Mitochondrial heteroplasmy and the evolution of insecticide resistance: Non-Mendelian inheritance in action

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Genes encoded by mitochondrial DNA (mtDNA) exist in large numbers per cell but can be selected very rapidly as a result of unequal partitioning of mtDNA between germ cells during embryogenesis. However, empirical studies of this ''bottlenecking'' effect are rare because of the apparent scarcity of heteroplasmic individuals possessing more than one mtDNA haplotype. Here, we report an example of insecticide resistance in an arthropod pest (*Tetranychus urticae*) being controlled by mtDNA and on its inheritance in a heteroplasmic mite strain. Resistance to the insecticide bifenazate is highly correlated with remarkable mutations in cytochrome *b*, a mitochondrially encoded protein in the respiratory pathway. Four sites in the Q_o site that are absolutely conserved across fungi, protozoa, plants, and animals are mutated in resistant mite strains. Despite the unusual nature of these mutations, resistant mites showed no fitness costs in the absence of insecticide. Partially resistant strains, consisting of heteroplasmic individuals, transmit their resistant and susceptible haplotypes to progeny in highly variable ratios consistent with a sampling bottleneck of ≈180 copies. Insecticide selection on heteroplasmic individuals favors those carrying resistant haplotypes at a frequency of 60% or more. This combination of factors enables very rapid evolution and accounts for mutations being fixed in most field-collected resistant strains. The results provide a rare insight into non-Mendelian mechanisms of mitochondrial inheritance and evolution, relevant to anticipating and understanding the development of other mitochondrially encoded adaptations in arthropods. They also provide strong evidence of cytochrome *b* being the target site for bifenazate in spider mites.

bifenazate | *Tetranychus urticae* | cytochrome *b* | mtDNA

Compared with cell nuclei, mitochondria contain few genes, but they exhibit some intriguing evolutionary characteristics. Their inheritance is essentially non-Mendelian in that recombination through meiosis is lacking and transmission to offspring is predominantly uniparental. In contrast to nuclear DNA, in which there are only two copies of each gene per cell, the mitochondrial DNA (mtDNA) copy number in somatic cells is generally in the range of 10^3–10^4 copies per cell (1). Higher mutation rates in animal mtDNA together with limited DNA repair mechanisms render these genes susceptible to rapid evolution through random drift or natural selection. As a consequence, mtDNA is highly polymorphic and is widely used for studies in systematics and population genetics. However, most genetic variation within a species exists between individuals, and the occurrence of more than one mtDNA sequence variant in a single individual (heteroplasmy) is relatively rare (2). Empirical work has shown that genotype frequencies in heteroplasmic individuals can shift greatly in a single transmission from mother to offspring, a phenomenon that has been ascribed to ''genetic bottlenecking'' caused by selective replication of DNA templates or unequal partitioning of mtDNA between germ cells

during embryogenesis (2–4). As a result of these characteristics, traits encoded by mtDNA have the potential to evolve, and reach fixation, very rapidly. We report here on a case of insecticide resistance encoded by mtDNA in which the occurrence of heteroplasmy has disclosed relationships between intracellular genetic variation and inheritance of the resistance phenotype, a rare example of non-Mendelian inheritance in action.

The ability of arthropods to develop insecticide resistance has led in extreme cases to catastrophic crop failures or resurgence of vector-borne diseases. Considerable progress has been made with characterizing the underlying mechanisms, including enhanced detoxification or structural changes to target-site proteins (5). To date, however, there have been no reports of resistance encoded by mtDNA in arthropods. Several insecticides do target biochemical processes in mitochondria, but cases of resistance resolved so far have involved mutations in the nuclear genome. A possible example of mtDNA-controlled resistance arose from work involving the insecticide bifenazate and the two-spotted spider mite, *Tetranychus urticae* Koch. *T. urticae* is a cosmopolitan and polyphagous crop pest with a long history of developing resistance to pesticides (6). Bifenazate is a member of the new insecticidal group of hydrazine carbazate derivatives, whose mode of action has so far been unknown. Selection of *T. urticae* with bifenazate in the laboratory generated >100,000-fold resistance (7). Of particular note, reciprocal crosses between susceptible and resistant mites showed resistance to be inherited only maternally; offspring of resistant females were strongly resistant, but those of susceptible females remained fully susceptible. Such complete uniparental inheritance is unprecedented for insecticide resistance and suggested mitochondrial control as one possible explanation.

