Combination of restriction endonuclease digestion with the ΔΔCt method in real-time PCR to monitor etoxazole resistance allele frequency in the two-spotted spider mite

Running title: ΔΔCt method to monitor etoxazole resistance

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Abstract

Monitoring resistance allele frequency at the early stage of resistance development is important for the successful acaricide resistance management. Etoxazole is a mite growth inhibitor to which resistance is conferred by an amino acid substitution in the chitin synthase 1 (CHS1; I1017F) in *T. urticae*. If the susceptible allele can be specifically digested by restriction endonuclease, the ΔΔCt method using real-time PCR for genomic DNA (RED-ΔΔCt method) may be available for monitoring the resistance allele frequency. We tested whether the etoxazole resistance allele frequency in a pooled sample was accurately measured by the RED-ΔΔCt method and validated whether the resistance variant frequency was correlated with etoxazole resistance phenotype in a bioassay. Finally, we performed a pilot test using field populations. Strong linearity of the measures by the RED-ΔΔCt method with practical resistance allele frequencies; resistance allele frequency in the range between 0.5% to at least 0.75% was strictly represented. The strong linear relationship between hatchability of haploid male eggs after the etoxazole treatments (phenotype) and resistance allele frequencies in their mothers provided direct evidence that I1017F is a primary resistance factor to etoxazole in the strains used for experiments. The pilot test revealed a significant correlation between egg hatchability (including both diploid female eggs and haploid male eggs) and estimators in field populations. Consequently, we concluded that the RED-ΔΔCt method is a powerful tool for monitoring a resistance allele in a pooled sample.

Keywords: Acaricide resistance; Chitin synthase 1; CHS1; qPCR; *Tetranychus urticae*; Tetranychidae; Acari

1. Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, has developed resistance to most acaricides that are currently available. The resistance allele frequency in a field population is an important factor that determines the rate of resistance development against new chemicals or the effectiveness of current ones. Therefore, monitoring resistance allele frequency in the early stage of resistance development is essential for the successful management of acaricide resistance. The traditional toxicological bioassay is a simple and convenient means to determine susceptibility, namely resistance level, of a mite population. However, because mortalities are affected by the hereditary mode of resistance, gene frequencies are not precisely determined by this method in samples collected from fields. This is particularly the case when evaluating ovicidal and larvicidal activities against *T. urticae* because of their arrhenotokous parthenogenesis and because the sexes of mites at those developmental stages are indeterminable.

Etoxazole is a mite growth inhibitor that was placed on the market in 1998. Although this acaricide is currently used worldwide, etoxazole-resistant *T. urticae* were found in northern Japan in 1997 before etoxazole was commercially available [1]. Etoxazole resistance inheritance is completely recessive in *T. urticae* [2,3], which makes it difficult to estimate resistance gene frequency. Molecular studies have revealed that simple amino acid substitutions confer resistance to many acaricides [4,5,6,7]. A single base substitution in *tetur03g08510* causing an amino acid substitution in the transmembrane region of chitin synthase 1 (CHS1; I1017F) confers etoxazole resistance on *T. urticae* [3]. This single nucleotide polymorphism (SNP) in CHS1 may also cause hexythiazox and clofentezine resistance [8], in which resistance to etoxazole is indicated by cross

73 experiments [9].

74 A pesticide resistance gene may be present at a low frequency in the natural
75 population, and gene frequency increases in recovering population sizes after chemical
76 spraying is logistically accelerated [10]. To predict future resistance development for pest
77 management, it is desirable to develop a monitoring technology in which a low frequency
78 (< 1%) SNP (resistance allele) can be detected. Many molecular techniques have been
79 developed to diagnose of SNPs associated with pesticide resistance, e.g. polymerase chain
80 reaction (PCR) amplification of specific alleles (PASA) [11], restriction fragment length
81 polymorphism (RFLP) assay on PCR products (PCR-RFLP) [12], and TaqMan SNP
82 genotyping assay (TM-SNP) [13]. PASA and PCR-RFLP may provide accurate results
83 for the diagnosis of resistance allele frequency when those are used for individual-based
84 detection. However, because spider mites are patchily distributed in their habitats, we
85 need to assess numerous individuals to accurately estimate resistance allele frequency in
86 a local population. Therefore, methods that can evaluate resistance allele frequency in a
87 pooled sample, such as TM-SNP, are preferable.

88 Van Leeuwen et al. [14] applied quantitative sequencing (QS) to assess frequency of
89 haplotypes in mitochondrial DNA (cytochrome *b*) associated with bifenazate resistance
90 in *T. urticae*. Kwon et al. [15] established a monitoring method based on QS for several
91 acaricide resistance mutations. However, because QS is only reliable when the resistance
92 allele frequency is higher than 10% [15], the authors recommended real-time PASA
93 (rtPASA [16]) as an alternative monitoring method, which is an individual-based method,
94 when the frequency was low. Similarly, the accurate estimation of resistance allele
95 frequency may be possible with TM-SNP when the allele frequency is higher than 5%
96 (and lower than 95%) [13]. The resistance allele frequency was estimated based on the

97 calibration curve between measured and practical allele frequencies in standardized
98 samples in both QS and TM-SNP. Because the calibration curve can be affected by
99 experimental conditions, standardized samples may be required frequently.

100 The $\Delta\Delta C_t$ method was developed as an analytic method to obtain relative gene
101 expression data from quantitative polymerase chain reaction (qPCR) assays [17,18,19].
102 In the method, PCR amplification of a gene of interest is normalized by comparison with
103 an internal control gene, such as a housekeeping gene. Then, fold changes compared to
104 the normalized data are calibrated using a calibrator sample, such as an untreated control
105 sample, representing 1-fold expression of the gene of interest. We considered that if a
106 susceptible allele-specific restriction endonuclease recognition site was present, the ΔΔCt
107 method after qPCR for genomic DNA digested by the restriction endonuclease (RED-
108 ΔΔCt method) could be used to monitor the resistance allele frequency based on
109 calibration with an undigested DNA sample as the calibrator.

110 Van Leeuwen et al. [3] reported four types of variants, including I1017F, which are
111 associated with etoxazole susceptibility and the adjoining upstream codon. We found
112 etoxazole-susceptible strain-specific restriction endonuclease recognition sites in this
113 region. Therefore, we determined if SNPs associated with resistance were accurately
114 estimated by the RED-ΔΔCt method, and validated whether the resistance variant
115 frequency was practically correlated with etoxazole resistance phenotype in a bioassay.
116 Finally, we also performed a pilot test using field populations.

117

118 ## 2. Materials and methods

119

120 ### *2.1. Mites*

121

122 An etoxazole resistant strain (SoOm1-etoR) was selected with commercial
123 formulations of etoxazole (10% suspension concentrate; Kyoyu Agri, Tokyo, Japan) at 50
124 mg l^{-1} in a laboratory from a field population collected from strawberry plants in a
125 greenhouse in Omaezaki City, Shizuoka Prefecture, Japan (34.7° N, 138.1° E) in January
126 2012. Although the selection was performed only once using an etoxazole solution at a
127 practical concentration, mortalities from etoxazole at 50 to 10,000 mg l^{-1} were from 0%
128 to 3.9% (Supplementary Table 1), indicating that this strain had already developed
129 resistance to etoxazole. An acaricide-susceptible strain (Kyoyu-S) was obtained from
130 Kyoyu Agri Co., Ltd. (Kanagawa, Japan). Four pairs of females and males were
131 separately mated on kidney bean leaf squares (2×2 cm) on water soaked cotton in Petri
132 dishes for SoOm1-etoR (pair designations: O2, O3, O4 and O6) and Kyoyu-S (pair
133 designations: K1, K2, K3 and K4). After egg production for 4 days, the 1017 codon of
134 CHS1 of each mite was sequenced. Then we selected offspring from two pairs of each
135 strain to evaluate the linearity of the interaction between practical resistance variant
136 frequencies (R variant frequency) and its estimators using the RED-ΔΔCt method.
137 Six field populations and two laboratory strains (Table 1) were used to validate the
138 effects of resistance variant frequencies on etoxazole resistant phenotypes in a bioassay.
139 The field populations were maintained on kidney bean leaf disks without acaricide
140 selection after collection. One laboratory strain, NS, was adversely selected with
141 etoxazole and hexythiazox [9] and then reared under acaricide-free conditions [20].
142 Another laboratory strain, Tsukuba, had been reared in a laboratory without acaricide
143 selection for >15 years.
144 Five field populations were newly collected from commercial strawberry

145 greenhouses in Nara Prefecture from November to December 2016 (Table 2) and used for
146 comparison of the RED-ΔΔCt method with a common toxicological bioassay. One (Uda)
147 of the five field populations was also used for sequencing analysis of CHS1. The mites
148 were reared on kidney bean leaves on water soaked cotton in Petri dishes in a laboratory
149 at 25°C with a 16 h light:8 h dark cycle, except the newly collected field populations,
150 which were reared in a laboratory at 25°C with natural day length condition (app. 10 h
151 light:14 h dark light cycle).
152
153 *2.2. Sequence analysis of mated mites from resistant and susceptible strains*
154
155 We prepared crude DNA extracts, which were directly used as DNA templates for
156 PCR amplification, following the method of Osakabe et al. [21]. A single adult female or
157 male of a mated pair described in section 2.1 was homogenized in 20 µl lysis buffer (10
158 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% Igepal CA-630 [Sigma, Tokyo, Japan], 10
159 mM NaCl, and 1 mg/ml proteinase K [Takara, Kusatsu, Japan]) with a plastic pestle
160 (Pellet mixer; Toho, Tokyo, Japan) in a 0.2 ml PCR tube. The homogenate was incubated
161 at 65°C for 20 min and then at 95°C for 10 min. The lysate was diluted with 380 µl
162 (female) or 180 µl (male) nuclease-free water (Qiagen, Tokyo, Japan).
163 A 1,277-base region, including the 1017 codon of CHS1, was amplified by PCR. The
164 PCR amplification was conducted using 1 µl of each DNA template in a total reaction
165 volume of 20 µl of PCR buffer for KOD FX Neo (Toyobo, Osaka, Japan) containing 0.4
166 mM of each dNTP, 0.25 µM of each primer, and 0.4 U of KOD FX Neo DNA polymerase
167 (Toyobo). PCR conditions were an initial 2 min at 94°C followed by 10 s at 98°C, 30 s at
168 60°C, and 77 s at 68°C for 40 cycles, and a final 7 min at 68°C. Primers used for

amplification were TuCHS1-R666628, 5'-CTTATGTTGGTCGGAGCTATGG-3', and TuCHS1-F667904b, 5'-CCGAATCGATGAAACAGCATAC-3'. After removal of the remaining PCR primers using MicroSpin S-400 HR Columns (GE Healthcare UK, Little Chalfont, UK), sequencing was performed in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) with an internal primer: TuCHS1-R666768, 5'-AATGTCCGCTTGTTATGCAC-3'. Primers used in this study were designed using GENETYX ver. 9.1.0 (GENETYX, Tokyo, Japan) referring to the *tetur03g08510* DNA sequence and that in the scaffold_3 of *T. urticae* genome DNA (http://bioinformatics.psb.ugent.be/orcae/overview/Tetur).

