

Combination of restriction endonuclease digestion with the $\Delta\Delta\text{Ct}$ method in real-time
PCR to monitor etoxazole resistance allele frequency in the two-spotted spider mite

Running title: $\Delta\Delta\text{Ct}$ method to monitor etoxazole resistance

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25 Abstract

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

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

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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

experiments [9].

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

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

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

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

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

2. Materials and methods

2.1. Mites

An etoxazole resistant strain (SoOm1-etoR) was selected with commercial formulations of etoxazole (10% suspension concentrate; Kyoyu Agri, Tokyo, Japan) at 50 mg l⁻¹ in a laboratory from a field population collected from strawberry plants in a greenhouse in Omaezaki City, Shizuoka Prefecture, Japan (34.7° N, 138.1° E) in January 2012. Although the selection was performed only once using an etoxazole solution at a practical concentration, mortalities from etoxazole at 50 to 10,000 mg l⁻¹ were from 0% to 3.9% (Supplementary Table 1), indicating that this strain had already developed resistance to etoxazole. An acaricide-susceptible strain (Kyoyu-S) was obtained from Kyoyu Agri Co., Ltd. (Kanagawa, Japan). Four pairs of females and males were separately mated on kidney bean leaf squares (2×2 cm) on water soaked cotton in Petri dishes for SoOm1-etoR (pair designations: O2, O3, O4 and O6) and Kyoyu-S (pair designations: K1, K2, K3 and K4). After egg production for 4 days, the 1017 codon of CHS1 of each mite was sequenced. Then we selected offspring from two pairs of each strain to evaluate the linearity of the interaction between practical resistance variant frequencies (R variant frequency) and its estimators using the RED-ΔΔCt method.

Six field populations and two laboratory strains (Table 1) were used to validate the effects of resistance variant frequencies on etoxazole resistant phenotypes in a bioassay. The field populations were maintained on kidney bean leaf disks without acaricide selection after collection. One laboratory strain, NS, was adversely selected with etoxazole and hexythiazox [9] and then reared under acaricide-free conditions [20]. Another laboratory strain, Tsukuba, had been reared in a laboratory without acaricide selection for >15 years.

Five field populations were newly collected from commercial strawberry

greenhouses in Nara Prefecture from November to December 2016 (Table 2) and used for comparison of the RED- $\Delta\Delta$ Ct method with a common toxicological bioassay. One (Uda) of the five field populations was also used for sequencing analysis of CHS1. The mites were reared on kidney bean leaves on water soaked cotton in Petri dishes in a laboratory at 25°C with a 16 h light:8 h dark cycle, except the newly collected field populations, which were reared in a laboratory at 25°C with natural day length condition (app. 10 h light:14 h dark light cycle).

2.2. Sequence analysis of mated mites from resistant and susceptible strains

We prepared crude DNA extracts, which were directly used as DNA templates for PCR amplification, following the method of Osakabe et al. [21]. A single adult female or male of a mated pair described in section 2.1 was homogenized in 20 μ l lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% Igepal CA-630 [Sigma, Tokyo, Japan], 10 mM NaCl, and 1 mg/ml proteinase K [Takara, Kusatsu, Japan]) with a plastic pestle (Pellet mixer; Toho, Tokyo, Japan) in a 0.2 ml PCR tube. The homogenate was incubated at 65°C for 20 min and then at 95°C for 10 min. The lysate was diluted with 380 μ l (female) or 180 μ l (male) nuclease-free water (Qiagen, Tokyo, Japan).

A 1,277-base region, including the 1017 codon of CHS1, was amplified by PCR. The PCR amplification was conducted using 1 μ l of each DNA template in a total reaction volume of 20 μ l of PCR buffer for KOD FX Neo (Toyobo, Osaka, Japan) containing 0.4 mM of each dNTP, 0.25 μ M of each primer, and 0.4 U of KOD FX Neo DNA polymerase (Toyobo). PCR conditions were an initial 2 min at 94°C followed by 10 s at 98°C, 30 s at 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- $\Delta\Delta C_t$ method

2.3.1. Genomic DNA preparation

Genomic DNA samples were prepared from offspring lines of two pairs of SoOml-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 μ l of extraction buffer in a 1.5 ml sample tube, 20 μ l 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

concentration was adjusted to 1 ng μL^{-1} . We prepared two DNA samples for each offspring line from the two pairs and used them as four replications for each strain.