Results

Amplification of Whole Mitochondrial Genomes and Identification of Putative Resistance-Conferring Mutations. We amplified, sequenced, and compared complete mitochondrial genomes of susceptible and resistant strains of *T. urticae* (GenBank EU345430, susceptible strain). Despite being one of the most compact mitochondrial genomes (13,103 bp), all 13 protein-encoding genes expected in animal mitochondrial genomes and genes coding for S12 and S16 ribosomal RNA were represented
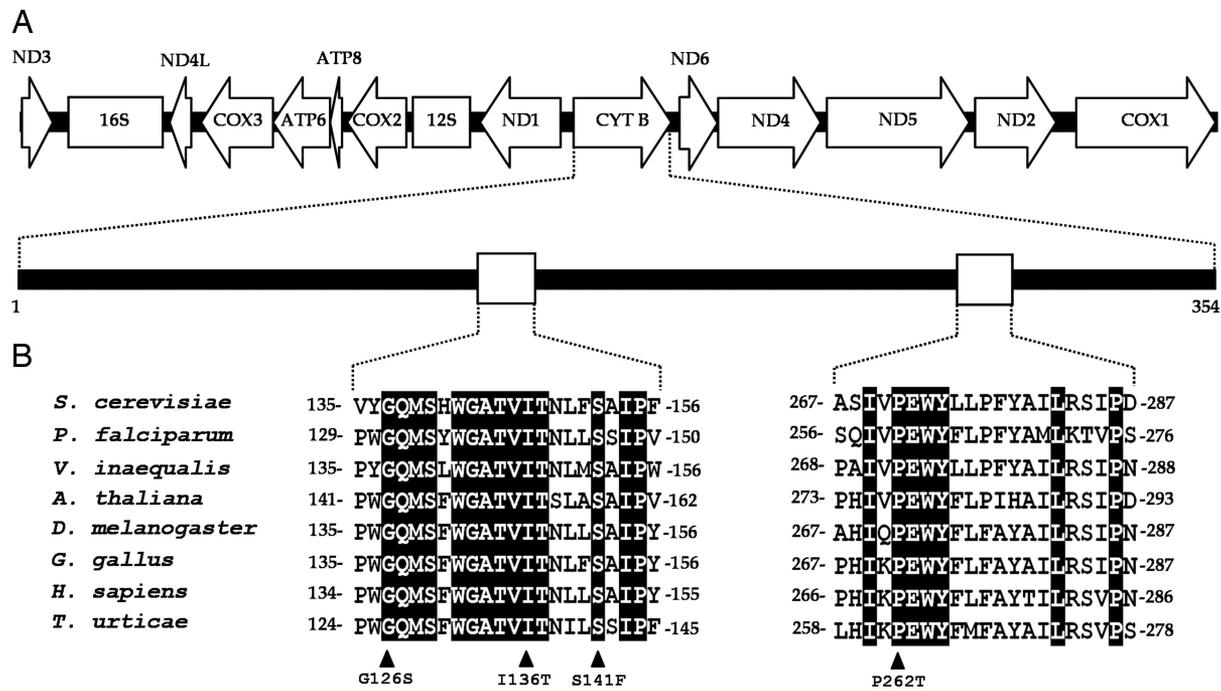
Fig. 1. *Cytb* resistance mutations in the mitochondrial genome. (*A*) Linear representation of the mitochondrial genome of *T. urticae* (13,103 bp) showing the position and orientation of the protein-encoding genes (*n* = 13) and the ribosomal RNA genes (*n* = 2). (*B*) Sequence alignment of conserved Q_o pocket residues positioned on the cytochrome *b* of *T. urticae* with those of *S. cerevisiae* (ABS28693), *P. falciparum* (NP_059668), *Venturia inaequalis* (AAC03553), *Arabidopsis thaliana* (CAA47966), *Drosophila melanogaster* (CAB91062), *Gallus gallus* (AAO44995), and *Homo sapiens* (AAX15094). Fully conserved residues in the alignment are marked in black. Point mutations linked to bifenazate resistance in *T. urticae* are indicated by triangles.

