumetofen (c). Dark gray square: PyflMixR-1; dark gray circle: PyflMixR-2; dark gray triangle: PyflMixR-3; light gray square: CyeMixR-1; light gray circle: CyeMixR-2; light gray triangle: CyeMixR-3; open square, open circle and open triangle: before selection.

Figure 3. Concentration–mortality plots of strains in which *B-I260V* and *C-S56L* were recoupled, tested using pyflubumide for PyflMixMT-1–2 (a) and cyenopyrafen for CyeMixMT-2–3 (b). Circles: PyflMixMT-1 and CyeMixMT-2; triangles: PyflMixMT-2 and CyeMixMT-3. Gray points indicate the data used for linear regression analysis (see Table 4), while data shown as open points were not used for analysis (see text).

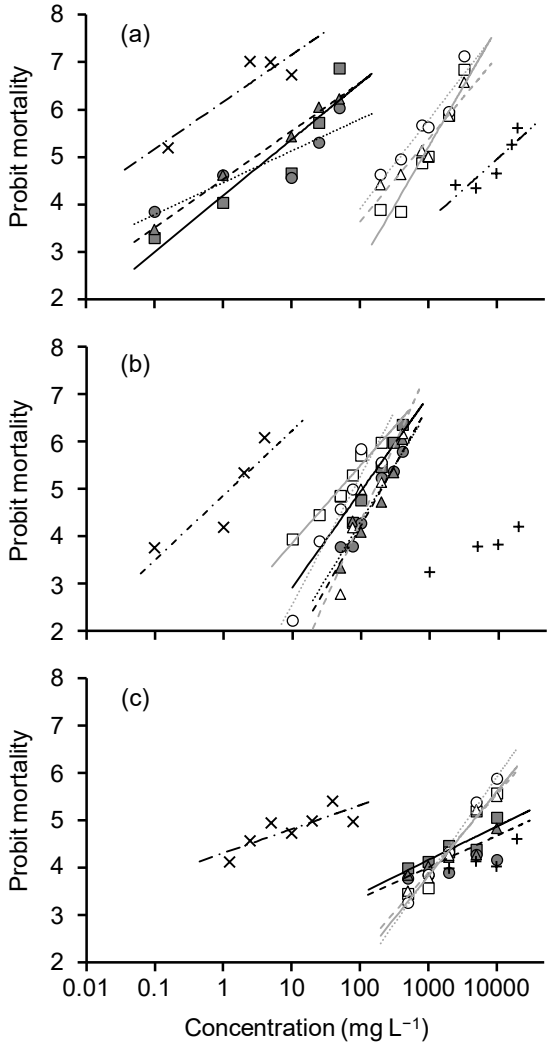
(a)
(b)
(c)
Probit mortality
Probit mortality
Probit mortality
8
7
6
5
4
3
2
0.01
0.1
1
10
100
1000
10000
Concentration (mg L⁻¹)

Figure 1.

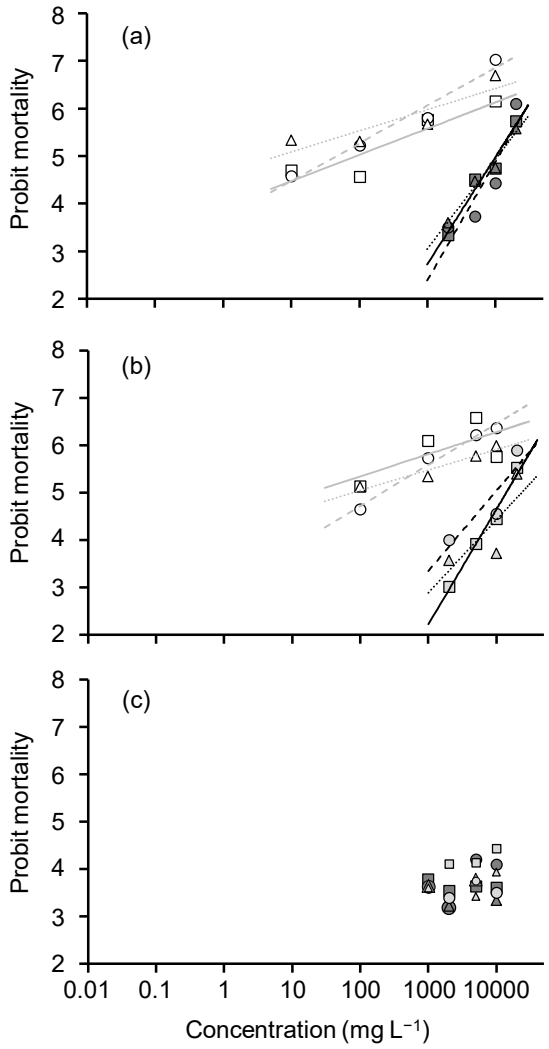
(a)
(b)
(c)
Probit mortality
Probit mortality
Probit mortality
8
7
6
5
4
3
2
0.01
0.1
1
10
100
1000
10000
Concentration (mg L⁻¹)

