Identification and characterization of new mutations in mitochondrial cytochrome b that confer resistance to bifenazate and acequinocyl in the spider mite *Tetranychus urticae*

Short running title: Novel target-site mutations cause Q90I resistance in *Tetranychus urticae*

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Abstract

BACKGROUND: In spider mites, mutations in the mitochondrial cytochrome b Q$_0$ pocket have been reported to confer resistance to the Q$_0$ inhibitors bifenazate and acequinocyl. In this study we surveyed populations of the two-spotted spider mite Tetranychus urticae for mutations in cytochrome b, linked newly discovered mutations with resistance and assessed potential pleiotropic fitness costs.

RESULTS: We identified two novel mutations in the Q$_0$ site: G132A (equivalent to G143A in fungi resistant to strobilurins) and G126S + A133T (previously reported in Panonychus citri to cause bifenazate and acequinocyl resistance). Two T. urticae strains carrying G132A were highly resistant to bifenazate but not acequinocyl, while a strain with G126S + A133T displayed high levels of acequinocyl resistance, but only moderate levels of bifenazate resistance. Bifenazate and acequinocyl resistance inherited maternally, providing strong evidence for the involvement of these mutations in the resistance phenotype. Near isogenic lines carrying G132A revealed several fitness penalties in T. urticae: a lower net reproductive rate (R0), the intrinsic rate of increase (rm), and the finite rate of increase (LM), a higher doubling time (DT), and a more male biased sex ratio.

CONCLUSIONS: Several lines of evidence were provided to support the causal role of newly discovered cytochrome b mutations in bifenazate and acequinocyl resistance. Due to the fitness costs associated with the G132A mutation, resistant T. urticae populations might be less competitive in a bifenazate free environment, offering opportunities for resistance management.

Keywords: spider mites; complex III inhibitor; cytochrome b; mutation; cross-resistance; fitness cost
1. INTRODUCTION

The spider mite *Tetranychus urticae* Koch (Arthropoda: Acari: Tetranychidae) is an important cosmopolitan pest damaging many agricultural crops. Frequent acaricide applications are needed to control this species which inevitably led to the development of resistance. This species is considered as one of the most pesticide resistant arthropods based on the number of active ingredients to which resistance has been reported.\(^1\), \(^2\) Pesticide resistance evolves via two main mechanisms: (1) toxicodynamic changes, such as the reduction in the sensitivity or availability of the target-site due to point mutation(s), gene knock-out or amplification, (2) toxicokinetic changes that reduce the amount of pesticides that reaches the target-site through changes in exposure, penetration, transportation, metabolism and excretion.\(^3\), \(^4\) Resistance mechanisms are often costly, for example point mutations in essential target genes can convey pleiotropic effects and affect other phenotypic traits in addition to pesticide resistance.\(^5\)-\(^7\) Reproduction, dispersal, generation time, and longevity have been reported to be negatively affected by target-site resistance mutations.\(^8\)-\(^12\) Also, for the spider mite *T. urticae*, fitness costs have been reported after marker assisted back-crossing, but not for all resistance mutations.\(^11\)

Although environmentally friendly methods such as biological control increase in importance, especially in greenhouse crops,\(^13\), \(^14\) spider mites as *T. urticae* are still mainly controlled by acaricide applications.\(^15\) The hydrazine carbazate acaricide bifenazate is one of the most recently developed and frequently used acaricides with excellent selectivity to all life stages of *Tetranychus* spp. and *Panonychus* spp.,\(^16\), \(^17\) Bifenazate was first classified as a neurotoxin,\(^18\) but later studies revealed a mitochondrial mode of action via inhibition of electron transport.\(^12\), \(^19\) Bifenazate resistance was shown to inherit maternally and high levels of resistance were tightly linked with

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mutation(s) at highly conserved regions (the cd1-helix and ef-helix) of the cytochrome b Q$_0$ site of the mitochondrial complex III (bc1 complex, ubiquinone: cytochrome c oxidoreductase enzyme complex).