2.3.2. Evaluation of amplification efficiency

We tested the efficiency of amplification in qPCR with a primer set for CHS1 (tu03CHS1 forward: 5'-GGCACTGCTTCATCCACAAG-3', and reverse: 5'-GTGTTCCCCAAGTAACAACGTTC-3') and for an internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]; tu25GAPDH forward: 5'-GCACCAAGTGCTAAAGCATGGAG-3', and reverse: 5'-GAACTGGAACACGGAAAGCCATAC-3'). DNA (1 ng μL^{-1}) from O6 and K4 were separately diluted 10-fold to 1×10^{-4} ng μL^{-1} with nuclease free water, and 8 μL of the DNA solution was used for each qPCR amplification. qPCR analysis using the intercalator method was performed using the LightCycler Nano System (Roche Diagnostics, Basel, Switzerland) with SYBR Fast qPCR Mix (Takara). The reaction mix contained 0.4 μM each of forward and reverse primers and 8 μL of diluted DNA samples. PCR conditions were an initial 30 s at 95°C followed by 10 s at 95°C, 10 s at 62°C, and 15 s at 72°C for 45 cycles, with a melting curve analysis. Although the LightCycler Nano System produced C_q values instead of C_t values, we phrased the C_q (quantification cycle) value as C_t (cycle threshold) values because C_q values have similar semantic content to C_t values. Regression lines and amplification efficiency were analyzed using LightCycler Nano Software ver. 1.1.0 (Roche). Parallelism between regression lines for CHS1 and GAPDH were statistically analyzed based on an interaction in a two-way analysis of variance (ANOVA) using the “aov” and “lm” modules in R version 3.2.1 [22] for each strain.

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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^qI* (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- $\Delta\Delta\text{Ct}$ 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^qI* 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

the Ct value of GAPDH from that of CHS1 and $\Delta\Delta C_t$ values by subtracting ΔC_t values for an undigested intact DNA sample containing the R variant at 100% (intact SoOm1-etoR DNA; calibrator) from the ΔC_t values for corresponding digested DNA samples. Finally, resistant variant frequencies were calculated as $2^{-\Delta\Delta C_t}$. Given a regression line through the origin, a linear regression between the practical R variant frequency and $2^{-\Delta\Delta C_t}$ was analyzed using the “lm” module of R version 3.2.1 [22].

2.4. Validation of the effects of resistant variant frequencies in the etoxazole resistant phenotype

Because of the completely recessive inheritance of etoxazole resistance, we analyzed the relationship between resistant variant frequencies of virgin females and hatchability of their haploid male eggs after etoxazole treatment. Virgin females (34–43 ♀♀ for each population; ~4 days after the last molt) from six field populations and two laboratory strains (Table 1) were introduced to four kidney bean leaf squares (2×2 cm) on water soaked cotton in a Petri dish and kept in a laboratory at 25°C with a 16 h light:8 h dark light cycle. The next day, surviving females (31–42 ♀♀ for each population) were collected, and their DNA was prepared and cleaned using the DNeasy Blood and Tissue kit and NucleoSpin gDNA Clean-up XS, respectively, as described above. Eggs laid on the leaf squares were sprayed with etoxazole at 50 mg l⁻¹, which is a practical concentration (adhesion amount: 1.91 ± 0.16 mg cm⁻²). We used commercial formulations of etoxazole (10% suspension concentrate). Hatchability was determined by observation with a binocular microscope after 5 days.

The DNA samples were used for qPCR after double digestion with *MluC* I and *Taq*[®]I.

Estimators of R variant frequencies were calculated as $2^{-\Delta\Delta C_t}$. We performed digestion and qPCR twice for each population as technical replications, and an averaged $2^{-\Delta\Delta C_t}$ 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 $2^{-\Delta\Delta C_t}$ and egg hatchability was analyzed using the “lm” module of R version 3.2.1 [22].

2.5. Comparison of the RED- $\Delta\Delta C_t$ method with a common toxicological bioassay

Preparation of DNA samples for the RED- $\Delta\Delta C_t$ 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 μ l 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

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

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

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

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

3. Results

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

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

3.2. Evaluation of linearity in the relationship between practical resistance variant frequencies and measures by the RED- $\Delta\Delta C_t$ method

3.2.1. Evaluation of amplification efficiency

Amplification efficiencies (e) for CHS1 were 1.075 and 0.997 for SoOm1-etoR (O6) and Kyoyu-S (K4), respectively, indicating that about 100% amplification was realized with the primer set. Similarly, e for GAPDH was also 1.073 for O6 and 1.019 for K4. The contribution ratios (R^2) of regression lines were high (> 0.99) in all of the cases. Parallelism of the CHS1 and GAPDH regression lines was not significant in both O6 (two-way ANOVA [gene $\times$ DNA concentration]: $df = 1$, $F = 0.001$, $P = 0.262$) and K4 ($df = 1$, $F = 1.767$, $P = 0.232$). Two-way ANOVA was also used to evaluate the effects of genes and DNA concentrations. In O6, no significant differences were detected in genes ($df = 1$, $F = 1.532$, $P = 0.262$), but there were differences in DNA concentrations ($df = 1$, $F = 1303.8$, $P = 3.01 \times 10^{-8}$). Significant differences were observed in both DNA concentrations ($df = 1$, $F = 28274.4$, $P = 2.98 \times 10^{-12}$) and the effects of genes ($df = 1$, $F = 39.04$, $P = 7.79 \times 10^{-4}$) in K4. This may be caused by extremely high linearity; R^2 was 1 for both CHS1 and GAPDH in K4 and the lines were also close to each other in O6, indicating that a combination of these primer sets would work for subsequent qPCR analyses.