2.3. Evaluation of linearity in the relationship between practical resistance variant frequencies and measurements using the RED-ΔΔCt method

2.3.1. Genomic DNA preparation

Genomic DNA samples were prepared from offspring lines of two pairs of SoOm1-etoR (O4 and O6) and Kyoyu-S (K1 and K4) selected by sequencing data obtained by the method described above. DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol. Briefly, 50 adult females were homogenized in 180 µl of extraction buffer in a 1.5 ml sample tube, 20 µl of proteinase K were added to the homogenate, and the mixture was incubated at 56°C overnight. After incubation with RNase A for 2 min, the DNA sample was purified from the resulting sample mixture on the following day. The resulting DNA sample was cleaned using NucleoSpin gDNA Clean-up XS (Macherey-Nagel, Düren, Germany) and the

193 concentration was adjusted to 1 ng μl^{-1}. We prepared two DNA samples for each offspring
194 line from the two pairs and used them as four replications for each strain.
195
196 *2.3.2. Evaluation of amplification efficiency*
197 We tested the efficiency of amplification in qPCR with a primer set for CHS1
198 (tu03CHS1 forward: 5'-GGCACTGCTTCATCCACAAG-3', and reverse: 5'-
199 GTGTTCCCCAAGTAACAACGTTC-3') and for an internal reference gene,
200 glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde 3-phosphate dehydrogenase
201 [GAPDH]; tu25GAPDH forward: 5'-GCACCAAGTGCTAAAGCATGGAG-3', and
202 reverse: 5'-GAACTGGAACACGGAAAGCCATAC-3'). DNA (1 ng μl^{-1}) from O6 and
203 K4 were separately diluted 10-fold to 1×10^{-4} ng μl^{-1} with nuclease free water, and 8 µl
204 of the DNA solution was used for each qPCR amplification. qPCR analysis using the
205 intercalator method was performed using the LightCycler Nano System (Roche
206 Diagnostics, Basel, Switzerland) with SYBR Fast qPCR Mix (Takara). The reaction mix
207 contained 0.4 µM each of forward and reverse primers and 8 µl of diluted DNA samples.
208 PCR conditions were an initial 30 s at 95°C followed by 10 s at 95°C, 10 s at 62°C, and
209 15 s at 72°C for 45 cycles, with a melting curve analysis. Although the LightCycler Nano
210 System produced Cq values instead of Ct values, we phrased the Cq (quantification cycle)
211 value as Ct (cycle threshold) values because Cq values have similar semantic content to
212 Ct values. Regression lines and amplification efficiency were analyzed using LightCycler
213 Nano Software ver. 1.1.0 (Roche). Parallelism between regression lines for CHS1 and
214 GAPDH were statistically analyzed based on an interaction in a two-way analysis of
215 variance (ANOVA) using the "aov" and "lm" modules in R version 3.2.1 [22] for each
216 strain.

217

218 *2.3.3. qPCR for DNA samples digested by restriction endonucleases*

219 We deliberately mixed the DNA samples from the SoOm1-etoR and Kyoyu-S groups
220 as four discrete combinations. We dealt with those four combinations as biological
221 replications in the subsequent statistical analysis. Resistance variant frequencies in the
222 mixed samples (total DNA concentration: 1 ng μl^{-1}) were set at 0%, 0.1%, 0.5%, 1%, 5%,
223 10%, 25%, 50%, 75% and 100%. A total of 15 ng of the mixed sample were incubated in
224 the manufacturer's buffer (20 µl) for restriction endonucleases, including *MluC* I (10
225 units) and *Taq*$^{\alpha}$I (20 units; New England BioLabs, Ipswich, MA, USA), at 37°C for 3 h
226 followed by incubation at 65°C for 3 h. Then the enzymes were inactivated by an
227 incubation at 80°C for 20 min. The buffer was removed using MicroSpin S-200 HR
228 columns (GE Healthcare). Prior to testing for the linearity of the relationship between
229 practical R variant frequencies and measurement by the RED-ΔΔCt method, we tested
230 the efficiency of incubation times at 15 min, 1 h, and 3 h to determine an appropriate
231 incubation time to estimate resistance variant frequencies.

232 The 1017 codon of *T. urticae* includes ATT (susceptible) and TTT (etoxazole
233 resistant) variants, and both variants adjoin the upstream synonymous TCG and TCA
234 variants (1016 codon) [3]. Thus, the susceptible strain possibly has TCGATT or TCAATT,
235 whereas the resistant strain has TCGTTT or TCATTT (Fig. 1a). Because recognition site
236 sequences of *MluC* I and *Taq*$^{\alpha}$I are AATT and TCGA, only the susceptible variant (S
237 variant) should be digested by double digestion with these restriction endonucleases,
238 meaning that only resistant variants should be amplified by qPCR amplification.

239 qPCR analysis for CHS1 and GAPDH (internal control) was performed using 8 µl of
240 digested and undigested intact DNA samples. We calculated the ΔCt value by subtracting

241 the Ct value of GAPDH from that of CHS1 and ΔΔCt values by subtracting ΔCt values
242 for an undigested intact DNA sample containing the R variant at 100% (intact SoOm1-
243 etoR DNA; calibrator) from the ΔCt values for corresponding digested DNA samples.
244 Finally, resistant variant frequencies were calculated as $2^{-\Delta\Delta Ct}$. Given a regression line
245 through the origin, a linear regression between the practical R variant frequency and
246 $2^{-\Delta\Delta Ct}$ was analyzed using the "lm" module of R version 3.2.1 [22].
247
248 *2.4. Validation of the effects of resistant variant frequencies in the etoxazole resistant*
249 *phenotype*
250
251 Because of the completely recessive inheritance of etoxazole resistance, we analyzed
252 the relationship between resistant variant frequencies of virgin females and hatchability
253 of their haploid male eggs after etoxazole treatment. Virgin females (34–43 ♀♀ for each
254 population; ~4 days after the last molt) from six field populations and two laboratory
255 strains (Table 1) were introduced to four kidney bean leaf squares (2×2 cm) on water
256 soaked cotton in a Petri dish and kept in a laboratory at 25°C with a 16 h light:8 h dark
257 light cycle. The next day, surviving females (31–42 ♀♀ for each population) were
258 collected, and their DNA was prepared and cleaned using the DNeasy Blood and Tissue
259 kit and NucleoSpin gDNA Clean-up XS, respectively, as described above. Eggs laid on
260 the leaf squares were sprayed with etoxazole at 50 mg l^{-1}, which is a practical
261 concentration (adhesion amount: 1.91 ± 0.16 mg cm^{-2}). We used commercial
262 formulations of etoxazole (10% suspension concentrate). Hatchability was determined by
263 observation with a binocular microscope after 5 days.
264 The DNA samples were used for qPCR after double digestion with *MluC* I and *Taq*$^{\alpha}$I.

Estimators of R variant frequencies were calculated as $2^{-\Delta\Delta Ct}$. We performed digestion and qPCR twice for each population as technical replications, and an averaged $2^{-\Delta\Delta Ct}$ value between the technical replications for each population was used in subsequent linear regression analyses. Given a regression line through the origin, a linear regression between $2^{-\Delta\Delta Ct}$ and egg hatchability was analyzed using the "lm" module of R version 3.2.1 [22].

2.5. Comparison of the RED-ΔΔCt method with a common toxicological bioassay

Preparation of DNA samples for the RED-ΔΔCt method was fundamentally the same as in section 2.4, but DNA extraction from 100 adult females was performed using the Wizard Genomic DNA Purification kit (Promega, Fitchburg, WI, USA) following the method of Hinomoto et al. [23] with some modifications. Females were homogenized in a 1.5-ml microtube and crushed using several zirconium dioxide beads in 100 μl of the manufacturer supplied Nuclei Lysis Solution. Then the resulting DNA sample solution was cleaned using NucleoSpin gDNA Clean-up XS. qPCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Toxicological tests were also performed as in section 2.4. Briefly, 20 adult females were randomly collected from a stock culture and introduced to a kidney bean leaf square (5×5 cm) in a Petri dish, allowed to lay eggs for 48 h in a laboratory at 25°C with a 16 h light:8 h dark light cycle, and then removed. We prepared three Petri dishes for etoxazole treatment and three as control (sprayed with distilled water) per population. Eggs on the leaves were sprayed with etoxazole at 50 mg l^{-1} (adhesion amount: 4 mg cm^{-2}) immediately after the removal of adult females. Hatchability was observed on day

289 9 (Sakurai population) or 10 (others). We corrected mortality using Abbott's correction
290 as in following the formula [24]:

291 $$M = \frac{X - Y}{X},$$

292 where M is the corrected mortality, X is egg hatchability in the control sprayed with water,
293 and Y is that of eggs sprayed with etoxazole.