The mitochondrial complex III is an essential enzyme complex in the electron transport chain and plays a critical role in the biochemical generation of adenosine triphosphate (ATP) via oxidative phosphorylation. The catalytic core of this enzyme complex is composed of three subunits in eukaryotes which are cytochrome b, Rieske iron–sulphur protein (ISP) and cytochrome c1 proteins. Cytochrome b is encoded by the mitochondrial genome while the other subunits are encoded by the nuclear genome. Electrons are transported from low-potential ubiquinol to a higher potential cytochrome c via the Q-cycle pathway.$^{20,21}$ This pathway requires two separate quinone-binding sites: the quinol oxidation site (Q$_0$ site) and the quinone reduction site (QI site). These two sites are located on opposite sides of the membrane and linked by a transmembrane electron-transfer pathway. Pesticides that inhibit the normal functioning of Q$_0$ sites have been developed from different chemical classes including, in addition to the carbazate bifenzate, the 2-hydroxynaphthoquinones (HONQs) and the b-methoxyacrylates (MOAs) with the strobilurins as a commercially successful family of potent fungicides.$^{22-24}$ Acequinocyl is the only commercialized acaricide of the naphthoquinone analogue group$^{25}$ and is commonly used against all stages of T. urticae and other spider mite species.$^{18}$ Cross-resistance between bifenzate and acequinocyl associated with cytochrome b mutations has been reported from T. urticae and Panonychus citri populations.$^{19,24}$ The strobilurin fungicides were originally isolated from the mycelium of the basidiomycete Strobilurus tenacellus strain No. 21602$^{26}$ and are currently considered as one of the most important classes of agricultural fungicides.$^{27,28}$ The first field
resistance to strobilurin fungicides was reported in wheat powdery mildew populations in northern Germany in 1998. Later studies revealed that resistance to this group of fungicide in plant pathogenic fungi is most often due to point mutation(s) in the Qo region of mitochondrial cytochrome b. 

In this study we discovered a G132A mutation in cytochrome b of T. urticae, equivalent to G143A in fungi, which has been reported as the most frequent mutation associated with strobilurin resistance. During a survey investigating the frequency of G132A in T. urticae field strains, we also uncovered for the first time the combination of G126S + A133T in T. urticae, previously reported in the spider mite P. citri. We provide strong evidence of the causal role of these resistance mutations by revealing maternal inheritance and determined the strength of the phenotype by introgression of the mitochondrial haplotype in a susceptible genomic background. Last, we used the generated isogenic lines to assess potential fitness costs associated with G132A in T. urticae.

2. Materials and Methods

2.1 Chemicals and mite strains

Commercial formulations of the mitochondrial complex III electron transport inhibitors bifenazate (Floramite® 240 g litre$^{-1}$ SC) and acequinocyl (Cantack® 150 g litre$^{-1}$ SC) were purchased from Intergrow (Aalter, Belgium). All chemicals were analytical grade and purchased from Sigma-Aldrich, unless stated otherwise. The JP-R strain and the laboratory susceptible Wasatch strain were previously described. In addition, twenty-three field strains were collected from different geographical areas across Europe between 2016 and 2019 for resistance mutation screening (Table
1) All mites were reared on kidney bean plants *Phaseolus vulgaris* L. cv. ‘Speedy’ or ‘Prelude’ at 25 ± 1°C, 60% RH, and 16/8 h (L/D) photoperiod.

### 2.2 Survey of cytochrome b variants

DNA extraction and PCR amplification of cytochrome b was performed as described by Van Leeuwen *et al.* Briefly, approximately 200 adult females were collected and homogenized in 800 μL SDS buffer (200 mM Tris-HCl, 400 mM NaCl, 10 mM EDTA, 2% SDS at pH = 8.3) followed by phenol-chloroform extraction. For single mite DNA extraction, a single adult female was homogenized by hand in 20 μl mixture of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and proteinase K (10 mg/ml, 2 ml) in a 1.5 ml Eppendorf tube. Then, the mixture was incubated at 37°C for 30 min followed by 95°C for 5 minutes. PCR was performed using the Expand Long Range PCR kit (Roche) and the primers Cytbdia2F (5’-TTAAGAACTCCTAAAAACTTTTCGTTC) and Cytbdia2R (5’-GAAACAAAAATTATTATTCC-CAAC). PCR products were purified with a Cycle-Pure Kit (E.Z.N.A.) and sequenced with the original PCR primers and four internal sequencing primers (cytbWTF, 5’-CGGAATAATTTTACAATAACTCATGC; cytbWTR, 5’-TGGTACAGATCGTAGAATTGCG; PEWYF1, 5’-AAAGGCTCATCTAACCAATAGG; PEWYR2, 5’-AATGAAATTTCTGTAAAAARGG-TATTC). Sequence data were analyzed with BioEdit software. Sequences have been submitted to the NCBI repository (Table 1).

### 2.3 Generation of isofemale and introgressed lines

Isofemale lines were established from the FS1 and FS8 strains and were labeled as iso-FS1 and iso-FS8, respectively. Approximately 500 mated female mites were transferred to detached bean leaves on wet cotton wool in petri-dishes and were sprayed with 200 mg/L bifenazate. Five Petri-
dishes were prepared per strain. After 72 h, ten alive females were selected randomly from the sprayed arenas and transferred to 9 cm² square bean leaf discs individually. Mites were allowed to lay eggs for 3-4 days. DNA of each single female was extracted as described above. Progeny of a single female with the G132A (iso-FS1) and G126S + A133T (iso-FS8) mutations was used to create the isofemale lines.Introgressed lines were established using the back-crossing methods described by Bajda et al. 2017.40,41 Briefly, JP-R and iso-FS8 virgin females were crossed with susceptible Wasatch males. A virgin F1 female was back-crossed to Wasatch males, and the back-crossing was repeated seven times. After back-crossing, mites were transferred to full bean plants and were allowed to expand their population size for toxicity and fitness costs experiments. Introgressed lines that carry G132A and G126S + A133T are labeled as JP-R-BC (1-3) and iso-FS8-BC (1-3), respectively.