3.2.2. qPCR for DNA samples digested by restriction endonucleases

Digestion times for genomic DNA of 15 min and 1 h for each endonuclease, *MluC* I and *Taq*^{HI}, did not ensure the separation of ΔC_t values for practical resistance variant frequencies at 0.01 or lower (Fig. 3). We made sure that ΔC_t 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, $2^{-\Delta\Delta\text{Ct}}$, produced by the RED- $\Delta\Delta\text{Ct}$ method.

There was a strong correlation between practical R variant frequencies in the genomic DNA samples and $2^{-\Delta\Delta\text{Ct}}$ (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- $\Delta\Delta\text{Ct}$ 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

$$y = 1.015x \ (R^2 = 0.982, P < 2.2 \times 10^{-16}),$$

where y represents $2^{-\Delta\Delta\text{Ct}}$ 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- $\Delta\Delta\text{Ct}$ 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- $\Delta\Delta Ct$ 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- $\Delta\Delta Ct$ 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 ($2^{-\Delta\Delta C_t}$) 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 $2^{-\Delta\Delta C_t}$. 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 ΔC_t of the intact sample from the same pooled DNA in the RED- $\Delta\Delta C_t$ method.

The strong linearity in the correlation between practical frequencies of the resistant variant and estimator, $2^{-\Delta\Delta C_t}$, supported a high degree of accuracy. In addition to the general $\Delta\Delta C_t$ 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 ΔC_t of a sample consisting of a 100%

susceptible variant with the ΔC_t in a test of amplification efficiency calculated using the C_t for GAPDH at 1 ng l^{-1} as a reference (Supplementary Fig. 6). The averaged ΔC_t 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 ΔC_t of 9.29 was calculated to be $0.00137 \pm 0.00024 \text{ ng l}^{-1}$ (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 ($2^{-\Delta\Delta C_t}$) demonstrated the reliability of the RED- $\Delta\Delta C_t$ 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 $2^{-\Delta\Delta C_t}$ 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- $\Delta\Delta C_t$ 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

480

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

486

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Figure legends

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

Fig. 2 Amplification efficiency for CHS1 (solid circle) and GAPDH (open circle) for the SoOm1-etoR (O6) (a) and Kyoyu-S (K4) (b) strains. DNA concentration: $\log_{10}(\text{ng } \mu\text{L}^{-1})$. For O6 regression line equations for CHS1 and GAPDH were $y = -3.15x + 19.98$ ($E = 2.075$, $R^2 = 0.997$) and $y = -3.16x + 20.27$ ($E = 2.073$, $R^2 = 0.994$), respectively. For K4 regression line equations for CHS1 and GAPDH were $y = -3.33x + 19.37$ ($E = 1.997$, $R^2 = 1$) and $y = -3.28x + 19.82$ ($E = 2.019$, $R^2 = 1$), respectively. E : amplification efficiency (e) + 1 (if 100% amplification was achieved E would be “2”).

Fig. 3 Effects of restriction enzyme digestion periods on ΔCt values of various practical frequencies of the resistance variant (R variant frequency). Digestion time shows the digestion times for *MluC* I and *Taq^a* I. $\Delta\text{Ct} = (\text{Ct for CHS1}) - (\text{Ct for GAPDH})$.

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

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^{-ΔΔCt} 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^{-ΔΔCt}. 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