(Fig. 1*A*). A comparison of the complete genomic sequences of the laboratory-selected bifenazate-resistant strain (GenBank accession no. EU5567754, BR-VL) with that of the original unselected strain (LS-VL) showed only three nucleotide substitutions, all nonsynonymous, in the cytochrome *b* (*cytb*) gene, resulting in amino acid substitutions G126S, S141F, and D161G (Fig. 1*B* and Table 1). Cytochrome *b* is the only mitochondrially encoded component of the ubihydroquinone:cytochrome *c* oxidoreductase (cytochrome bc_1, complex III) that catalyzes electron transfer from reduced ubiquinone to cytochrome *c*, coupled to proton translocation across the membrane. Comparison of *cytb* alignment of translated sequences from the susceptible and laboratory-resistant strains with those from several model organisms showed that these mutations were located in the Q_o

oxidation site of the protein, more precisely in the cd1 helix aligning the enzyme pocket (8).

Amplification of *cytb* from Collected Field Strains. To reinforce the likelihood of these mutations being implicated in resistance, we sequenced and assayed several field strains of *T. urticae* from regions in The Netherlands where loss of susceptibility to bifenazate was observed. Three more strains (HOL1, HOL2, and HOL4) contained the G126S mutation found in the laboratory-selected strain. In two strains (HOL1 and HOL4) this mutation was accompanied by a second I136T mutation, also located in the cd1 helix of the Q_o pocket (Fig. 1*B* and Table 1). Combination of these mutations gave extreme resistance to bifenazate (Table 1). Interestingly, the strain with G126S alone (HOL2) showed only low resistance to bifenazate (Table 1), suggesting

Table 1. Relationship between genotype (*cytb* sequence) and bifenazate susceptibility for laboratory and field-derived strains of *T. urticae*

Strain	Accession no.	Origin	*Cytb* genotype,* protein/gene	LC_{50} bifenazate, mg a.i. per liter (95% fiducial limits)
LS-VL	EU345430	Laboratory, susceptible	WT/WT	1.6 (1.5–1.7)
GSS	EU556751	Laboratory, susceptible	WT/WT	3.6 (3.3–3.9)
MR-VL	EU556752	Ghent, Belgium	WT/WT	10.5 (9.7–11.3)
MR-VP	EU556753	Brussels, Belgium	WT/WT	7.3 (5.6–9.4)
BR-VL	EU556754	Selected from LS-VL	G126S/G376A S141F/C422T	>10,000
HOL1	EU556747	Hoek van Holland, The Netherlands	G126S/G376A I136T/T407C	>10,000
HOL 2	EU556748	Hoek van Holland, The Netherlands	G126S/G376A	43.4[†] (38.1–49.7)
HOL3	EU556749	Nieuwveen, The Netherlands	P262T/C784A	>10,000[‡]
HOL4	EU556750	Tuil, The Netherlands	G126S/G376A I136T/T407C	>10,000

a.i., active ingredient.

*WT, wild-type sequence corresponding to the alignment in the bottom row of Fig 1*B*.

[†]Mixed strain with individuals fixed for G126S or WT. Mites surviving 50 mg of a.i. per liter of bifenazate consistently had the G126S genotype.