2.4 Toxicity bioassays

To determine bifenazate and acequinocyl toxicity, dose-response bioassays were conducted with female adult mites as described by Van Leeuwen et al.42 Briefly, we tested a minimum of five concentrations in four replicates. For each replicate 20-35 adult females were transferred to 9 cm² bean leaf discs on wet cotton wool. Arenas were sprayed with 1 ml of acaricide solution or deionized water (as control) at 1 bar pressure in a Potter spray tower resulting in 2 mg aqueous deposit per cm². Mortality was recorded after 24 h. The LC₅₀-values and their 95% confidence limits were calculated from probit regressions using the POLO-Plus software (LeOra Software, 2006).

2.5 Reciprocal crosses
To elucidate the mode of inheritance of bifenazate and acequinocyl resistance, reciprocal crosses were set up between the JP-R (G132A), iso-FS1(G132A) and iso-FS8 (G126S + A133T) resistant lines and the susceptible Wasatch strain. To create hybrid F1 females, approximately 80 teleiochrysalid females and 100 adult males were placed on detached bean leaves on wet cotton wool and were allowed to mate. After two days, females were collected and transferred daily to a fresh 9 cm² square bean leaf disc and allowed to lay eggs. F1 adult females were used for toxicity bioassays. The degree of dominance (D) was calculated with the Stone (1968) formula: D = (2X₂ – X₁ – X₃) / (X₁ – X₃), where X₁ = log₁₀ LC₅₀ of the resistant strain, X₃ = log₁₀ LC₅₀ of the susceptible strain and X₂ = log₁₀ LC₅₀ of the F1 females obtained from the reciprocal cross.

2.6 Fitness cost of G132A

To explore potential fitness costs associated with the G132A mutation, demographic experiments were conducted with the three independent JP-R back-crossed lines in comparison with the parental Wasatch line as control.

Developmental time, immature stage survivorship (ISS), and sex ratio

From each introgressed line and the Wasatch control, 100 females were randomly collected from stock cultures and transferred to a detached bean leaf on wet cotton in three replicates. Females were allowed to lay eggs for 4-5 h and the numbers of eggs were recorded. After eight days, the development of the offspring was followed every 12h, and the eclosion time and sex of the adults were recorded.

Oviposition and adult longevity

From the three introgressed lines and the Wasatch control, 40 female teleiochrysalids were placed individually with an adult male on a 2 cm² leaf disk (in total 4 × 40 = 160 leaf disks each with a
mite couple). Every 12 h, all disk arenas were checked for female oviposition and death. Every 24 h each mite couple was transferred to a fresh leaf disk until the female died. Pre-oviposition, oviposition and post-oviposition periods were determined as the time spanning between adult female emergence and the first egg, the time between the first and last day of oviposition, and the day when no eggs were deposited until her death, respectively.

2.7 Statistical analysis

Statistical analysis was conducted within the R framework [R Core Team (2014), version 3.1.2] for all data. Normality of variances was tested using a Shapiro-Wilk test. A generalized linear model with a negative binomial error distribution was used to analyse the data of female longevity, pre-oviposition period, oviposition period, post-oviposition period and the number of eggs. Sex ratio data was analysed using a generalized linear model with a binomial error distribution. A general linear model was used to analyse ISS data that were normally distributed. Differences between the introgressed lines were determined using the Tukey’s HSD test at 95% confidence level. Life table analysis was performed based on the lifetable R script. The intrinsic rate of increase (rm) was calculated with the equation

\[ L(x) = \frac{\sum \Omega_g}{x_0} e^{-r mx x} = 1 \]

where \( L(x) \) is the proportion of females surviving to age \( x \) and \( m_x \) is the mean number of female progeny per adult female at age \( x \). The net reproductive rate or mean number of daughters produced per female was calculated from \( R_0 = \sum \Omega_g \ L(x) m_x \) and the mean generation time from \( T = \frac{\ln(R_0)}{rm} \). The finite rate of increase and doubling time were inferred from the equations \( L = \frac{\ln(R_0)}{rm} \) and \( DT = \frac{\ln2}{rm} \), respectively. Variance for the life table (LT) parameters was estimated with Jackknife resampling method. As the Jackknife method is an asymptotic procedure that is sensitive to a highly skewed distribution,
the symmetry of our dataset was measured with the function skewness from package moments prior to the final analysis. Subsequently, mean Jackknife values and their standard errors (SE) were calculated for the five LT parameters. Mean jackknife values for lines carrying mutations were then compared to Wasatch using Dunnett’s test (adjusted p-value <0.05).