294 For sequencing analysis of CHS1, we prepared crude DNA extracts from 10
295 individual adult females of the Uda population. The methods for DNA preparation and
296 sequencing analysis were the same as those in section *2.2*.

297

298 ## 3. Results

299

300 ### *3.1. Sequencing analysis of mated mites in resistant and susceptible strains*

301

302 The nucleotide sequences analyzed in this section are available from the
303 DDBJ/EMBL/GenBank database under accession numbers LC218436–LC218439. All
304 females and males of Kyoyu-S were Haplotype I for the 1016 and 1017 codons (Fig. 1,
305 Supplementary Fig. 1). The O4 female and all males of SoOm1-etoR were Haplotype III.
306 The O3 and O6 females were heterozygotes of Haplotypes III and IV. The O2 female was
307 also a heterozygote, but we could not determine between the heterozygote of Haplotypes
308 I and IV or Haplotypes II and III (Fig. 1, Supplementary Fig. 1). We chose two pairs from
309 SoOm1-etoR (O4 and O6) and from Kyoyu-S (K1 and K4) to establish offspring lines for
310 use in the following experiments to evaluate the RED-ΔΔCt method.

311

312 *3.2. Evaluation of linearity in the relationship between practical resistance variant*
313 *frequencies and measures by the RED-ΔΔCt method*
314
315 *3.2.1. Evaluation of amplification efficiency*
316 Amplification efficiencies (*e*) for CHS1 were 1.075 and 0.997 for SoOm1-etoR (O6)
317 and Kyoyu-S (K4), respectively, indicating that about 100% amplification was realized
318 with the primer set. Similarly, *e* for GAPDH was also 1.073 for O6 and 1.019 for K4. The
319 contribution ratios (R^2) of regression lines were high (> 0.99) in all of the cases.
320 Parallelism of the CHS1 and GAPDH regression lines was not significant in both O6
321 (two-way ANOVA [gene×DNA concentration]: $df = 1$, $F = 0.001$, $P = 0.262$) and K4 (df
322 $= 1$, $F = 1.767$, $P = 0.232$). Two-way ANOVA was also used to evaluate the effects of
323 genes and DNA concentrations. In O6, no significant differences were detected in genes
324 ($df = 1$, $F = 1.532$, $P = 0.262$), but there were differences in DNA concentrations ($df = 1$,
325 $F = 1303.8$, $P = 3.01\times10^{-8}$). Significant differences were observed in both DNA
326 concentrations ($df = 1$, $F = 28274.4$, $P = 2.98\times10^{-12}$) and the effects of genes ($df = 1$, $F =$
327 39.04, $P = 7.79\times10^{-4}$) in K4. This may be caused by extremely high linearity; R^2 was 1
328 for both CHS1 and GAPDH in K4 and the lines were also close to each other in O6,
329 indicating that a combination of these primer sets would work for subsequent qPCR
330 analyses.
331
332 *3.2.2. qPCR for DNA samples digested by restriction endonucleases*
333 Digestion times for genomic DNA of 15 min and 1 h for each endonuclease, *MluC* I
334 and *Taq*$^{\alpha}$I, did not ensure the separation of ΔCt values for practical resistance variant
335 frequencies at 0.01 or lower (Fig. 3). We made sure that ΔCt values increased by

decreasing the practical R variant frequencies after digestion for 3 h for each endonuclease. As PCR amplification occurs (allowing to calculate the ΔCt value) even if the DNA sample only includes a susceptible variant (practical R variant frequency was 0) that should be completely digested by endonucleases, digestion efficiencies might become rate-limiting. Therefore, we used a digestion time of 3 h in the following analyses of the linearity of correlation between the practical resistance gene frequencies and estimators, $2^{-\Delta\Delta Ct}$, produced by the RED-ΔΔCt method.

There was a strong correlation between practical R variant frequencies in the genomic DNA samples and $2^{-\Delta\Delta Ct}$ (Fig. 4, Supplementary Fig. 2), and no serious non-specific amplification was observed (Supplementary Fig. 3). A slope of the linear regression line given through the origin was approximately 1 (Fig. 4), ensuring accurate estimation of resistant variant frequencies by the RED-ΔΔCt method. However, the plots for R variant frequencies of 0 and 0.001 tended to deviate from the regression line. This may be due to the influence of rate-limitation by digestion efficiency described above. The plot for an R variant frequency of 1 also tended to deviate from the regression line, although the reason was not clear. We also performed linear regression analyses using the same dataset above, but excluding data in the R variant frequencies of 0, 0.001, and 1. The resulting regression line formula was

$$y = 1.015x \ (R^2 = 0.982, P < 2.2\times10^{-16}),$$

where y represents $2^{-\Delta\Delta Ct}$ and x is the practical R variant frequency. The slope was 1 and R^2 increased. Therefore, we can accurately estimate R variant frequencies in the range from 0.005 to at least 0.75 using the RED-ΔΔCt method.

3.3. Validation of the effects of resistance variant frequencies on etoxazole resistant

phenotypes

After spraying with etoxazole, no eggs hatched in the two laboratory strains. Their R variant frequencies ($2^{-\Delta\Delta Ct}$) were measured to be 0.2% and 0.5% in Tsukuba and NS, respectively (Supplementary Table 2). Because the numbers of females introduced to kidney bean leaves were 41 and 35, minimum R variant frequencies (assuming the presence of one heterozygote in susceptible individuals) were expected to be 1.2% and 1.4% in Tsukuba and NS, respectively. Therefore, it is possible that the R variant was not present in these laboratory strains. Egg hatchability and estimated R variant frequencies ($2^{-\Delta\Delta Ct}$) ranged from 33.5% to 97.7% and from 30.2% to 94.5% in the field populations, respectively (Supplementary Table 2). A strong correlation was detected between $2^{-\Delta\Delta Ct}$ and hatchability over the field populations and laboratory strains (Fig. 5, solid regression line). Moreover, the slope of the regression line (0.963) was close to 1 and supported by a high R^2 value (0.987), indicating compatibility between the estimator by the RED-ΔΔCt method and results of the bioassay with haploid male eggs.

One sample, Yokote, obviously deviated from the regression line. The number of eggs laid per female was lowest for Yokote (1.9 eggs), then Izu2 (3.6 eggs) and Yawata (4 eggs; Supplementary Table 2). The number of eggs produced per female ranged from 4.8 to 9.4 in other field populations and laboratory strains. The low number of eggs may be due to bias of oviposition in that a substantial number of females did not lay eggs. Therefore, we also performed linear regression analyses excluding Yokote, obtaining a regression line slope of 1 and an increased R^2 (Fig. 5, dashed regression line).

3.4. Comparison of the RED-ΔΔCt method with a common toxicological bioassay

Prior to the experiments, we tentatively confirmed the height of amplification efficiency (*e*) and parallelism of regression lines between CHS1 and GAPDH because we conducted the analysis using distinct equipment. *e* for CHS1 and GAPDH was 0.933 and 0.945, respectively, in the Uda population (Supplementary Fig. 5). Parallelism was not rejected (two-way ANOVA, [gene×DNA concentration]: $df = 1$, $F = 0.112$, $P = 0.75$). The difference between genes was not significant ($df = 1$, $F = 0.006$, $P = 0.942$), while the difference among concentrations was significant ($df = 1$, $F = 4540.6$, $P = 0.942$).

The R variant frequencies ($2^{-\Delta\Delta Ct}$) were 16.3% in the Kashihara population and ranged from 79% to 86.5% in the remaining four populations. Egg hatchability after etoxazole treatment was lowest in Kashihara at 41.4% and ranged from 73.9% to 100% in the remaining four populations (Supplementary Table 3). Although the slope of the linear regression line (1.104) was greater than 1 and R^2 (0.956) was lower than in the validation (*3.4*; Fig. 5), significant correlation was detected between egg hatchability and $2^{-\Delta\Delta Ct}$. In this experiment, we used gravid females, so that diploid female and haploid male eggs were mixed in the toxicological bioassay. This may be a reason for the increased regression variation; residual standard error, which is a possible indicator of variation, was 0.174 in this experiment, higher than 0.073 in the case of validation (*3.4*).

The nucleotide sequences analyzed in this section are available from the DDBJ/EMBL/GenBank database under accession numbers LC218440–LC218442. Sequencing analysis of the Uda population revealed a high frequency of synonymous SNPs at the 1016 codon (Fig. 1). Of the 10 adult females, 9 were homozygotes with synonymous SNPs (TCA) and only 1 was a heterozygote (TCG/TCA; Supplementary Fig. 4). With regard to the 1017 codon, seven females were homozygotes of the etoxazole resistant variant (TTT), while the remaining three females were heterozygotes

(ATT/TTT) and their 1016 codons were all TCA. Consequently, the Uda population consisted of at least Haplotypes II, III, and IV (Fig. 1).

4. Discussion

We developed a monitoring method that enabled the detection of a variant associated with etoxazole resistance in a pooled DNA sample of *T. urticae*. Resistance allele frequency in a pooled DNA sample is estimated by referring to a calibration curve in ongoing monitoring methods [13,15]. By contrast, R variant detection in a pooled DNA sample was enabled by restriction endonuclease digestion of an S variant and was calibrated using the ΔCt of the intact sample from the same pooled DNA in the RED-ΔΔCt method.

The strong linearity in the correlation between practical frequencies of the resistant variant and estimator, $2^{-\Delta\Delta Ct}$, supported a high degree of accuracy. In addition to the general ΔΔCt method in the analysis of gene expression level with RT-PCR, correspondence between a gene of interest and reference and height of *e* are indispensable requirements [25]; thus, the accuracy of the estimator largely depends on the design of primer sets and purification of the DNA sample. The amplification efficiency test evidenced correspondence between the height of the efficiencies of the primers for CHS1 and GAPDH used in this study.