Figure 2.

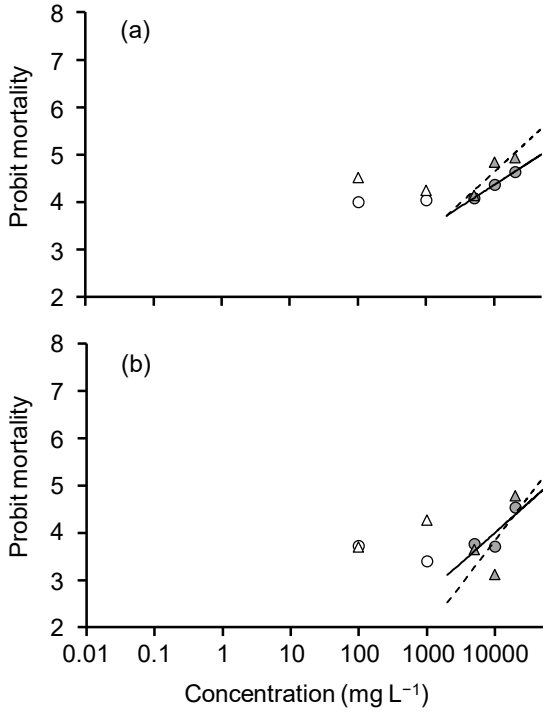
(a)
(b)
Probit mortality
Probit mortality
8
7
6
5
4
3
2
0.01
0.1
1
10
100
1000
10000
Concentration (mg L⁻¹)

Figure 3.

Supplementary Data 1:

Alignment with location of primer set for high resolution melting analysis and DNA sequencing data for *SdhB* of ML1 (SoKg_PflR_MT_male and TsukubaS_WT_female), decoupled strains (I260V-1–3 and S56L-1–3), and recoupled strains (PflMixMT-1,2 and CyeMixMT-2,3).

- No base substitution was found among three strains of I260V-1, 2 and 3 (I260V-1–3) and that of S56L-1–3 and between two strains of PflMixMT-1 and 2 (PflMixMT-1,2) and that of CyeMixMT-2,3. Therefore, the consensus sequences are shown for these strains.
- In CyeMixMT-2,3, the primer set tuSdhB-gF and tuSdhB-gR did not work in PCR amplification. This failure was likely due to degradation of the DNA samples. Therefore, we used two newly designed primer pairs to amplify shorter fragments. One of the primer pairs was tuSdhB-459F 5′-GGTTCCTGATATGAACCATTTCTACGAG-3′ and tuSdhB-826R (see Section 2.4) (products: 368 bp), and the other pair was tuSdhB-gFS 5′-TGATGAGTAGAT CAAGACAACTCTGAC-3′ and tuSdhB-656F 5′-GATACTTGGGACCTGCTGTACTC-3′ (480 bp). For subsequent cycle sequencing, we used tuSdhB-459F and tuSdhB-900R (see Section 2.3) for the former and the latter, respectively.
- The sequence data of *tetur01g15710* was obtained from Online Resource for Community Annotation of Eukaryotes (https://bioinformatics.psb.ugent.be/orcae/).