3. Results

3.1 Cytochrome b genotypes of JP-R and the field strains

During a cross-resistant screen of the Japanese JP-R strain selected for cyenopyrafen resistance, we found strong bifenazate resistance, and therefore sequenced the complete cytochrome b gene. Aligning the cytochrome b sequences of JP-R against that of the susceptible strains Wasatch and GSS revealed a novel amino acid substitution (G132A) (Table 1 and Figure 1). To explore the spread of this mutation in Europe, several field-collected strains were screened (Table 1). We found four mutations in the conserved cd1 and ef-helix of the Qo pocket of cytochrome b of mitochondrial complex III (G126S, G132A, A133T and P262T). The novel G132A uncovered in JP-R was also identified in FS1, a strain from the Netherlands. In addition, a novel mutation combination (G126S + A133T) was identified in strain FS8 from the UK. This combination of mutations has already been reported from P. citri, but was so far never encountered in T. urticae (Table 1 and Figure 1). Last, the well characterized P262T was found in a population from strawberry in the UK. Additional substitutions were also found in non-conserved regions. The G126S mutation was found by itself in five strains collected from the Netherlands and the UK (Table 1 and Figure 1), but whether the mutation alone confers resistance remains to be investigated.
3.2 Resistance to bifenaizate and acequinocyl

Results of all toxicity tests are listed in Table 2. The JP-R and FS1 strain that carry the G132A mutation were resistant to bifenaizate ($LC_{50} > 150$ mg/L), but not to acequinocyl. In contrast, the FS8 strain with the G126S + A133T haplotype showed high levels of acequinocyl resistance ($LC_{50} > 600$ mg/L), and only very moderate resistance to bifenaizate (Table 2). The levels of resistance between parental and introgressed lines were comparable across all independent replicates for G132A (Table 2), strongly suggesting that the cytochrome b mutation alone completely determines the resistance phenotype. For the G126S + A133T haplotype, resistance ratios for acequinocyl were two-fold lower after introgression, but $LC_{50}$ values were still very high (Table 2). This suggests a strong effect of the combination of these mutations in acequinocyl resistance, but also implies that additional factors might be involved in the very high resistance of the non-introgressed strain iso-FS8.

3.3 Mode of inheritance of bifenaizate and acequinocyl resistance

Reciprocal crosses revealed a complete maternal inheritance of bifenaizate resistance in the G132A lines (Table 3, Figure 2), linking the mutation to the phenotype. The limited bifenaizate resistance observed in iso-FS8 with the G126S + A133T haplotype also inherited completely maternal. There was a very strong maternal effect in the inheritance pattern of acequinocyl resistance in the reciprocal cross of iso-FS8 × Wasatch. In contrast, the very low resistance to acequinocyl in G132A lines did not inherit maternally (Table 3, Figure 2), indicating that G132A does not confer acequinocyl resistance. The $LC_{50}$ values and dominance levels for all reciprocal crosses are specified in Table 3.

3.4 Fitness costs
Adult males and females of Wasatch emerged earlier than the introgressed lines JP-R-BC (1-3) (female: df = 3, F= 11.12, \( P < 0.001 \) and male: df= 3, F= 7.29, \( P < 0.001 \)) (Supplemental Figure 1 and Figure 3). Significant differences were observed between the three introgressed resistant lines JP-R-BC and the bifenazate susceptible strain Wasatch in terms of ISS (\( F = 4.13; \) df = 3, \( P = 0.015 \)), sex-ratio (\( \chi^2 = 9.30; \) df = 3; \( P = 0.023 \)), longevity (\( \chi^2 = 17.76; \) df = 3; \( P < 0.001 \)), oviposition period (\( \chi^2 = 17.62; \) df = 3; \( P < 0.001 \)), total number of eggs laid per female (\( \chi^2 = 12.61; \) df = 3; \( P = 0.005 \)), and post-oviposition (\( \chi^2 = 7.97; \) df = 3; \( P = 0.46 \)), but not pre-oviposition period (\( \chi^2 = 0.12; \) df = 3; \( P = 0.989 \)) (Figure 3).

**Fertility life table parameters**

All LT parameters, net reproductive rate (\( R_0 \)), the intrinsic rate of increase (\( r_m \)), the finite rate of increase (\( L_M \)), mean generation time (\( T \)) and the doubling time (\( DT \)) of the three introgressed lines carrying resistance mutations JP-R-BC (1-3) and Wasatch, are summarized in Table 4. All three introgressed lines of JP-R showed significantly smaller values for \( R_0 \), \( r_m \) and \( L_M \) and significantly longer DT compared with their congenic line, Wasatch (Table 4 and Figure 4). No significant difference was found in \( T \).