Experiments on the effectiveness of digestion time showed that the accuracy of the estimator at low frequency for the variant of interest, a SNP associated with etoxazole resistance, depended on the efficiency of digestion by restriction endonucleases. The accuracy was also estimated by comparing the ΔCt of a sample consisting of a 100%

susceptible variant with the ΔCt in a test of amplification efficiency calculated using the Ct for GAPDH at 1 ng l^{-1} as a reference (Supplementary Fig. 6). The averaged ΔCt value in the linearity test (for O6 and K4 in *3.2.2*) was 9.29 for a sample of the S variant at 100%. The DNA concentration corresponding with a ΔCt of 9.29 was calculated to be 0.00137 ± 0.00024 ng l^{-1} (averaged among regression lines for O6, K4, and Uda; Supplementary Fig. 6), implying that 0.1–0.2% of the susceptible variant remained undigested by restriction endonucleases. This may be the reason why 0.1% of the R variant frequency was accurately undetectable in the conditions of this study. Conversely, modification to complete digestion, such as elongating the time for digestion, may enable the detection of etoxazole R variants at very low frequencies.

Strong correlation of haploid egg hatchability after etoxazole treatment with the estimation of R variant frequencies ($2^{-\Delta\Delta Ct}$) demonstrated the reliability of the RED-ΔΔCt method as a monitoring method for etoxazole resistance alleles in a pooled DNA sample. Association of the R variant was probed from the population bulk segregation and the common presence over resistant strains of the SNP in CHS1 [3,8,26]). The strong correlation between survivability and the variant frequency provides direct evidence that I1017F is a primary factor of life and death for *T. urticae* in the place where sprayed with etoxazole in the strains used for experiments.

Results of ovicidal toxicological tests may deviate from the proper resistance allele frequency in the population due to factors whether resistance inheritance is dominant or recessive and whether egg production ability varies based on the condition of the female (young or aged, well-fed or undernourished, and fertile or infertile). Etoxazole resistance inheritance is completely recessive in *T. urticae* [2]. A common toxicological test on a field population is usually performed using females in various conditions. These

potentially weaken the correlation between practical resistance allele frequencies and egg hatchability after acaricide treatment as the correlation between the common toxicological test and $2^{-\Delta\Delta Ct}$ in this study, demonstrating the significance of an accurate monitoring method for resistance allele frequency. The mutation (I1017F) in CHS1 also confers cross-resistance to clofentezine and hexythiazox [8], although another mechanism also suggested in hexythiazox resistance [9]. Therefore, this method is relevant to resistance against those chemicals.

Greenhouse populations of *T. urticae* fundamentally divide into breeding patches when the population density is low, which may be the usual condition, rather than high density in a commercial greenhouse [27,28,29]. It is important to monitor resistance allele frequency when the population density remains under the control threshold. In such cases, we need to increase the number of monitored breeding patches in a greenhouse to avoid missing the rare resistance allele, because even if the resistance allele frequency is very low in a greenhouse, its frequency may be at mid or high level in a few local breeding patches due to the founder effect. The RED-ΔΔCt method is an appropriate monitoring method to meet these demands.

To the best of our knowledge, etoxazole resistance mechanisms other than the mutation in CHS1 in *T. urticae*, whereas the resistance in a phytoseiid mite *Phytoseiulus persimilis* Athias-Henriot was suggested to have a relevance to the activities of detoxification enzymes by Salman et al. [30]. Application of this method to such metabolic resistance system may mostly be difficult, and the application to the detection of SNPs is also limited by the availability of restriction enzyme as well as PCR-RFLP.

5. Conclusions

The RED-ΔΔCt method is a powerful tool for monitoring resistance alleles in a pooled sample and analyzing the spatial distribution and dynamics of the resistance allele. Therefore, studies on the expansion of the RED-ΔΔCt method to monitor resistance allelic variation against other acaricides are worthwhile. However, lack of proper restriction endonucleases to digest the S variant will limit the scope of its application.

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590

591 Figure legends

592

593 Fig. 1 Haplotypes of CHS1 reported by Van Leeuwen et al. [6] and endonuclease
594 recognition sites in susceptible haplotypes (I and II) (a) and SNP detected in mated
595 SoOm1-etoR (O2, O3, O4 and O6) and Kyoyu-S (K1, K2, K3 and K4) females and
596 males (b). R: G/A, W: A/T.

597

598 Fig. 2 Amplification efficiency for CHS1 (solid circle) and GAPDH (open circle) for the
599 SoOm1-etoR (O6) (a) and Kyoyu-S (K4) (b) strains. DNA concentration: log10(ng
600 μl^{-1}). For O6 regression line equations for CHS1 and GAPDH were $y = -3.15x +$
601 19.98 ($E = 2.075$, $R^2 = 0.997$) and $y = -3.16x + 20.27$ ($E = 2.073$, $R^2 = 0.994$),
602 respectively. For K4 regression line equations for CHS1 and GAPDH were $y =$
603 $-3.33x + 19.37$ ($E = 1.997$, $R^2 = 1$) and $y = -3.28x + 19.82$ ($E = 2.019$, $R^2 = 1$),
604 respectively. *E*: amplification efficiency (*e*) + 1 (if 100% amplification was acheived
605 *E* would be "2").

606

607 Fig. 3 Effects of restriction enzyme digestion periods on ΔCt values of various practical
608 frequencies of the resistance variant (R variant frequency). Digestion time shows the
609 digestion times for *MluC* I and Taq^{α} I. ΔCt = (Ct for CHS1) − (Ct for GAPDH).

610

611 Fig. 4 Correlation between practical resistance variant frequency (R variant frequency)
612 and the estimator, $2^{-\Delta\Delta Ct}$. Regression line: $y = 1.077x$, $R^2 = 0.980$, $P < 2.2 \times 10^{-16}$.
613 Vertical lines on the plots indicate standard error. ΔΔCt = (ΔCt in digested sample) −
614 [ΔCt in undigested corresponding intact DNA samples (calibrator)].

615

616 Fig. 5 Correlation between hatchability of male haploid eggs of local populations after
617 treatment with etoxazole (50 mg l^{-1}) and $2^{-\Delta\Delta Ct}$ of mother females. Solid regression
618 line includes Yokote: $y = 0.963x$, $R^2 = 0.987$, $P < 4.661 \times 10^{-8}$. Dashed gray
619 regression line excludes Yokote: $y = 1.009x$, $R^2 = 0.997$, $P < 7.37 \times 10^{-9}$.

620

621 Fig. 6 Correlation between egg hatchability (mixture of female and male eggs) in newly
622 collected local populations after treatment of females with etoxazole (50 mg l^{-1}) and
623 $2^{-\Delta\Delta Ct}$. Solid regression line: $y = 1.104x$, $R^2 = 0.956$, $P < 4.670 \times 10^{-4}$. The dashed
624 gray line represents the regression line excluding Yokote in Figure 5.

625

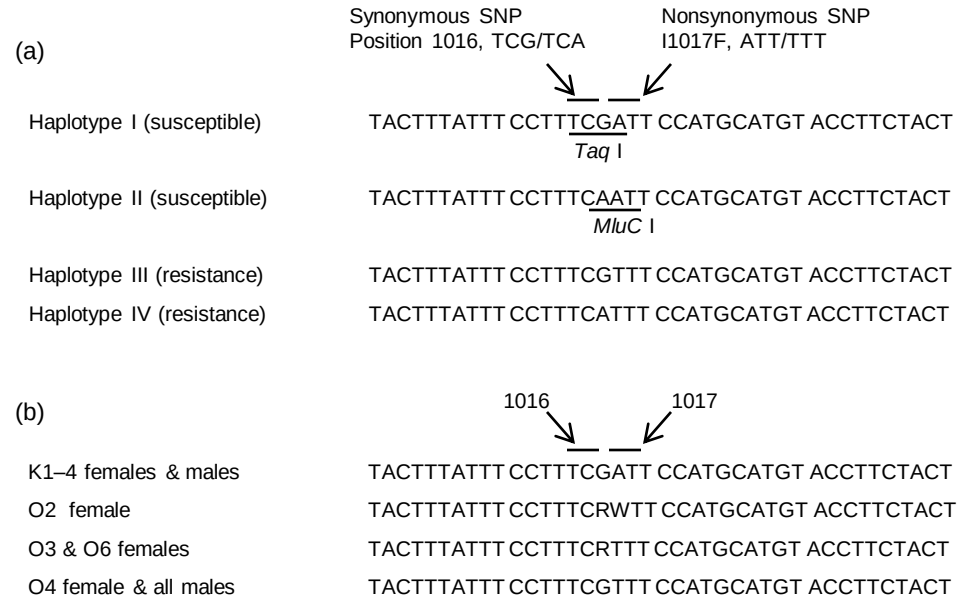

(a)
Synonymous SNP
Position 1016, TCG/TCA
Nonsynonymous SNP
I1017F, ATT/TTT
Haplotype I (susceptible)
TACTTTATTT CCTTTCGATT CCATGCATGT ACCTTCTACT
Taq I
Haplotype II (susceptible)
TACTTTATTT CCTTTCAATT CCATGCATGT ACCTTCTACT
MluC I
Haplotype III (resistance)
TACTTTATTT CCTTTCGTTT CCATGCATGT ACCTTCTACT
Haplotype IV (resistance)
TACTTTATTT CCTTTCATTT CCATGCATGT ACCTTCTACT
(b)
1016
1017
K1–4 females & males
TACTTTATTT CCTTTCGATT CCATGCATGT ACCTTCTACT
O2 female
TACTTTATTT CCTTTCRWTT CCATGCATGT ACCTTCTACT
O3 & O6 females
TACTTTATTT CCTTTCRTTT CCATGCATGT ACCTTCTACT
O4 female & all males
TACTTTATTT CCTTTCGTTT CCATGCATGT ACCTTCTACT