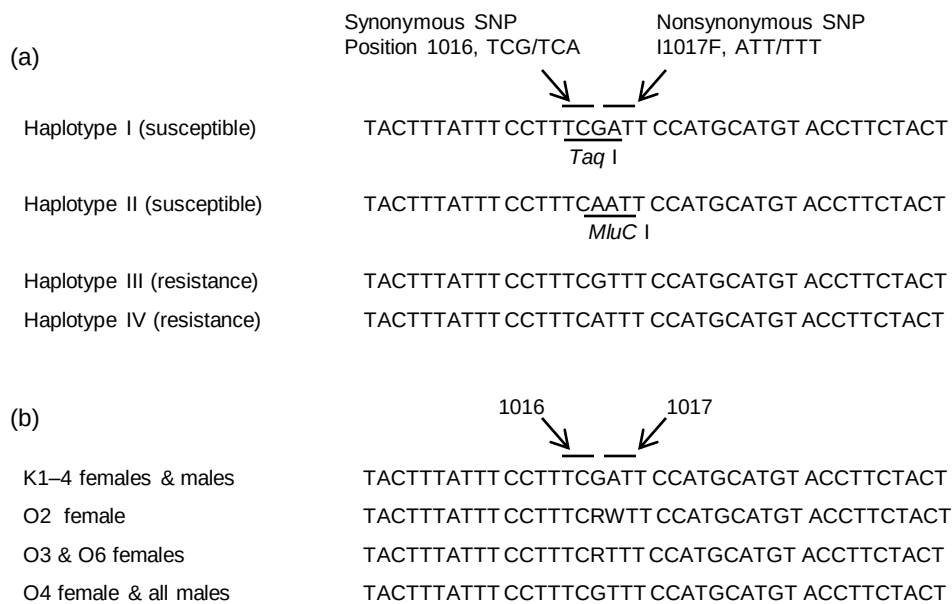


Fig. 1

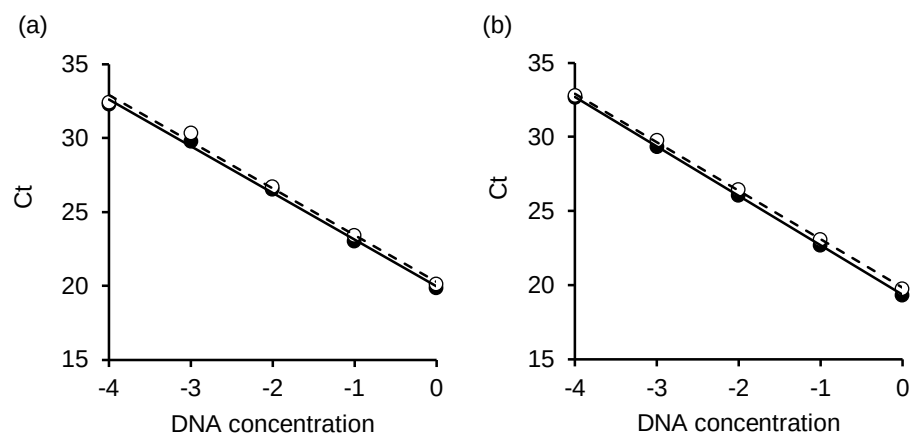


Fig. 2

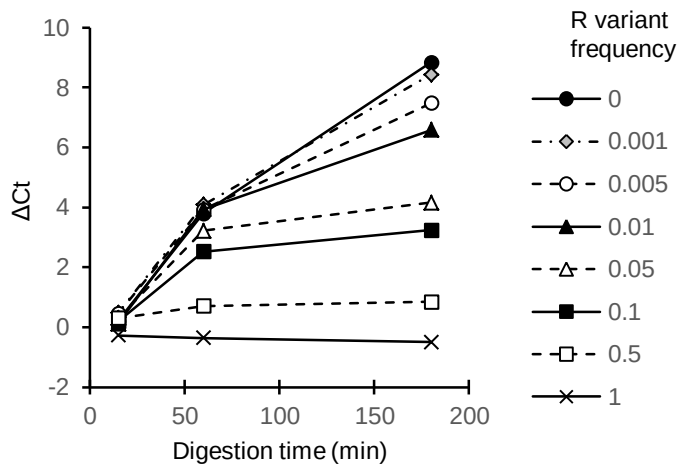


Fig. 3

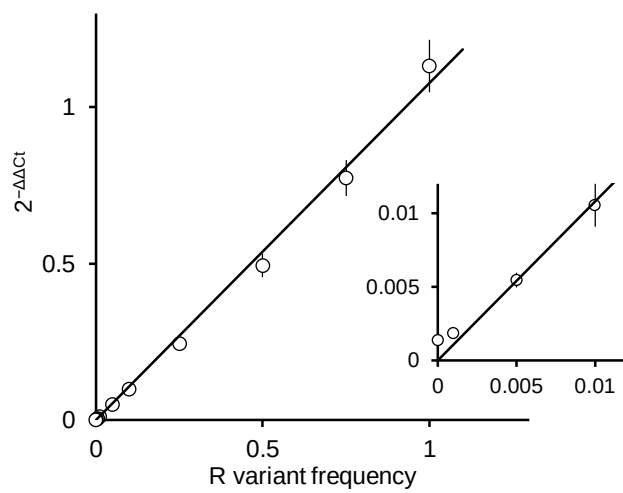


Fig. 4

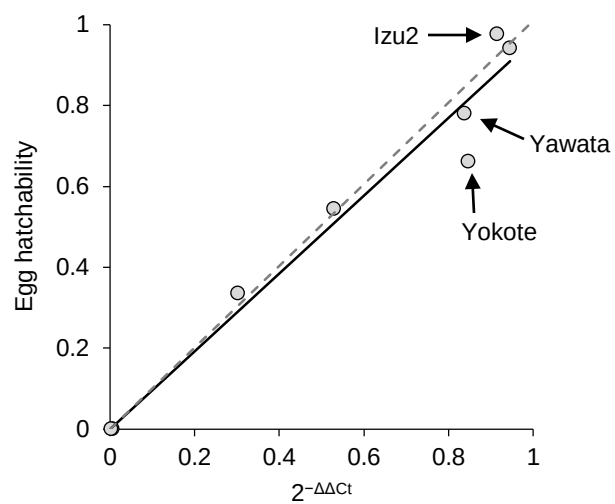


Fig. 5

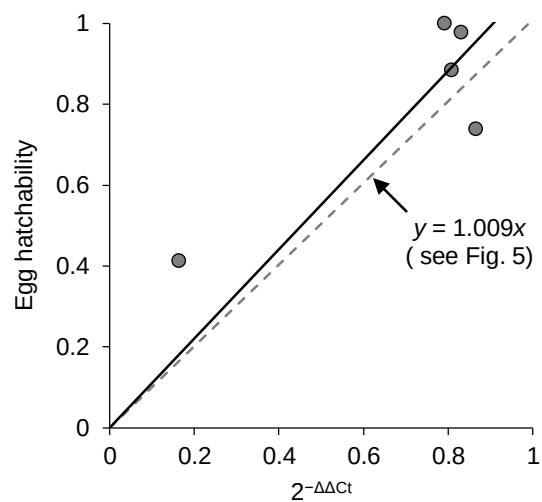


Fig. 6

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)

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tetur03g08510 3241:TATGCACTACTCATGATGGCTGTCTTTGTTGGTACCGCTATTCAAATGGCTGAAGATGGT 3300
K4M      1:-----GATGGT 6
K3M      1:-----GATGGT 6
K2M      1:-----GATGGT 6
K1M      1:-----GATGGT 6
K4F      1:-----GATGGT 6
K3F      1:-----GATGGT 6
K2F      1:-----GATGGT 6
K1F      1:-----GATGGT 6
O6M      1:-----GATGGT 6
O4M      1:-----GATGGT 6
O3M      1:-----GATGGT 6
O2M      1:-----GATGGT 6
O6F      1:-----GATGGT 6
O4F      1:-----GATGGT 6
O3F      1:-----GATGGT 6
O2F      0:----- 0

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tetur03g08510 3301:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 3360
K4M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K3M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K2M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K1M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K4F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K3F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K2F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
K1F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O6M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O4M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O3M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O2M      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O6F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O4F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O3F      7:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 66
O2F      1:-----GGGTCTTTTGTAGTGGCGGCA 21
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tetur03g08510 3361:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 3420
K4M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K3M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K2M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K1M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K4F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K3F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K2F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
K1F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O6M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O4M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O3M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O2M      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O6F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O4F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O3F      67:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 126
O2F      22:CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCTTTCGATT 81
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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

TuCHS1_cyber-R

tetur03g08510 3421:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 3480

K4M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K3M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K2M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K1M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K4F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K3F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K2F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

K1F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O6M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O4M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O3M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O2M 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O6F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O4F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O3F 127:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 186

O2F 82:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 141

tetur03g08510 3481:GGAACACGTGAAGTTCAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 3540

K4M 187:GGAACACGTGAAGTTCAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K3M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K2M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K1M 187:GGAACACGTGAAGTTCAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K4F 187:GGAACACGTGAAGTTCAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K3F 187:GGAACACGTGAAGTTCAGWCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K2F 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

K1F 187:GGAACACGTGAAGTTCAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O6M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O4M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O3M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O2M 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O6F 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O4F 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

O3F 187:GGAACACGTGAAGTTCAGACTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 246

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K4F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

K3F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

K2F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

K1F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

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O4M 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O3M 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O2M 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O6F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O4F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O3F 247:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 306

O2F 202:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAACGCTAAA 261

Supplementary Fig. 1 continue

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tetur03g08510 3721:ATGACTGACAAACTTGGTGCTCTTGAAAAGTATCTTGACCCACTTGGTGGCCCCAGACGT 3780
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Supplementary Fig. 1 continue