**4. Discussion**

Because of its excellent efficacy and safety toward biological control agents such as predatory mites,\textsuperscript{15,16,50} bifenazate has been frequently used worldwide. Soon after its introduction in the EU, resistance was reported in *T. urticae* populations from greenhouse roses in the Netherlands.\textsuperscript{12} Surprisingly, bifenazate resistance inherits maternally and investigation of resistance mechanisms lead to the discovery of a mitochondrial mode of action,\textsuperscript{12} instead of the earlier proposed interaction with GABA gated chloride channels.\textsuperscript{51,52} Mitochondrial genome sequencing revealed...
mutations at conserved sites in the mitochondrial cytochrome b gene, suggesting that bifenazate acts as a Qo inhibitor.\textsuperscript{12, 19, 53} In spider mites, reciprocal genetic crosses between populations can be easily conducted, and should thus be the standard in validating the role of specific mutations in cytochrome b in QoI resistance. As cytochrome b is encoded by the mitochondrial genome, maternal inheritance is uniquely associated with these resistance conferring mutations. In addition, for a number of cytochrome b mutations, repeated back-crossing to a susceptible line has confirmed the very potent resistant phenotype in bifenazate resistance.\textsuperscript{40} Over the years, a number of mutations conferring bifenazate and acequinocyl resistance have been validated by revealing a maternal inheritance, both in \textit{T. urticae} as \textit{P. citri} populations (Figure 1). Although a number of other mutations has been reported, formal evidence of their contribution to bifenazate resistance is still lacking.\textsuperscript{54} The same is true for G126S, which was initially reported in combination with other cd1 helix mutations, but the mutation alone has never been validated to confer a resistant phenotype, despite a recent report.\textsuperscript{54} This is in contrast with mutations in (or close to) the ef helix, where P262T and I256V alone confer bifenazate and acequinocyl resistance respectively (Figure 1).\textsuperscript{12, 55}

In this study, we report for the first time a single mutation in the cd1-helix, G132A, that confers resistance to bifenazate. The mutation was uncovered after a cross-resistance screen of JP-R,\textsuperscript{37} a strain of Japanese origin, and was subsequently also detected in a Dutch field strain, FS1. Both lines harboring the G132A mutation, as well as back-crossed lines, displayed similar LC\textsubscript{50}'s and RR and resistance inherited perfectly maternal. This strongly validates the role of the G132A mutation in bifenazate resistance. However, the mutation did not confer acequinocyl cross-resistance. Bifenazate resistance levels of 30-fold with LC\textsubscript{50} values of 150-200 mg/L are very
significant in the light of field rate (e.g. Floramite at 96 mg active ingredient/L in EU) and could
cause field failure, but nevertheless are much lower than those previously reported in the cd1 helix
(LC50s > 10,000).\textsuperscript{12, 19} This suggests that a combination of mutations is needed to attain these very
high resistance levels. Interestingly, this mutation is the main resistance factor in pathogenic fungi
resistant to strobilurins, which are classified as MOAs and QoI inhibitor fungicides,\textsuperscript{22, 23, 56, 57}
providing a strong example of convergent evolution across kingdoms. Screening of field-collected
European $T. urticae$ populations also led to the discovery of another novel combination of
mutations: G126S + A133T. This Qo pocket haplotype is associated with high levels of
acequinocyl and bifenazate resistance in $P. citri$.\textsuperscript{24} In our study, the combination of G126S and
A133T in $T. urticae$ conferred only moderate levels of resistance to bifenazate but high resistance
to acequinocyl. It is surprising that this combination of substitutions confers such different levels
of bifenazate resistance in $P. citri$ and $T. urticae$, especially because the resistant phenotype
inherited maternally in both species, and additional (nuclear) factors in resistance can thus be ruled
out. For G132A, it is clear that bifenazate must be the most relevant selective force in $T. urticae$
field populations, as it does not confer acequinocyl resistance. The opposite is likely true for
G126S + A133T, as the effect seems to be much more pronounced on acequinocyl toxicity, and
hence it is tempting to speculate that frequent acequinocyl use lies at the basis of resistance
development.

After repeated back-crossing to the susceptible Wasatch strain, we obtained congenic lines
harboring the mitochondrial haplotype of JP-R (G132A) and the nuclear background of Wasatch.
As this uncouples the mitochondrial resistance mutations from confounding genomic factors, it is
not only a validation of the phenotypic strength, but also a powerful approach to assess fitness
costs. Our analyses of the G132A congenic lines revealed a lower $R_0$, rm, LM, and a higher DT compared with Wasatch. It therefore seems that in an acaricide-free environment the resistant genotypes might be less competitive and will grow slower than susceptible genotypes. In addition, we found that the resistant genotype is more male biased, which could further reduce the frequency of the transmission of G132A. Our findings could be important for the management of G132A conferred resistance in the field. It appears that the management of G132A resistance might be easier than that of the mutations without fitness costs, such as G126S + S141F and P262T.11