[‡]After fixation of the mutation through laboratory selection. The initial LC_{50} after collection was 72 mg of a.i. per liter (fiducial limits: 64–82).
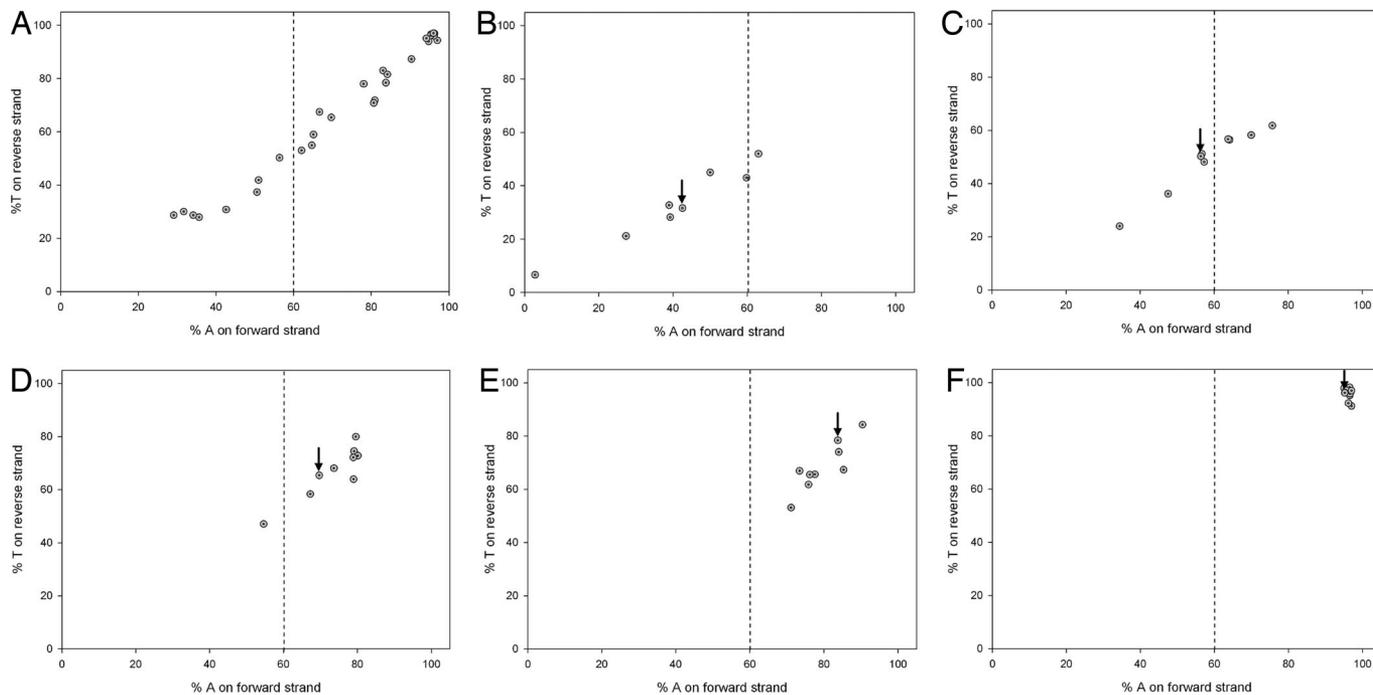
Fig. 2. Inheritance of mtDNA heteroplasmy for the P262T mutation in the HOL3 strain of *T. urticae*. Frequencies of resistant haplotypes are shown as corresponding relative peak heights of nucleotides on forward and reverse strands. (*A*) Frequency of resistant haplotypes in 25 randomly selected individual mites on collection from the field. (*B–F*) Frequency of resistant haplotypes in single mothers (shown by arrows) and in eight of their first-generation offspring. Dotted lines show the estimated mutation frequency (60%) needed to survive field-applied concentrations of bifenazate.