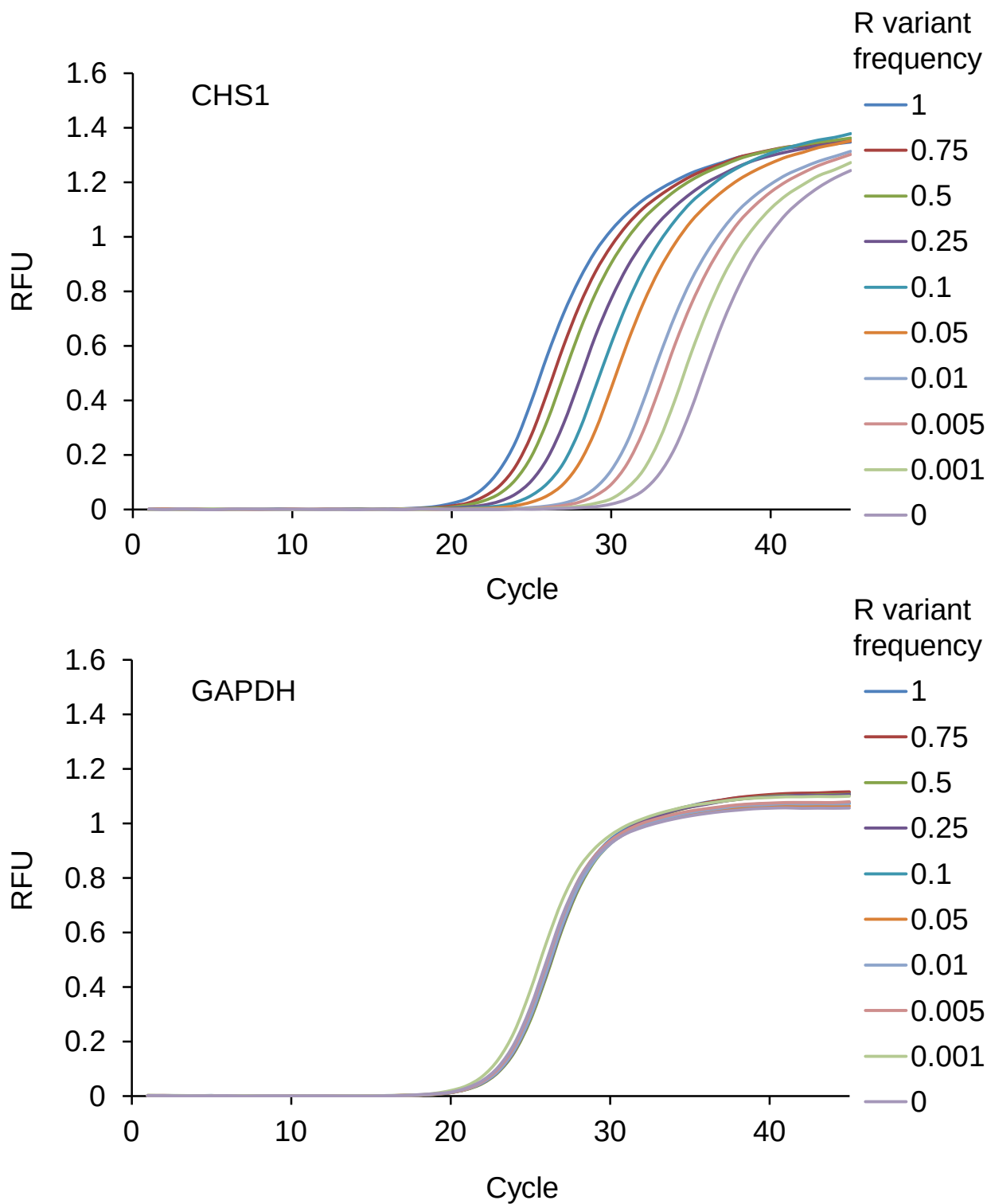
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O3F 487:AAAGGATCTTCRATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 546
O2F 442:AAAGGATCTTCGATTGGACGTAATGCTAGATTTTCAGATAATTTATCTACTGTAACAGAA 501