There are several reports on the fitness of resistant fungal species that carry the G143A (G132A in spider mites). Some species, such as *Plasmopara viticola*58, 59, and *Magnaporthe oryzae*60 show lower fitness. For example, conidia production of the field G143A azoxystrobin-resistant mutant of *M. oryzae* is lower than that of the susceptible wild-types.60 Other studies failed to find fitness costs in resistant species such as *Blumeria graminis*61, *Alternaria alternata*62, *Botrytis cinerea*63, and *Colletotrichum acutatum*64. These fitness studies, however, did not provide direct evidence for the association of fitness consequences with the G143A mutation. To evaluate the role of G143A in fungicide resistance and its impact on the fitness of fungi, the mutation was introduced into the cytochrome b of the yeast species *Saccharomyces cerevisiae* as a model system65. While confirming the involvement of the mutation in resistance, they showed that the mutation has a slightly deleterious effect on the bc1 function of the site mimic of some pathogenic fungi species but not all. The authors therefore argued that a small variation in the Qo site can affect the impact of the G143A mutation on bc1 activity, and can differentially affect the fitness between species. In this light, it is not surprising that different spider mite mutations can confer different levels of fitness penalties.

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5. Conclusion

In conclusion, new cytochrome b mutations were uncovered and several lines of evidence support the causal role of these mutations in bifenazate or acequinocyl resistance. Patterns of maternal inheritance and introgression experiments identified G132A as tightly linked with high levels of bifenazate resistance. In *T. urticae*, G126S + A133T conferred very high acequinocyl resistance, with only limited levels of bifenazate cross-resistance. Investigation into the fitness costs revealed that strains harboring G132A might be more easily managed.

6. Acknowledgments

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7. References


For Peer Review

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Table 1. The cytochrome b Q\(_0\) genotypes of the surveyed T. urticae strains.

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<td>FS14</td>
<td>Hop</td>
<td>Germany</td>
<td>-</td>
<td>MN029038</td>
</tr>
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<td>FS15</td>
<td>Hop</td>
<td>Germany</td>
<td>-</td>
<td>MN029039</td>
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<td>FS16</td>
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<td>Germany</td>
<td>-</td>
<td>MN029040</td>
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<tr>
<td>FS17</td>
<td>Carnation</td>
<td>Italy</td>
<td>-</td>
<td>MN276070</td>
</tr>
<tr>
<td>FS18</td>
<td>Rose</td>
<td>Italy</td>
<td>-</td>
<td>MN276071</td>
</tr>
<tr>
<td>FS19</td>
<td>Citrus</td>
<td>Italy</td>
<td>-</td>
<td>MN276072</td>
</tr>
<tr>
<td>FS20</td>
<td>Strawberry</td>
<td>Spain</td>
<td>-</td>
<td>MN029045</td>
</tr>
<tr>
<td>FS21</td>
<td>Cucumber</td>
<td>Spain</td>
<td>-</td>
<td>MN029046</td>
</tr>
<tr>
<td>FS22</td>
<td>Rose</td>
<td>Romania</td>
<td>-</td>
<td>MN029047</td>
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</table>