Fig. 1

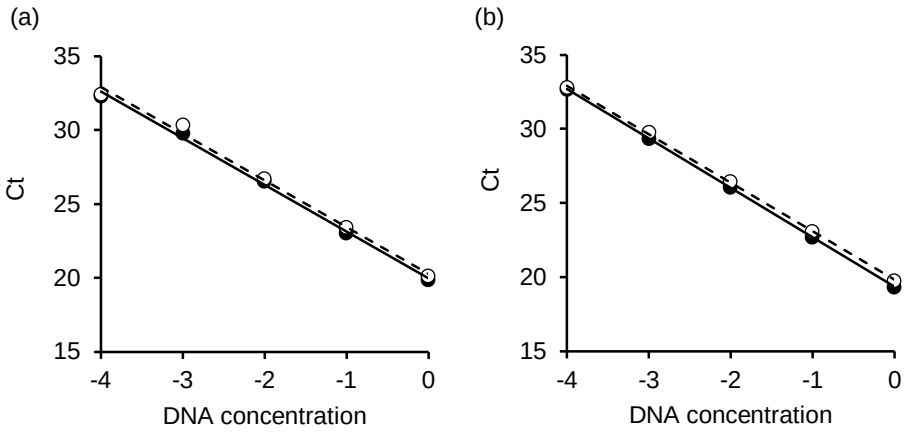

(a)
35
30
25
20
15
Ct
-4
-3
-2
-1
0
DNA concentration
(b)
35
30
25
20
15
Ct
-4
-3
-2
-1
0
DNA concentration

Fig. 2

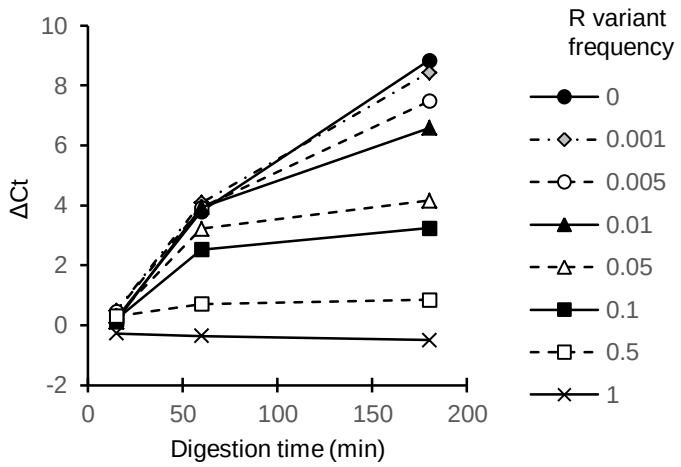

10
8
6
4
2
0
-2
ΔCt
0
50
100
150
200
Digestion time (min)
R variant frequency
0
0.001
0.005
0.01
0.05
0.1
0.5
1

Fig. 3

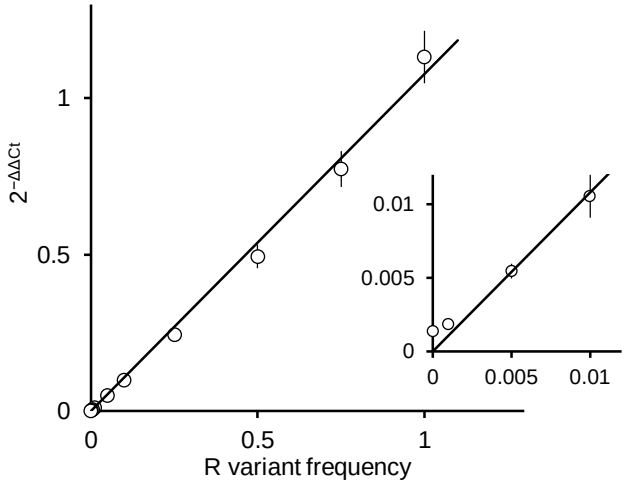


Fig. 4

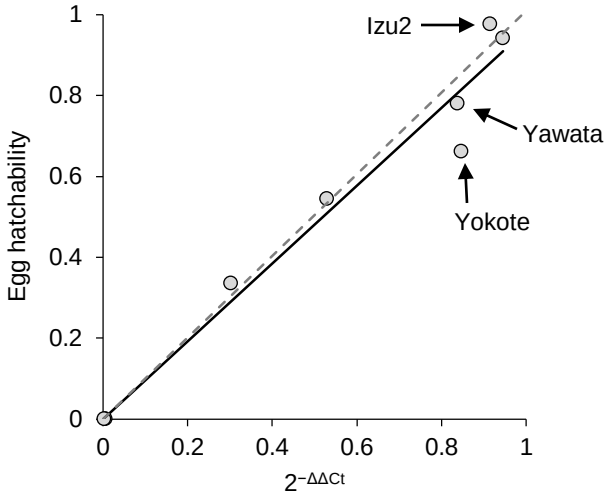

Izu2
Yawata
Yokote
Egg hatchability
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
2−ΔΔCt

Fig. 5

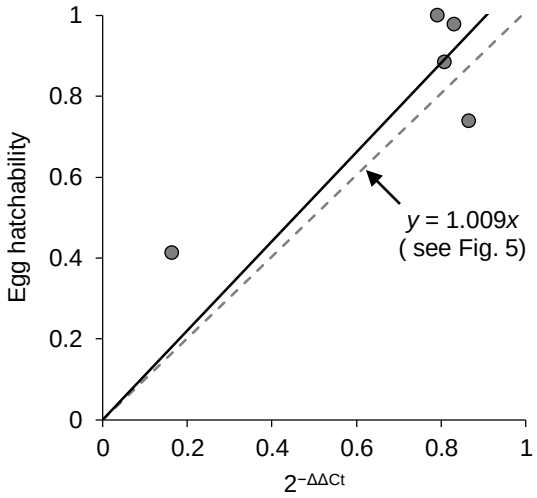

Egg hatchability
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
2−ΔΔCt
y = 1.009x
(see Fig. 5)

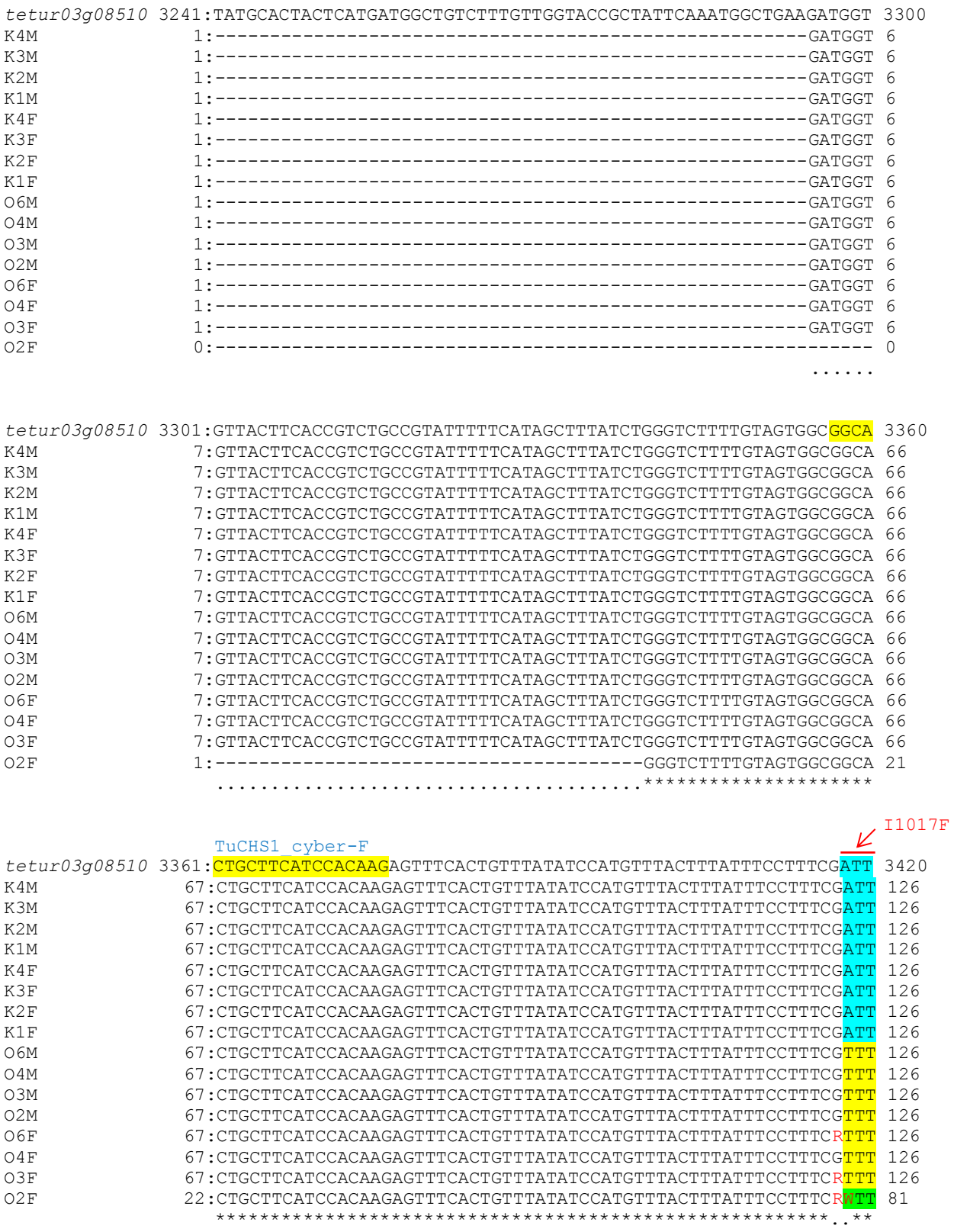
Fig. 6

Table 1 Collection records of field populations and laboratory strains

Population	Date	Host plant	Site
Field population			
Iwate	Oct. 1999	Apple	Shimokuriyagawa, Morioka, Iwate Pref., Japan (39.8° N, 141.1° E)
Yokote	Jun. 2014	Apple	Hiraga, Yokote, Akita Pref., Japan (39.2° N, 140.6° E)
Izu2	Feb. 2013	Strawberry	Nagasaki, Izunokuni, Shizuoka Pref., Japan (35.1° N, 139.0° E)
Masu	Jan. 2012	Strawberry	Shimizu, Shizuoka, Shizuoka Pref., Japan (35.0° N, 138.5° E)
Komagoe	Jan. 2012	Strawberry	Shimizu, Shizuoka, Shizuoka Pref., Japan (35.0° N, 138.5° E)
Yawata	Oct. 2014	Japanese pear	Uchizato, Yawata, Kyoto Pref., Japan (34.9° N, 135.7° E)
Laboratory strain			
Tsukuba	unknown	Kidney bean	Laboratory strain
NS	1998	Chrysanthemum	Katsuragi, Nara Prefecture, Japan (34.5° N, 135.7° E)