tetur03g08510 3841:AATGAAGAACATGAAGACATGGATTCAATTGGCTCAGAATCACAACTGATGATATGTCA 3900
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K3M 547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAACTGATGATATGTCA 606
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O3F 547:AATGAAGAACATGAAGACATGGATTCACTTGGCTCAGGATCACAACTGATGATATGTCA 606
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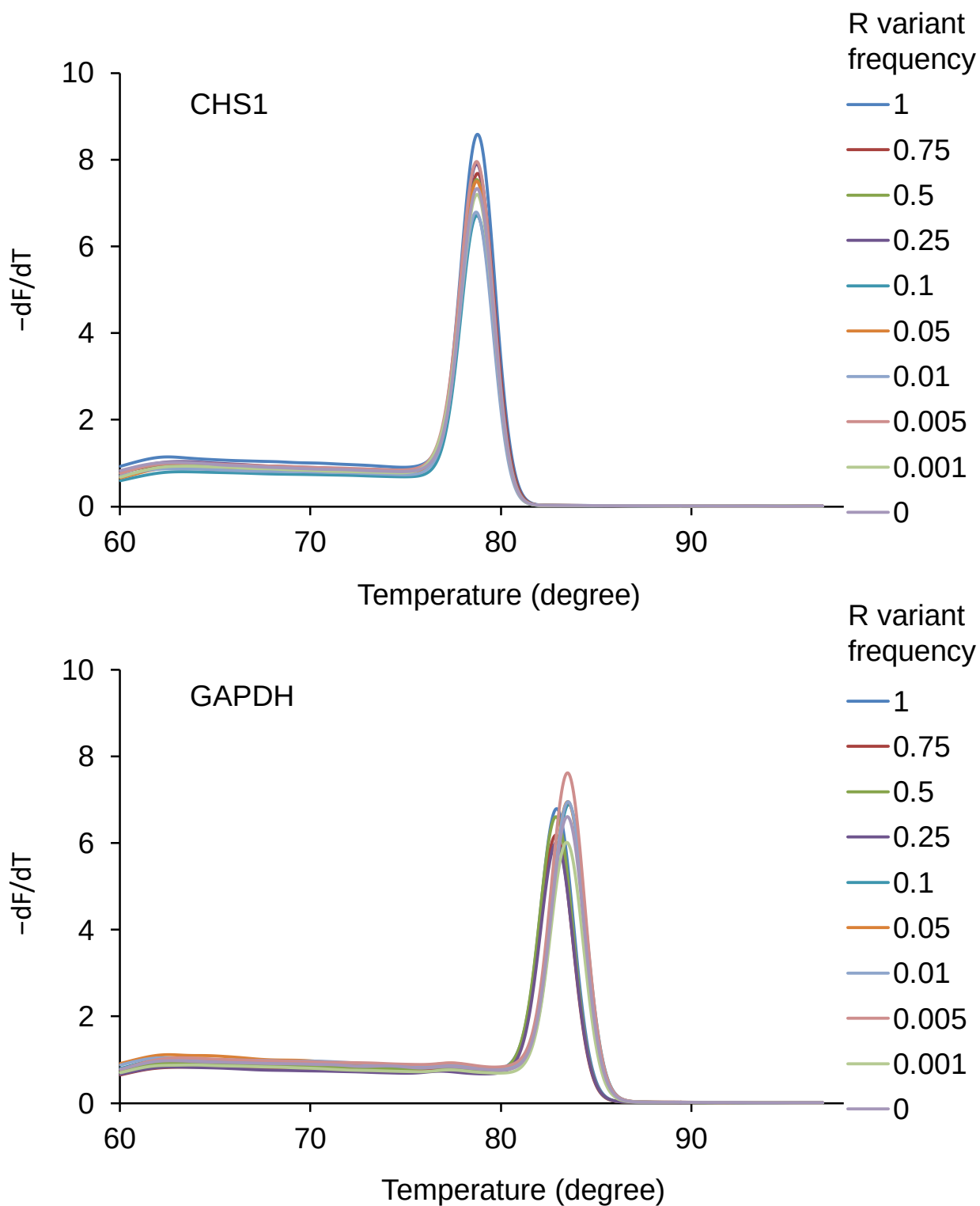
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K3M 607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
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K1F 607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
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O6F 607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O4F 607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O3F 607:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 666
O2F 562:ATTAAGGATAATGAAGAAGTTGCTCCACGATTTGATGAAGACCATCCATACTGGATTGAA 621

Supplementary Fig. 1 continue

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K3M      667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K2M      667:GATAAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K1M      667:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
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K3F      667:GATRAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K2F      667:GATRAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
K1F      667:GATGAAGATTTGAGAGATGGTGAAATCAAGCAGCTTGCAGAAAATGAAATCGCATTTT-- 724
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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*^qI, 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*^qI, for 3 h. dF/dT : derivative of melting curve.

tetur03g08510 3301:GTTACTTCACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGC **GGCA** 3360
 Uda-F1 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F2 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F3 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F4 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F5 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F6 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F7 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F8 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F9 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53
 Uda-F10 1:-----CACCGTCTGCCGTATTTTTCATAGCTTTATCTGGGTCTTTTGTAGTGGCGGCA 53

1016 I1017F


TuCHS1 cyber-F

tetur03g08510 3361: **CTGCTTCATCCACAAG**AGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTCG**ATT** 3420
 Uda-F1 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F2 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F3 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F4 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F5 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F6 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F7 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F8 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F9 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113
 Uda-F10 54: CTGCTTCATCCACAAGAGTTTCACTGTTTATATCCATGTTTACTTTATTTCCCTTTC**ATT** 113

TuCHS1 cyber-R

tetur03g08510 3421:CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTT **GAACGTTGTTACTTGG** 3480
 Uda-F1 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F2 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F3 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F4 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F5 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F6 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
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 Uda-F8 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F9 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173
 Uda-F10 114: CCATGCATGTACCTTCTACTTATGATCTATTCTCTGGTCAACTTGAACGTTGTTACTTGG 173

tetur03g08510 3481: **GGAACAC**GTGAAGTTCAAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 3540
 Uda-F1 174: GGAACACGTGAAGTTCAAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 233
 Uda-F2 174: GGAACACGTGAAGTTCAAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 233
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 Uda-F10 174: GGAACACGTGAAGTTCAAGTCTAAAAAGACCAAGGCTGAGCTGGAAGAGGAAAAGAAAGCT 233

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- $\Delta\Delta$ Ct method.