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tetur01g15710      181:TGGAATCCTGAAACACCTGAAGTTAAACCCTACATGCAGACATATGAGGTTGATCTAAAT 240
SoKg_PflR_MT_male    1:------------------------------TACATGCAGACATATGAGGTTGATCTAAAT  30
TsukubaS_WT_female   1:------------------------------TACATGCAGACATATGAGGTTGATCTAAAT  30
I260V-1-3            1:------------------------------TACATGCAGACATATGAGGTTGATCTAAAT  30
S56L-1-3             1:------------------------------TACATGCAGACATATGAGGTTGATCTAAAT  30
PflMixMT-1,2         1:------------------------------TACATGCAGACATATGAGGTTGATCTAAAT  30
CyeMixMT-2,3         0:------------------------------------------------------------  0
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tetur01g15710      241:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT 300
SoKg_PflR_MT_male   31:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT  90
TsukubaS_WT_female  31:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT  90
I260V-1-3           31:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT  90
S56L-1-3            31:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT  90
PflMixMT-1,2        31:ACATGCGGTCCTATGGTGCTTGATGCTTTGATTAAAATCAAAAATGAATTGGATCCTACT  90
CyeMixMT-2,3         0:------------------------------------------------------------  0
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tetur01g15710      301:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 360
SoKg_PflR_MT_male   91:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 150
TsukubaS_WT_female  91:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 150
I260V-1-3           91:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 150
S56L-1-3            91:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 150
PflMixMT-1,2        91:TTGACTTTCAGACGATCTTGCAGGGAAGGTATATGTGGTTCTTGTGCTATGAATATTGGA 150
CyeMixMT-2,3         0:------------------------------------------------------------  0
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teturO1g15710       361:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACCAGTGTCGAAAAACCGATGAAG 420
SoKg_PflR_MT_male   151:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACCAGTGTCGAAAAACCGATGAAG 210
TsukubaS_WT_female  151:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACYAGTGTCGAAAAACCGATGAAG 210
I260V-1-3           151:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACCAGTGTCGAAAAACCGATGAAG 210
S56L-1-3            151:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACTAGTGTCGAAAAACCGATGAAG 210
PflMixMT-1,2        151:GGTGGTAATACTCTTGCCTGTATCAGCAGAATTGACACCAGTGTCGAAAAACCGATGAAG 210
CyeMixMT-2,3          0:------------------------------------------------------------ 0
                        ............................................................

teturO1g15710       421:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 480
SoKg_PflR_MT_male   211:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 270
TsukubaS_WT_female  211:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 270
I260V-1-3           211:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 270
S56L-1-3            211:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 270
PflMixMT-1,2        211:ATCTATCCATTACCTCATATGTATGTGGTTAAGGACTTGGTTCCTGATATGAACCATTTC 270
CyeMixMT-2,3          0:------------------------------------------------------------ 0
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teturO1g15710       481:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 540
SoKg_PflR_MT_male   271:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 330
TsukubaS_WT_female  271:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 330
I260V-1-3           271:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 330
S56L-1-3            271:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 330
PflMixMT-1,2        271:TACGAGCAATATCGATCGATTCAGCCTTGGCTACAAAAGAAAGATGAAACTGGTTTTGGT 330
CyeMixMT-2,3          1:------------------------------------------ACTGGTTTTGGT 12
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SoKg_PflR_MT_male   331:AAGAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 390
TsukubaS_WT_female  331:AAAAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 390
I260V-1-3           331:AAGAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 390
S56L-1-3            331:AAAAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 390
PflMixMT-1,2        331:AAGAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 390
CyeMixMT-2,3         13:AAGAAACAAAATCTTCAAAGTGTTGATGATCGTAAGAAACTTGATGGTCTATATGAATGT 72
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SoKg_PflR_MT_male   391:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 450
TsukubaS_WT_female  391:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 450
I260V-1-3           391:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 450
S56L-1-3            391:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 450
PflMixMT-1,2        391:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 450
CyeMixMT-2,3         73:ATATTATGCGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATAC 132
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teturO1g15710       661:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCAAGAGATGAATCA 720
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TsukubaS_WT_female  451:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCGAGAGATGAATCA 510
I260V-1-3           451:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCAAGAGATGAATCA 510
S56L-1-3            451:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCGAGAGATGAATCA 510
PflMixMT-1,2        451:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCAAGAGATGAATCA 510
CyeMixMT-2,3        133:TTGGGACCTGCTGTACTCATGCAAGCATACCGATGGGTAATTGATTCAAGAGATGAATCA 192
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SoKg_PflR_MT_male   511:ACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAGTC 570
TsukubaS_WT_female  511:ACTGAAAAAAGGTTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAATC 570
I260V-1-3           511:ACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAGTC 570
S56L-1-3            511:ACTGAAAAAAGGTTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAATC 570
PflMixMT-1,2        511:ACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAGTC 570
CyeMixMT-2,3        193:ACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATGTCATACAGTC 252
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                                                tuSdhB-826R
tetur01g15710       781:ATGAATTGTTCCAGGACCTGTCCTAAGAACTTGAATCCTGGTCGAGCAATCGGTGAACTG 840
SoKg_PflR_MT_male   571:ATGAATTGT---------------------------------------------------- 579
TsukubaS_WT_female  571:ATGAATT----------------------------------------------------- 577
I260V-1-3           571:ATGAATTGT---------------------------------------------------- 579
S56L-1-3            571:ATGAATTGT---------------------------------------------------- 579
PflMixMT-1,2        571:ATGAATTGT---------------------------------------------------- 579
CyeMixMT-2,3        253:ATGAATTGTTCCAGGACCTGTCCTAAGAACTTGAATCCTGGTCGAGCAATCGGTGA---- 308
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>TsukubaS_WT_female | SdhB partial
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CGATGGGTAATTGATTCGAGAGATGAATCAACTGAAAAAAGGTTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATG
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>I260V-1-3 consensus | SdhB partial
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>S56L-1-3 consensus | SdhB partial
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CGATGGGTAATTGATTCGAGAGATGAATCAACTGAAAAAAGGTTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATG
TCATACAATCATGAATTGT