All strains were field collected in Europe, except JP-R and Wasatch, which were laboratory strains. The substitutions in the conserved regions of the cytochrome b Q\(_0\) pocket (the cd1-helix and ef-helix) are described using the GSS genotype as reference (EU556751.1). *: Selected by cyenopyrafen, a complex II inhibitor, under laboratory conditions.
Table 2. Bifenazate and acequinocyl resistance in *T. urticae* strains with novel Q<sub>0</sub> mutations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Q&lt;sub&gt;0&lt;/sub&gt; genotype</th>
<th>Bifenazate</th>
<th></th>
<th>Acequinocyl</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</td>
<td>Slope ± SE</td>
<td>RR</td>
<td>LC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</td>
<td>Slope ± SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(95%CI)</td>
<td></td>
<td></td>
<td>(95%CI)</td>
<td></td>
</tr>
<tr>
<td>Wasatch</td>
<td>wild-type</td>
<td>6.93 (6.31 - 7.51)</td>
<td>4.88 ± 0.49</td>
<td>-</td>
<td>10.71 (10.23 - 11.15)</td>
<td>-</td>
</tr>
<tr>
<td>JP-R</td>
<td></td>
<td>221.29 (192.80 - 250.93)</td>
<td>2.49 ± 0.19</td>
<td>31.93</td>
<td>39.86 (34.37 - 45.00)</td>
<td>3.36 ± 0.40</td>
</tr>
<tr>
<td>JP-R-BC1</td>
<td></td>
<td>164.13 (144.41 - 185.17)</td>
<td>3.15 ± 0.27</td>
<td>23.68</td>
<td>23.19 (20.45 - 25.64)</td>
<td>4.68 ± 0.48</td>
</tr>
<tr>
<td>JP-R-BC2</td>
<td></td>
<td>153.84 (136.16 - 173.02)</td>
<td>3.14 ± 0.25</td>
<td>22.2</td>
<td>23.86 (21.01 - 26.24)</td>
<td>5.31 ± 0.59</td>
</tr>
<tr>
<td>JP-R-BC3</td>
<td>G132A</td>
<td>180.13 (148.97 - 211.24)</td>
<td>3.17 ± 0.34</td>
<td>26</td>
<td>18.09 (16.13 - 19.85)</td>
<td>4.92 ± 0.49</td>
</tr>
<tr>
<td>FS1</td>
<td></td>
<td>126.8 (113.5 - 141.28)</td>
<td>3.26 ± 0.25</td>
<td>18.3</td>
<td>-</td>
<td>-</td>
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<tr>
<td>iso-FS1</td>
<td></td>
<td>261.35 (229.37 - 295.09)</td>
<td>3.12 ± 0.27</td>
<td>37.71</td>
<td>37.97 (34.04 - 41.81)</td>
<td>3.59 ± 0.29</td>
</tr>
<tr>
<td>FS8</td>
<td></td>
<td>51.42 (46.02 - 56.12)</td>
<td>4.87 ± 0.51</td>
<td>7.42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iso-FS8</td>
<td></td>
<td>79.22 (72.20 - 85.72)</td>
<td>5.47 ± 0.46</td>
<td>11.43</td>
<td>1340.51 (1053.38 - 1636.39)</td>
<td>1.67 ± 0.16</td>
</tr>
<tr>
<td>iso-FS8-BC1</td>
<td>G126S + A133T</td>
<td>42.32 (38.25 - 50.00)</td>
<td>5.10 ± 0.49</td>
<td>6.11</td>
<td>699.291 (584.34 - 824.97)</td>
<td>2.62 ± 0.21</td>
</tr>
<tr>
<td>iso-FS8-BC2</td>
<td></td>
<td>45.62 (39.98 - 52.08)</td>
<td>4.32 ± 0.40</td>
<td>6.58</td>
<td>617.56 (494.55 - 751.54)</td>
<td>2.92 ± 0.25</td>
</tr>
<tr>
<td>iso-FS8-BC3</td>
<td></td>
<td>49.00 (42.47 - 57.00)</td>
<td>4.64 ± 0.42</td>
<td>7.1</td>
<td>820.47 (695.79 - 956.49)</td>
<td>2.43 ± 0.2</td>
</tr>
</tbody>
</table>

D is the degree of dominance. Only adult females were used in the bioassays.

Table 3. Mode of inheritance of Q<sub>0</sub>I resistance in *T. urticae* strains with novel Q<sub>0</sub> mutations.

<table>
<thead>
<tr>
<th>Q&lt;sub&gt;0&lt;/sub&gt; genotype</th>
<th>Cross (♀ × ♂)</th>
<th>Bifenazate</th>
<th></th>
<th>Acequinocyl</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;1&lt;/f&gt; LC&lt;sub&gt;50&lt;/sub&gt; (95% CI) (mg/L)</td>
<td>D</td>
<td>F&lt;sub&gt;1&lt;/f&gt; LC&lt;sub&gt;50&lt;/sub&gt; (95% CI) (mg/L)</td>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP-R × Wasatch</td>
<td>300.04 (257.39 - 347.51)</td>
<td>1.14</td>
<td>24.39 (21.94 - 26.85)</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasatch × JP-R</td>
<td>7.59 (7.08 - 8.05)</td>
<td>-0.95</td>
<td>19.64 (17.88 - 21.38)</td>
<td>-0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-FS1 × Wasatch</td>
<td>167.04 (147.87 - 187.38)</td>
<td>0.75</td>
<td>36.71 (31.99 - 40.97)</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasatch × iso-FS1</td>
<td>7.45 (6.69 - 8.08)</td>
<td>-0.96</td>
<td>24.73 (21.62 - 27.4)</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-FS8 × Wasatch</td>
<td>61.84 (58.81 - 64.87)</td>
<td>0.80</td>
<td>555.74 (439.98 - 676.06)</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wasatch × iso-FS8</td>
<td>5.85 (5.43 - 6.25)</td>
<td>-1.14</td>
<td>28.72 (25.55 - 32.43)</td>
<td>-0.59</td>
<td></td>
<td></td>
</tr>
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</table>

Table 4. The effect of G132A on fertility life table parameters in *T. urticae*.