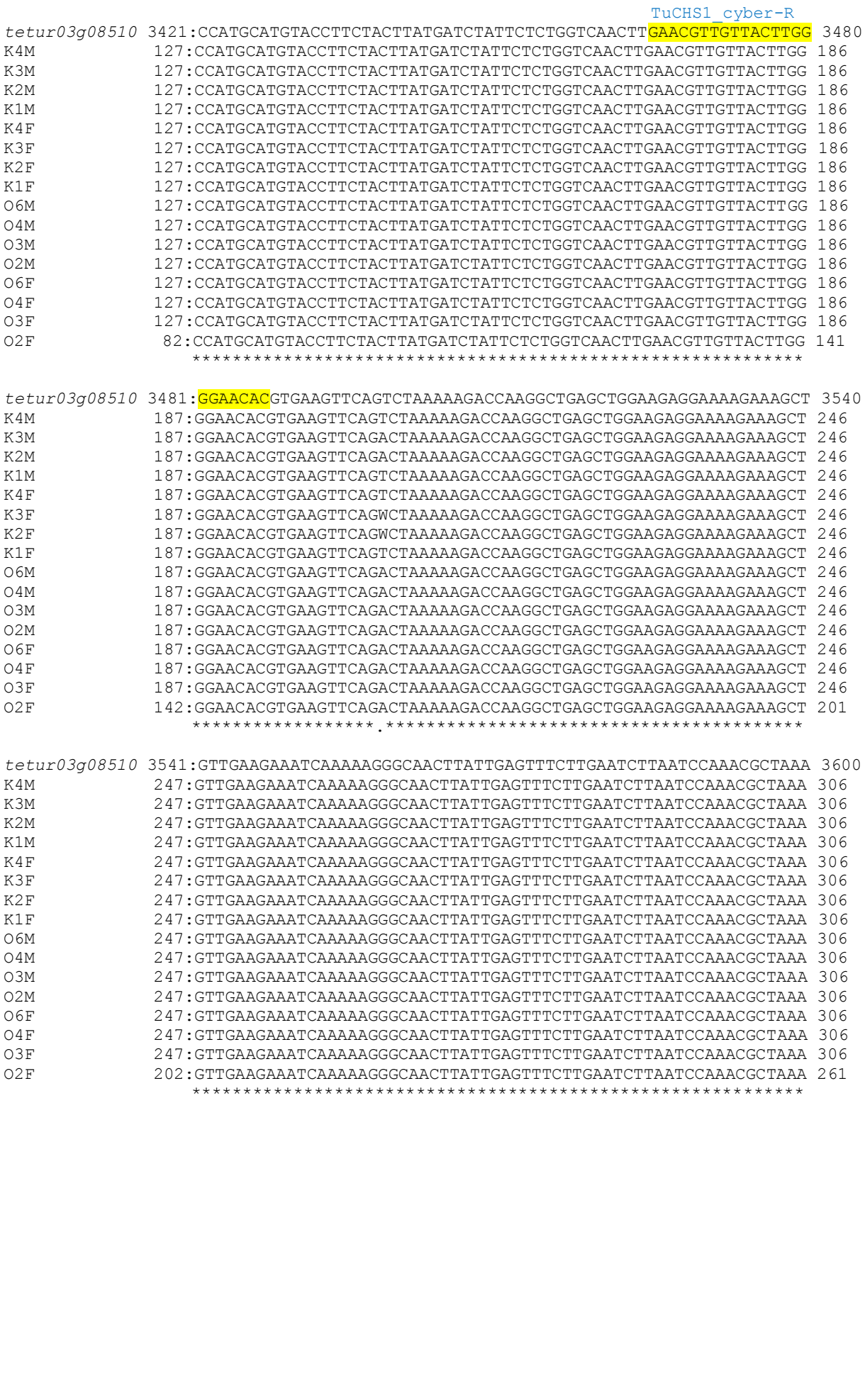
Table 2 Collection records of field populations newly collected for pilot experiments

Population	Date	Host plant	Site
Gojo_Ada	Nov. 2016	Strawberry	Ohno Shinden, Gojo, Nara Pref., Japan (34.4° N, 135.7° E)
Gojo_Oka	Nov. 2016	Strawberry	Oka, Gojo, Nara Pref., Japan (34.4° N, 135.7° E)
Sakurai	Dec. 2016	Strawberry	Higaida, Sakurai, Nara Pref., Japan (34.5° N, 135.8° E)
Kashihara	Nov. 2016	Strawberry	Toichi, Kashihara, Nara Pref., Japan (34.5° N, 135.8° E)
Uda	Dec. 2016	Strawberry	Ohuda Hirao, Uda, Nara Pref., Japan (34.5° N, 135.9° E)



Supplementary Fig. 1 Alignment of CHS1 DNA sequences of females (F) and males (M) in SoOm1-etoR (O2, O3, O4 and O6) and Kyoyu-S (K1, K2, K3 and K4). The first position of sequence except O2F are corresponding with 3295 b in *tetur03g08510*. TuCHS1_cyber-F (forward) and TuCHS1_cyber-R (reverse) are the primers designed for the RED-ΔΔCt method.

Supplementary Fig. 1 continue



Supplementary Fig. 1 continue

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tetur03g08510 3601:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 3660
K4M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K3M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K2M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K1M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K4F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K3F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K2F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
K1F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O6M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O4M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O3M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O2M            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O6F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O4F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O3F            307:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 366
O2F            262:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 321
                   ************************************************************

tetur03g08510 3661:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 3720
K4M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K3M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K2M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K1M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K4F            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K3F            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K2F            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
K1F            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
O6M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
O4M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
O3M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
O2M            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
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O3F            367:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 426
O2F            322:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 381
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tetur03g08510 3721:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 3780
K4M            427:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K3M            427:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K2M            427:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K1M            427:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K4F            427:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K3F            427:ATGACTGACAAACTTGGTKCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K2F            427:ATGACTGACAAACTTGGTKCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
K1F            427:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
O6M            427:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 486
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O2F            382:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 441
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Supplementary Fig. 1 continue

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tetur03g08510 3781:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 3840
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K3M            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K2M            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K1M            487:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K4F            487:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K3F            487:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K2F            487:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
K1F            487:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O6M            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O4M            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O3M            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
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O4F            487:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O3F            487:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O2F            442:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 501
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tetur03g08510 3841:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAAACTGATGATATGTCA 3900
K4M            547:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAAACTGATGATATGTCA 606
K3M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
K2M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
K1M            547:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAAACTGATGATATGTCA 606
K4F            547:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAAACTGATGATATGTCA 606
K3F            547:AATGAAGAACATGAAGACATGGATTCAMTTGGCTCAGRATCACAAACTGATGATATGTCA 606
K2F            547:AATGAAGAACATGAAGACATGGATTCAMTTGGCTCAGRATCACAAACTGATGATATGTCA 606
K1F            547:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAAACTGATGATATGTCA 606
O6M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O4M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O3M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O2M            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O6F            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O4F            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O3F            547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 606
O2F            502:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 561
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tetur03g08510 3901:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 3960
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K3M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K2M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K1M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K4F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K3F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K2F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
K1F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O6M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O4M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O3M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O2M            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O6F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O4F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O3F            607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O2F            562:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 621
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Supplementary Fig. 1 continue