>PflMixMT-1,2 consensus | SdhB partial
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CTCTTGCCTGTATCAGCAGAATTGACACCAGTGTCGAAAAACCGATGAAGATCTATCCATTACCTCATATGTATGTGGTT
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CTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATACTTGGGACCTGCTGTACTCATGCAAGCATAC
CGATGGGTAATTGATTCAAGAGATGAATCAACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGATG
TCATACAGTCATGAATTGT

>CyeMixMT-2,3 consensus | SdhB partial
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CGCTTGTTGTTCAACATCTTGTCCAAGCTATTGGTGGAATAGTGACCGATACTTGGGACCTGCTGTACTCATGCAAGCAT
ACCGATGGGTAATTGATTCAAGAGATGAATCAACTGAAAAAAGGCTGGATCAATTGAGGGATCCCTTCTCACTTTATCGA
TGTCATACAGTCATGAATTGTTCCAGGACCTGTCCTAAGAACTTGAATCCTGGTCGAGCAATCGGTGA

Supplementary Data 2:

Alignment with location of primer set for high resolution melting analysis and DNA sequencing data for *SdhC* of ML1 (SoKg_PflR_MT_male and TsukubaS_WT_female), decoupled strains (I260V-1–3 and S56L-1–3), and recoupled strains (PflMixMT-1,2 and CyeMixMT-2,3).