<table>
<thead>
<tr>
<th>Q&lt;sub&gt;0&lt;/sub&gt; genotype</th>
<th>Line</th>
<th>N</th>
<th>R&lt;sub&gt;0&lt;/sub&gt; ± SE</th>
<th>T ± SE</th>
<th>DT ± SE</th>
<th>rm ± SE</th>
<th>LM ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>Wasatch</td>
<td>38</td>
<td>28.96 ± 2.58a</td>
<td>17.83 ± 0.24a</td>
<td>3.66 ± 0.08a</td>
<td>0.19 ± 0.004a</td>
<td>1.21 ± 0.005a</td>
</tr>
<tr>
<td>G132A</td>
<td>JP-R-BC1</td>
<td>38</td>
<td>12.88 ± 0.96b</td>
<td>17.58 ± 0.17a</td>
<td>4.76 ± 0.12b</td>
<td>0.14 ± 0.004b</td>
<td>1.16 ± 0.004b</td>
</tr>
<tr>
<td></td>
<td>JP-R-BC2</td>
<td>39</td>
<td>19.61 ± 1.55b</td>
<td>17.72 ± 0.22a</td>
<td>4.12 ± 0.07b</td>
<td>0.17 ± 0.003b</td>
<td>1.18 ± 0.004b</td>
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<tr>
<td></td>
<td>JP-R-BC3</td>
<td>39</td>
<td>13.71 ± 1.10b</td>
<td>17.38 ± 0.17a</td>
<td>4.59 ± 0.12b</td>
<td>0.15 ± 0.004b</td>
<td>1.16 ± 0.005b</td>
</tr>
</tbody>
</table>
Net reproductive rate (R₀), the intrinsic rate of increase (rᵣ), the finite rate of increase (Lᵣ), mean generation time (T) and the doubling time (DT) of three near-isogenic lines of *T. urticae* (JP-R-BC1-3) and Wasatch were calculated. Means with different letters (a-b) within a column were significantly different at α = 0.05. N: Number of females

8. Figure Legends

Figure 1. The target-site mutations in the cd1- and ef-helices of mitochondrial cytochrome b in spider mites that confer QoI resistance. An amino acid alignment is shown of the cytochrome b cd1- and ef-helices of the spider mites *T. urticae* and *P. citri*, the fruit fly *Drosophila melanogaster*, human *Homo sapiens*, the fungi *Venturia inaequalis* and *Saccharomyces cerevisiae*, the protozoan *Plasmodium falciparum*, and the plant *Arabidopsis thaliana*. Fully conserved residues are shaded in grey. Asterisks indicate the locations of point mutations that are linked to QoI resistance in spider mites. The validated substitutions in the Qo pocket that cause bifenazate and acequinocyl resistance in spider mites are outlined below the alignment. °: these mutations were originally reported as I256V and N321S.

Figure 2. Bifenazate and acequinocyl dose-response toxicity data of susceptible reference and resistant strains carrying new QoI resistant mutations and their reciprocal crosses revealing the mode of inheritance. Panel A: Dose-response curves show that the JP-R and iso-FS1 strains that carry G132A were resistant to bifenazate, but susceptible to acequinocyl. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that bifenazate resistance is maternally inherited. The mother for each cross is indicated between brackets. Panel B: Dose-response curves show that the iso-FS8 strain carrying G126S + A133T showed high levels of acequinocyl resistance, and moderate resistance to bifenazate. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that both bifenazate and acequinocyl resistance is maternally inherited. The mother for each cross is indicated between brackets.
Figure 3. The effect of G132A on single-generation life-history traits in *T. urticae*. Three introgressed lines carrying the G132A substitution were compared to Wasatch in terms of female longevity, female oviposition, ISS (immature stage survivorship), sex ratio (proportion of males), pre-oviposition period, oviposition period, and post-oviposition period. Letters (a-b) indicate significant differences at $\alpha = 0.05$. The bottom and top of the boxplots depict the first and third quartiles. The central line shows the median, and the whiskers extend to the most extreme data points which are no more than 1.5 times the interquartile range from the box.

Figure 4. The effect of G132A on female longevity and oviposition in *T. urticae*. Panels A and B show the daily egg production per female and the proportion of alive females over the course of the experiment, respectively.
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### Table

<table>
<thead>
<tr>
<th></th>
<th>Q\textsubscript{o}-cd1</th>
<th>Q\textsubscript{o}-ef</th>
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<td>T. urticae</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>P. citri</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>V. inaequalis</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>PWCQSTFGATVITNLLRISFS</td>
<td>*PWCQSTFGATVITNLLRISFS</td>
</tr>
</tbody>
</table>

- **P. citri** G128S + A133T (Van Leeuwen et al. 2011)
- **P. citri** G126S + I136T (Van Leeuwen et al. 2008)
- **P. citri** G126S + S141T (Van Leeuwen et al. 2008)
- **P. citri** G126S + A133T (this study)
- **T. urticae** I260V + N326S (Kim et al. 2019)
- **T. urticae** P262T (Van Leeuwen et al. 2008)
- **T. urticae** G132A (this study)
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Panel A: Dose-response curves show that the JP-R and FS1 strains that carry G132A were resistant to bifenazate, but susceptible to acequinocyl. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that bifenazate resistance is maternally inherited. The mother for each cross is indicated between brackets.

Panel B: Dose-response curves show that the FS8 strain carrying G126S + A133T showed high levels of acequinocyl resistance, and moderate resistance to bifenazate. Wasatch was susceptible to both acaricides. Reciprocal crosses showed that both bifenazate and acequinocyl resistance is maternally inherited. The mother for each cross is indicated between brackets.
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177x150mm (300 x 300 DPI)