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tetur03g08510 3961:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTTGG 4019
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K3M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K2M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K1M            667:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K4F            667:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K3F            667:GATRAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K2F            667:GATRAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K1F            667:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O6M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O4M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O3M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O2M            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O6F            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O4F            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O3F            667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
O2F            622:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 679
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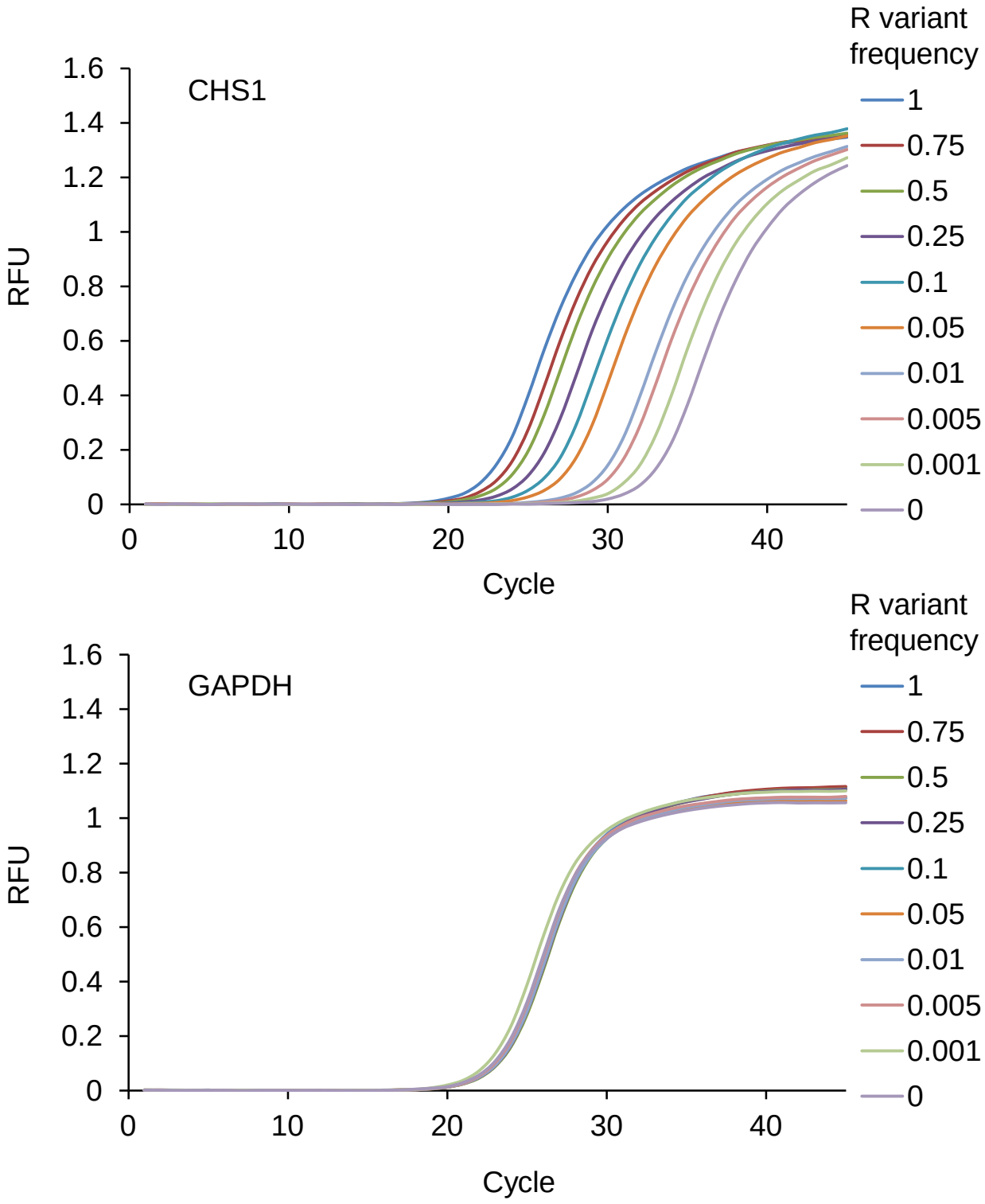
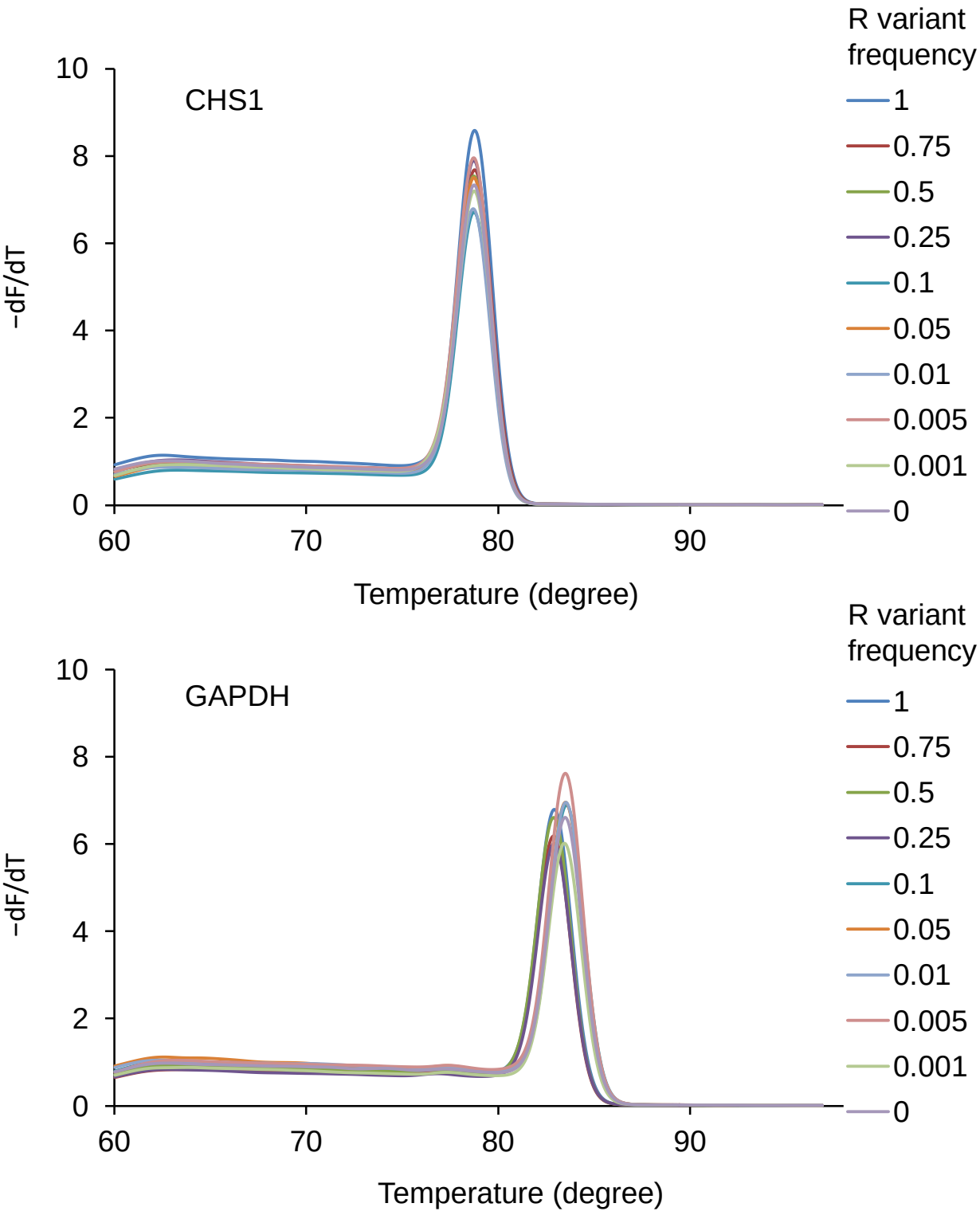
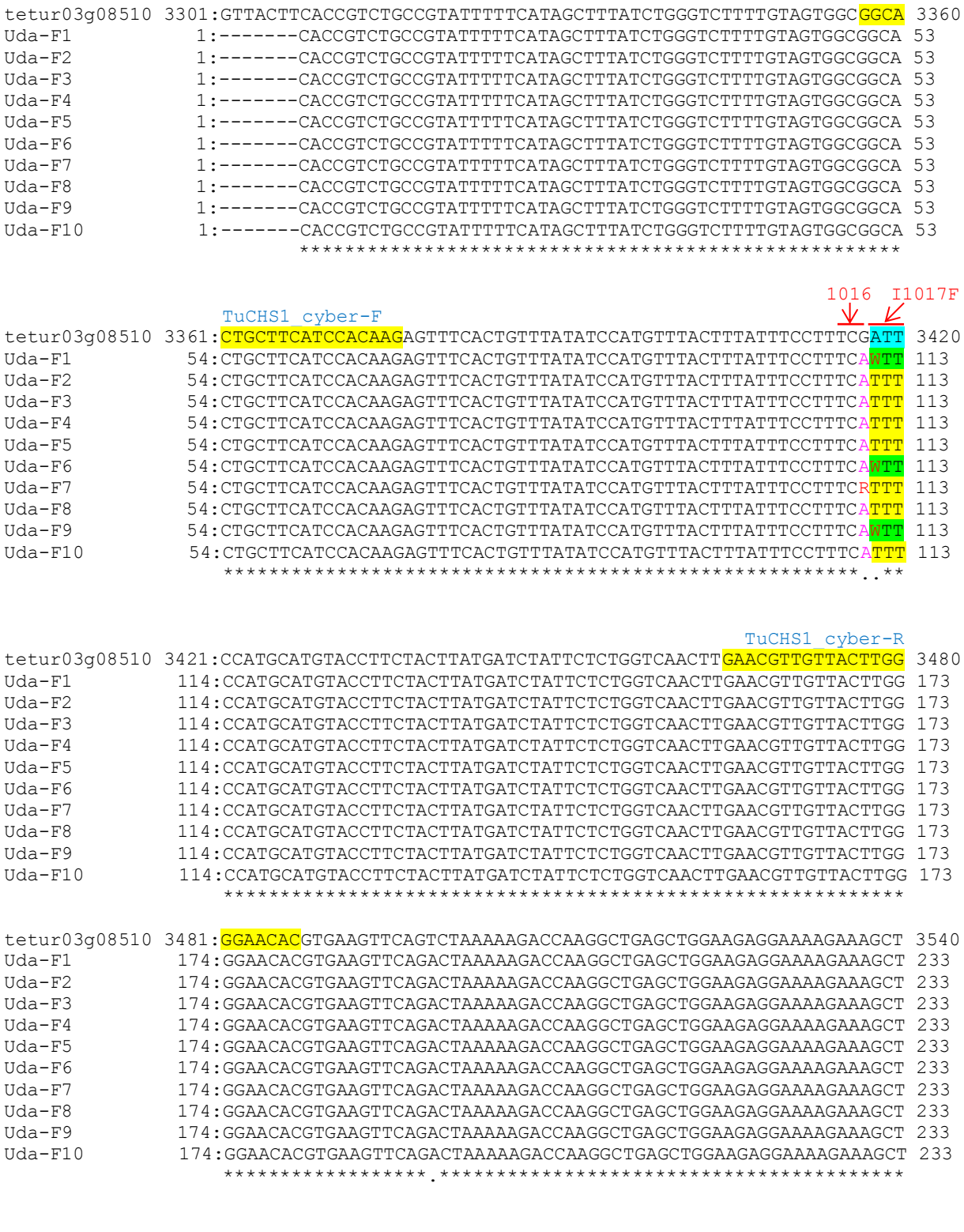


Supplementary Fig. 2 Amplification curves for CHS1 and GAPDH of genomic DNA with various practical R variant frequencies after digestion by restriction endonuclease, *MluC* I and *Taq*$^{\alpha}$I, for 3 h. RFU: relative fluorescent unit.