Supplementary Fig. 4 continue

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 Uda-F9 234:GTTGAAGAAATCAAAAAGGGCAACTTATTGAGTTTCTTGAATCTTAATCCAAAMGCTAAA 293
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tetur03g08510 3601:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACCTTGTTTCAGGTGCTCTTTCTGTACA 3660
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 Uda-F8 294:GAAGAAGAAGGATCTATTGAATTTAGTCTGGCAAACCTTGTTTCAGGTGCTCTTTCTGTACA 353
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 Uda-F2 354:TATCCCAAACCAAACGACGAAAAGATTTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
 Uda-F3 354:TATCCCAAACCAAACGACGAAAAGATTTCATCTACTTAAAATTGAACAACATCTTAGTGAA 413
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Supplementary Fig. 4 continue

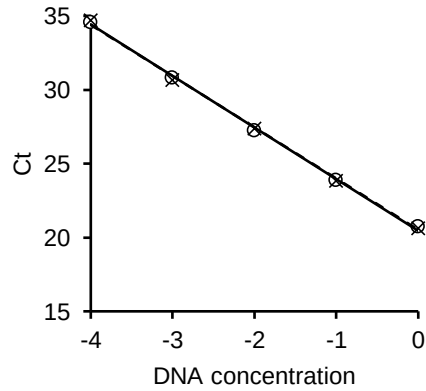
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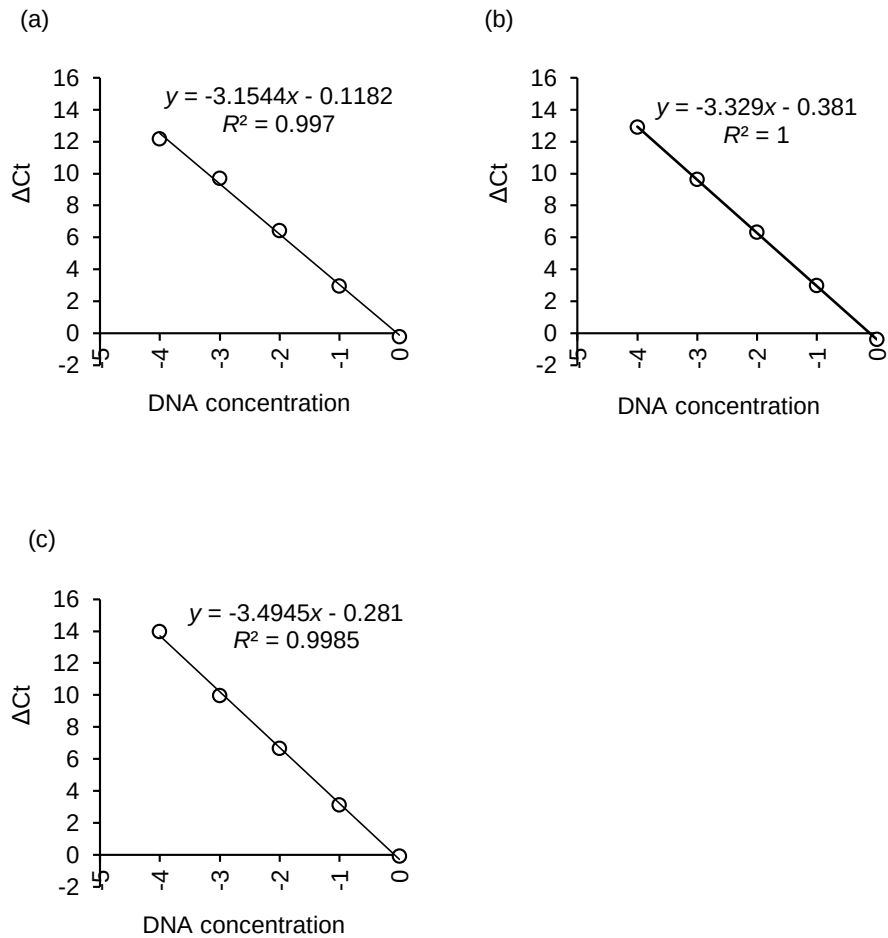
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Supplementary Fig. 5 Amplify efficiency for CHS1 (cross) and GAPDH (open circle) in Uda population. DNA concentration: $\log_{10}(\text{ng } \mu\text{l}^{-1})$. Equations of regression lines for CHS1 and GAPDH were $y = -3.49x + 20.46$ ($E = 1.933$, $R^2 = 0.985$) and $y = -3.46x + 20.54$ ($E = 1.945$, $R^2 = 0.997$). E : amplification efficiency (e) + 1 (if 100% amplification was achieved E would be “2”).



Supplementary Fig. 6 Correlation between DNA concentration ($\text{ng } \mu\text{l}^{-1}$) 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 $1 \text{ ng } \mu\text{l}^{-1}$ 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 ^{-ΔΔCt}) ^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