- No base substitution was found among three strains of I260V-1, 2 and 3 (I260V-1–3) and that of S56L-1–3 and between two strains of PflMixMT-1 and 2 (PflMixMT-1,2) and that of CyeMixMT-2,3. Therefore, the consensus sequences are shown for these strains.
- The sequence data of *tetur30g00210* was obtained from Online Resource for Community Annotation of Eukaryotes (https://bioinformatics.psb.ugent.be/orcae/).

```
tetur30g00210          1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGG 60
SoKg_PflR_MT_male      1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGA 60
TsukubaS_WT_female     1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGG 60
I260V-1-3              1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGG 60
S56L-1-3               1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGA 60
PflMixMT-1,2           1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGA 60
CyeMixMT-2,3           1:ATGTTATTTCCACGTTTGATTTGTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGA 60
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tetur30g00210         61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
SoKg_PflR_MT_male     61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
TsukubaS_WT_female    61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
I260V-1-3             61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
S56L-1-3              61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
PflMixMT-1,2          61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
CyeMixMT-2,3          61:GCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCATCGTCGCCATCACAACCT 120
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tetur30g00210        121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTCACCTTATACAATA 180
SoKg_PflR_MT_male    121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATA 180
TsukubaS_WT_female   121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTCACCTTATACAATA 180
I260V-1-3            121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTCACCTTATACAATA 180
S56L-1-3             121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATA 180
PflMixMT-1,2         121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATA 180
CyeMixMT-2,3         121:GAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATA 180
                         ************************************************..************
                                        tuSdhC-211R
tetur30g00210        181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGGGTTAGTGGTGTTGCATTA 240
SoKg_PflR_MT_male    181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTA 240
TsukubaS_WT_female   181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGGGTTAGTGGTGTTGCATTA 240
I260V-1-3            181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGGGTTAGTGGTGTTGCATTA 240
S56L-1-3             181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTA 240
PflMixMT-1,2         181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTA 240
CyeMixMT-2,3         181:TATCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTA 240
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tetur30g00210       241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