Supplementary Fig. 3 Melt peak chart for CHS1 and GAPDH of genomic DNA with various practical R variant frequencies after digestion by restriction endonuclease, *MluC* I and *Taq*$^{\alpha}$I, for 3 h. dF/dT: derivative of melting curve.



Supplementary Fig. 4 Alignment of CHS1 DNA sequences of females of Uda population. The first position of sequence is corresponding with 3308 b in *tetur03g08510*. TuCHS1_cyber-F (forward) and TuCHS1_cyber-R (reverse) are the primers designed for the RED-ΔΔCt method.

Supplementary Fig. 4 continue

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tetur03g08510 3541:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 3600
Uda-F1         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAAMGCTAAA 293
Uda-F2         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F3         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F4         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F5         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F6         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAAMGCTAAA 293
Uda-F7         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F8         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
Uda-F9         234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAAMGCTAAA 293
Uda-F10        234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 293
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tetur03g08510 3601:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 3660
Uda-F1         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F2         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F3         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F4         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F5         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F6         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F7         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F8         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F9         294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
Uda-F10        294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACTTGTTCAGGTGCTCTTTCTGTACA 353
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tetur03g08510 3661:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 3720
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Uda-F2         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F3         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F4         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F5         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F6         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F7         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F8         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F9         354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
Uda-F10        354:TATCCCAAACCAAACGACGAAAAGATTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
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tetur03g08510 3721:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 3780
Uda-F1         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F2         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F3         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F4         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F5         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F6         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F7         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F8         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F9         414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
Uda-F10        414:ATGACTGACAAACTTGGTTCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 473
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tetur03g08510 3781:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 3840
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Uda-F2         474:AAAGGATCTTCAATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 533
Uda-F3         474:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 533
Uda-F4         474:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 533
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Uda-F9         474:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 533
Uda-F10        474:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 533
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Supplementary Fig. 4 continue

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Uda-F2         534:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 593
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Uda-F5         534:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 593
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Uda-F9         534:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 593
Uda-F10        534:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAAACTGATGATATGTCA 593
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tetur03g08510 3901:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 3960
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Uda-F2         594:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 653
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Uda-F7         594:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 653
Uda-F8         594:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 653
Uda-F9         594:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 653
Uda-F10        594:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 653
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tetur03g08510 3961:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTTGG 4020
Uda-F1         654:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTTGG 713
Uda-F2         654:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTTGG 713
Uda-F3         654:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTTGG 713
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tetur03g08510 4021:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACCAAAATAAAGATCATCAAGCTCGT 4080
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Uda-F2         714:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACCAAAATAAAGATCATCAAGCTCGT 773
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Uda-F6         714:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACC-AAATAAAGATCATCAAGCTCGT 772
Uda-F7         714:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACCAAAATAAAGATCATCAAGCTCGT 773
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Uda-F9         714:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACCAAAATAAAGATCATCAAGCTCGT 773
Uda-F10        714:AAGGAACTGATTTCCAAATATCTCTATCCAATCGACCAAAATAAAGATCATCAAGCTCGT 773
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Uda-F2         774:GTAGCTGTTGATTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
Uda-F3         774:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
Uda-F4         774:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
Uda-F5         774:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
Uda-F6         773:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 809
Uda-F7         774:GTAGCTGTTGAGTTGAAAGAGCTRCGAAATAGAGTAG---------------------- 810
Uda-F8         774:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
Uda-F9         774:GTAGCTGTTGAGTTGAAAGAGCTGCGAAATAGAGTAG---------------------- 810
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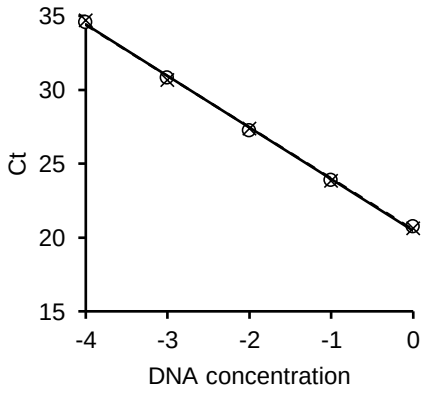
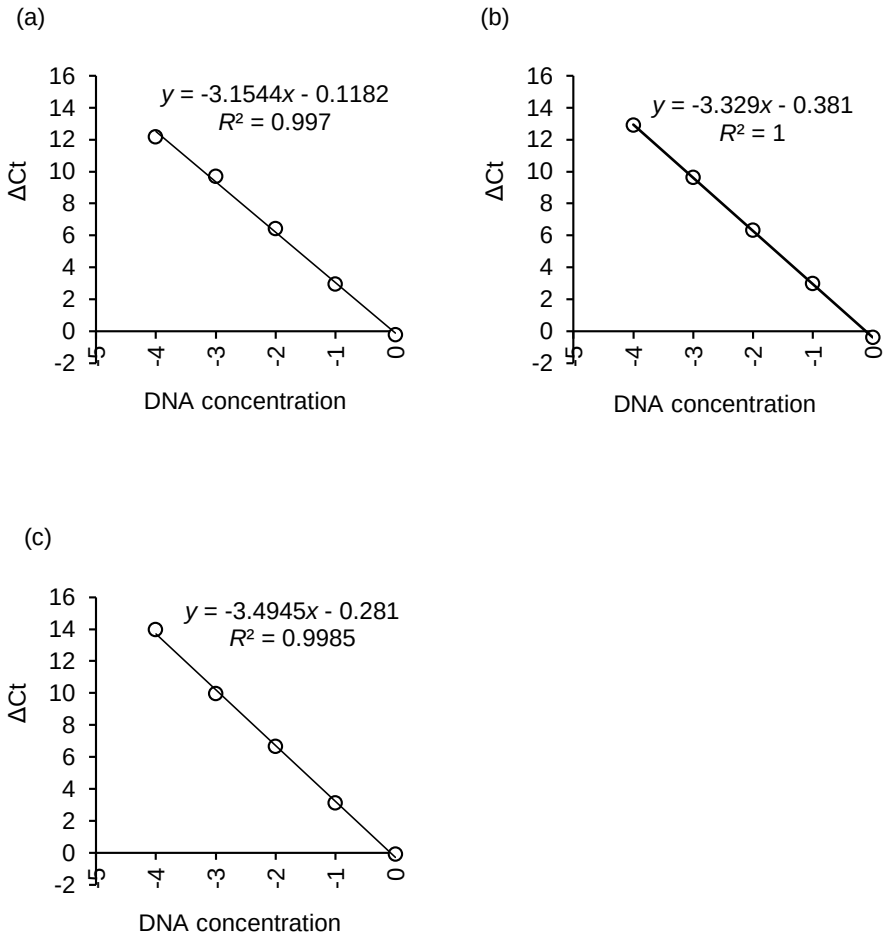


Supplementary Fig. 5 Amplify efficiency for CHS1 (cross) and GAPDH (open circle) in Uda population. DNA concentration: log10(ng μl^{-1}). Equations of regression lines for CHS1 and GAPDH were $y = -3.49x + 20.46$ ($E = 1.933$, $R^2 = 0.985$) and $y = -3.46x + 20.54$ ($E = 1.945$, $R^2 = 0.997$). *E*: amplification efficiency (*e*) + 1 (if 100% amplification was acheived *E* would be “2”).



Supplementary Fig. 6 Correlation between DNA concentration (ng μl^{-1}) and ΔCt value in amplify efficiency experiments in SoOm1-etoR (O6) (a) and Kyoyu-S (K4) (b) strains and Uda population (c) using the Ct values of GAPDH at the concentration of 1 ng μl^{-1} as references.

Supplementary Table 1 Susceptibility of SoOm1_etoR strain to etoxazole at 50–3,200 mg l^{-1} (a) and at 5,000 and 10,000 mg l^{-1} (b)

(a)

Etoxazole (mg l^{-1})	No. of eggs tested	No. of unhatched eggs	Hatchability (%)	Corrected mortality (%)
3,200	94	6	93.6	0
1,600	112	10	91.1	0.9
800	103	8	92.2	0
400	95	8	91.6	0.4
200	96	2	97.9	0
100	96	5	94.8	0
50	77	7	90.9	1.1
Control (DW)	99	8	91.9	—

(b)

Etoxazole (mg L^{-1})	No. of eggs tested	No. of unhatched eggs	Hatchability (%)	Corrected mortality (%)
10,000	110	11	90.0	1.9
5,000	135	20	88.1	3.9
Control (DW)	157	13	91.7	—

DW: distilled water

Supplementary Table 2 Record of virgin adult females laid eggs and used for DNA extraction, hatchability of their eggs after spraying with etoxazole at 50 mg l^{-1}, and estimator of resistant variant frequency (R variant frequency) in CHS1

Population	No. of females introduced to leaves	No. of females used for DNA extraction	No. of haploid male eggs tested	Egg hatchability	Estimator of R variant frequency ($2^{-\Delta\Delta Ct}$) [a]
Field population					
Iwate	42	42	202	0.545	0.529
Yokote	34	31	62	0.661	0.846
Izu2	36	36	129	0.977	0.914
Masu	43	41	392	0.941	0.945
Komagoe	42	42	346	0.335	0.302
Yawata	37	36	146	0.781	0.837
Laboratory strain					
Tsukuba	41	39	255	0	0.002
NS	35	34	217	0	0.005

Supplementary Table 3 Egg hatchability of newly collected field populations after spraying with etoxazole at 50 mg l^{-1} and CHS1 R variant frequency estimated in the populations

Population	No. of eggs for etoxazole	Egg hatchability in etoxazole	No. of eggs for control	Egg hatchability in control	Corrected hatchability	Estimator of R variant frequency ($2^{-\Delta\Delta Ct}$) [a]
Gojo_Ada	852	0.863	790	0.975	0.885	0.807
Gojo_Oka	1179	0.997	1046	0.996	1	0.790
Sakurai	432	0.951	802	0.973	0.978	0.829
Kashihara	469	0.386	434	0.933	0.414	0.163
Uda	997	0.737	1032	0.998	0.739	0.865