SoKg_PflR_MT_male   241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
TsukubaS_WT_female  241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
I260V-1-3           241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
S56L-1-3            241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
PflMixMT-1,2        241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
CyeMixMT-2,3        241:TCTGTGGGAATTTACGCTATGGGTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCAT 300
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tetur30g00210       301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
SoKg_PflR_MT_male   301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
TsukubaS_WT_female  301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
I260V-1-3           301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
S56L-1-3            301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
PflMixMT-1,2        301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
CyeMixMT-2,3        301:CAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATTATCACATCTAAAATATTA 360
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tetur30g00210       361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
SoKg_PflR_MT_male   361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
TsukubaS_WT_female  361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
I260V-1-3           361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
S56L-1-3            361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
PflMixMT-1,2        361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
CyeMixMT-2,3        361:GTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATG 420
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tetur30g00210       421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACA 480
SoKg_PflR_MT_male   421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACT 480
TsukubaS_WT_female  421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACA 480
I260V-1-3           421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACA 480
S56L-1-3            421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACT 480
PflMixMT-1,2        421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACT 480
CyeMixMT-2,3        421:GGATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACT 480
                        ************************************************************.

tetur30g00210       481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
SoKg_PflR_MT_male   481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
TsukubaS_WT_female  481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
I260V-1-3           481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
S56L-1-3            481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
PflMixMT-1,2        481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
CyeMixMT-2,3        481:GCTCTGGTGACCCTTTACGCTATTTTCAATCTTTAG                          516
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>SoKg_PflR_MT_male | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAACACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGAGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
ATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACTGCTCTGGTGACCCTTTACGCTA
TTTTCAATCTTTAG

>TsukubaS_WT_female | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAATACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGGGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTCACCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGGGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
ATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACAGCTCTGGTGACCCTTTACGCTA
TTTTCAATCTTTAG

>I260V-1-3 consensus | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAATACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGGGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTCACCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGGGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
ATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACAGCTCTGGTGACCCTTTACGCTA
TTTTCAATCTTTAG

>S56L-1-3 consensus | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAACACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGAGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
ATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACTGCTCTGGTGACCCTTTACGCTA
TTTTCAATCTTTAG

>PflMixMT-1,2 consensus | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAACACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGAGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
ATTTGGATTTGGCCTTAAAGAACTCTACACTTCAGGTTATGCAGTTCTCGGTATTACTGCTCTGGTGACCCTTTACGCTA
TTTTCAATCTTTAG

>CyeMixMT-2,3 consensus | SdhC partial
CTAATTTGGAACATTTAGCTTATTTATCTTCTGAGTAAAACACAACTGTTTTTAAATCATGTTATTTCCACGTTTGATTT
GTGCTCGAGCAACTATTAGCAATCAACTACTTGGCCGAGCTAATGCAGTTCTTCCCAGAATAGCCATGGCTTCAAGCTCA
TCGTCGCCATCACAACCTGAACAAGATTTTTTTACGAAGAATTCTCGGCTAAATCGTCCTATTTTGCCTTATACAATATA
TCAACCTCAATTAACCTCGGTCCTATCAATATCTCACCGAGTTAGTGGTGTTGCATTATCTGTGGGAATTTACGCTATGG
GTATTAGTCAGCTGTTTGCTTCAACTCCATTTGCTCATCAGCTGGAATCATTGTCGACTTCAATCCCATCTATATTAATT
ATCACATCTAAAATATTAGTTGCATCTGGCTTTGGGTTCCATTTCGCCAATGGCATACGTCATCTCTTTTGGGATATGGG
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TTTTCAATCTTTAG

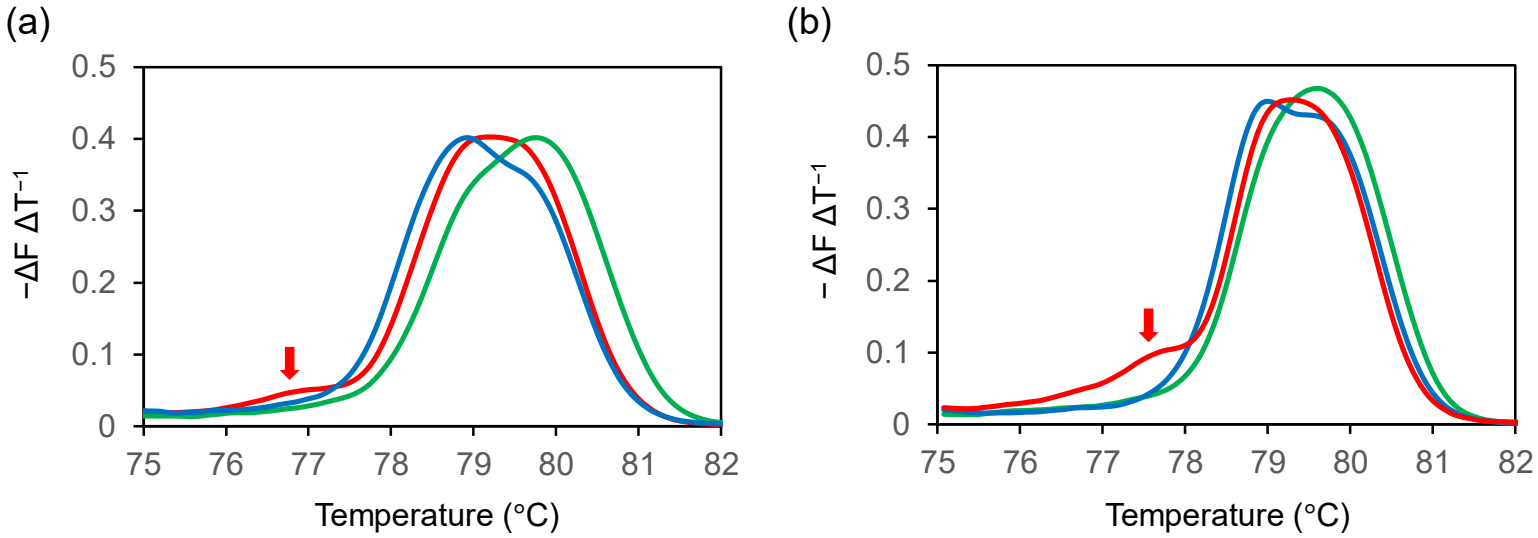

Fig. S1 Difference in melting curves of PCR products for *SdhB* (a) and *SdhC* (b) among genotypes. Vertical axis shows melting rate curves ($-\Delta F\ \Delta T^{-1}$; ΔF: descending fluorescence signals, ΔT: unit time). Blue line: wild type homozygote, red line: heterozygote, green line: mutant type homozygote. Red arrows show a small peak on the shoulder of that peak characteristic of heterozygote in these genes.

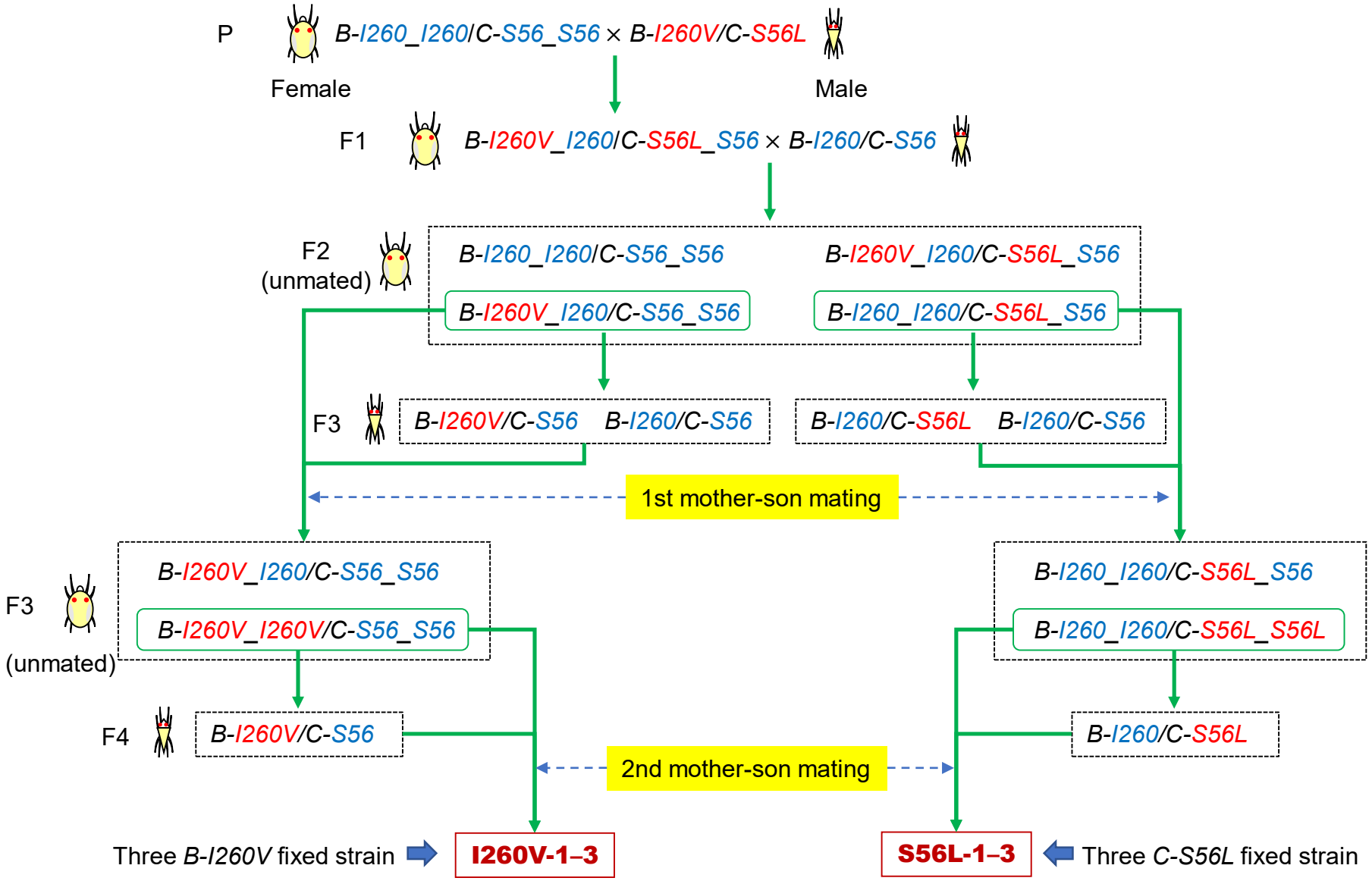

Fig. S2 Schematic diagram of cross experiments for decoupling of mutations in *SdhB* (*B-I260V*) and *SdhC* (*C-S56L*)

Table S1. Results of high-resolution melting (HRM) analysis for *SdhB* and *SdhC* genotyping in mother (F2)–son (F3) mating pairs

Genes	F2 famales					F3 males			
	n	SS	SR	RR	NA	*n*	S	R	NA
SdhB	48	22	24	0	2	48	35	10	3
SdhC	48	22	22	0	4	48	30	12	6

n: number of individuals tested

Genotypes SS: *B-I260_I260* or *C-S56_S56*, SR: *B-I260_I260V* or *C-S56_S56L*, RR: *B-I260V_I260V* or *C-S56L_S56L*, S: *B-I260* or *C-S56*, R: *C-I260V* or *C-S56L*, NA: undecidable due to unclear or confusing melting curves

Table S2. Results of HRM analysis for *SdhB* and *SdhC* genotyping in F3 females

F3 ♀ for strains of	Genotype		F3 ♀ for strains of	Genotype	
B-I260V alone	*SdhB*	*SdhC*	*C-S56L* alone	*SdhB*	*SdhC*
ML1-2-6-1 (I260V-1)	RR	SS	ML1-4-4-4	SS	SR
ML1-2-6-4 (I260V-2)	RR	SS	ML1-4-4-5	SS	RR
ML1-2-6-6	SR	SS	ML1-4-4-6	SS	RR
ML1-2-6-8 (I260V-3)	RR	SS	ML1-4-4-7 (S56L-1)	SS	RR
ML3-1-1-2	SR	SS	ML1-4-5-1	SS	SR
ML3-1-1-5	SR	SS	ML1-4-5-3 (S56L-2)	SS	RR
ML3-1-1-7	SR	SS	ML1-4-5-4 (S56L-3)	SS	RR
ML3-1-1-8	SR	SS	ML1-4-5-5	SS	SR

Branch numbers show identification of parent-F1-F2-F3 females. Genotype notation is the same as Table S1. Names of the decoupled strains generated from the F3 females are indicated in parentheses. Three F3 females with the genotype *B-I260V_I260V*/*C-S56_S56* and five with *B-I260_I260*/*C-S56L_S56L* were also applied for sequencing analysis, and no discrepancy were found (Data was not shown).

Table S3. Combination of strains with *B-I260V* fixed (I260V-1–3) and *C-S56L* fixed (S56L-1–3) for the establishment of intermixed strains and acaricides used for the selection of the intermixed strains

Intermixed strains	Combination of mating strains		Acaricides used for selection	Acaricide-selected strains
	B-I260V fixed	*C-S56L* fixed		
PflMix-1	I260V-1	S56L-1	Pyflubumide	PflMixR-1
PflMix-2	I260V-2	S56L-3		PflMixR-2
PflMix-3	I260V-3	S56L-2		PflMixR-3
CyeMix-1	I260V-3	S56L-3	Cyenopyrafen	CyeMixR-1
CyeMix-2	I260V-2	S56L-2		CyeMixR-2
CyeMix-3	I260V-1	S56L-1		CyeMixR-3

Table S4. Gene frequency of intermixed strains (PfMix-1–3) before acaricide selection

Genotypes [a]	Genotype frequencies (%)		
	PflMix-1	PflMix-2	PflMix-3
WbWb/WcWc	0.0	10.7	0.0
WbWb/WcMc	19.4	10.7	3.2
WbWb/McMc	16.1	17.9	0.0
WbMb/WcWc	16.1	10.7	22.6
WbMb/WcMc	22.6	25.0	29.0
WbMb/McMc	12.9	14.3	6.5
MbMb/WcWc	6.5	7.1	19.4
MbMb/WcMc	6.5	3.6	19.4
MbMb/McMc	0.0	0.0	0.0
Numbers of females used for sequencing	31	28	31

[a] Wild type *B-I260* and mutant type *B-I260V* of *SdhB* and wild type *C-S56* and mutant type *C-S56L* of *SdhC* are shown as Wb, Mb, Wc, and Mc, respectively.

Table S5. Results of HRM analysis for *SdhB* and *SdhC* genotyping in allele-selected recoupling

Intermixed strains	Parental crosses within each intermixed strain				F1 females		Recoupled strains
	Males		Females				
	No.	Genotypes	No.	Genotypes	No.	Genotypes	
PflMix-1	2	R/R	4	SR/SR	1	SR/RR	
					2	RR/SR	
					4	SR/RR	
					5	SR/NA	
					7	SR/NA	
					8	SR/RR	
					9	SR/SR	
					10	RR/RR	PflMixMT-1
PflMix-2	7	R/R	1	RR/SR	1	RR/RR	PflMixMT-2
					2	RR/SR	
					3	RR/RR	
					4	RR/SR	
CyeMix-2	5	R/R	4	SR/RR	2	NA/RR	
					3	RR/RR	
					4	RR/RR	
					5	SR/RR	
					6	RR/RR	
					7	RR/RR	CyeMixMT-2
					8	RR/RR	
CyeMix-3	6	R/R	5	RR/RR			CyeMixMT-3

'No.' indicates individual identification number. Genotypes are indicated as *SdhB*/*SdhC*, and the genotype notation is the same as Table S1. Data of parental male and females and their F1 females that were not used for generating recoupled strains were omitted. Genotypes of *SdhB* and *SdhC* in all of these individuals were confirmed by sequencing analysis later, and no discrepancy was found (Sequence data were not shown other than recoupled strains which sequence data are represent in Supplementary Data 1 and Data 2).