that a combination of at least two cd1 helix mutations (G126S and I136T in HOL1 and HOL4, or G126S and S141F in BR-VL) is necessary to confer a pronounced resistance phenotype. The third D161G mutation in BR-VL was located at a less conserved position not in contact with the Q_o pocket, and it is not clear whether this mutation is directly implicated in resistance. We also identified a field strain (HOL3) with a new mutation: a P262T substitution in the highly conserved PEWY motif at the ef helix aligning the Q_o pocket of *cytb* (Fig. 1*B*). This mutation was the only one detected in HOL3, which again exhibited at least 10,000-fold resistance to bifenazate. Other field and laboratory strains with a wild-type *cytb* sequence in the cd1 and ef helix positions showed no resistance to bifenazate. Collectively, these findings implicate bifenazate (when applied against spider mites) as a Q_o site inhibitor, whose activity can be severely compromised by mutations at the binding site.

Mitochondrial Heteroplasmy and Inheritance. In the first strain to be characterized (BR-VL), mutations conferring bifenazate resistance were ubiquitous, and no heteroplasmy was observed. However, the emergence of any new mtDNA haplotype implies a mutation in one female germ line cell and an intermediate heteroplasmic stage must follow. Under sustained selection, this stage may be of short duration because of rapid and unequal segregation of mitochondria in the female germ line or early embryo (the genetic bottleneck effect) (2). Thus, all subsequent field strains were carefully scrutinized for the occurrence of heteroplasmy and one such strain (HOL3) with the PEWY motif P262T mutation (Table 1) was identified. This strain had been collected in a glasshouse with a history of bifenazate treatment. The P262T mutation results from a C to A nucleotide substitution at position 784 in *cytb*. Heteroplasmy was suspected from a double peak at position 784 when analyzing a pooled sample of 100 mites with sequencing chromatographs. We subsequently amplified and investigated the complete *cytb* sequence of 26 individual mites and quantified the frequency of susceptible and resistant haplotypes as the relative peak heights at position 784 on chromatographs. The frequency of the mutation in these individuals varied in a continuous manner from 30 to 98% (Fig. 2*A*). The implications for inheritance were investigated by breeding offspring from five randomly selected females whose frequency of the C784A nucleotide substitution proved to be 37, 53, 68, 81, and 96% (Fig. 2 *B–F*). The first eight female offspring of each mother that reached adulthood were also analyzed for heteroplasmy, and a clear parent–offspring relationship emerged (Fig. 2 *B–F*). However, frequencies of the resistant haplotype in offspring varied substantially around the parental values with, for example, progeny of a mother with a 37% mutant frequency showing corresponding frequencies of 5–58% (Fig. 2*B*). Variation between progeny of a mother with 96% mutant haplotypes (i.e., approaching fixation for the resistance mutation) was much more limited, and in this case the mutation was transmitted more faithfully from parent to offspring (Fig. 2*F*).

Establishing a Mutation Threshold for Phenotypic Expression of Resistance. We divided mites of the heteroplasmic HOL3 strain into two groups: mites paralyzed at 100 mg/liter (equivalent to the field rate of bifenazate) and mites not paralyzed by this concentration of bifenazate. Paralyzed mites had an average percentage mutation of 55% (SE = 9, $n = 10$) and resistant mites 86% (SE = 5, $n = 8$). Mites surviving the field dose had a percentage mutation of at least 60%, defining a discriminating boundary for survival. We also tested third-generation offspring of single mothers with different initial mutation frequencies for toxicity at 100 mg/liter (Fig. 3), again revealing a threshold mutation frequency of ≈60% to result in field resistance.

Modeling the Inheritance of Heteroplasmy. By using the observed variance distributions for progeny, it is possible to estimate the magnitude of random processes influencing the segregation and partitioning of mitochondria during reproduction and embryogenesis (2, 9 and see *Materials and Methods*). The key parameter
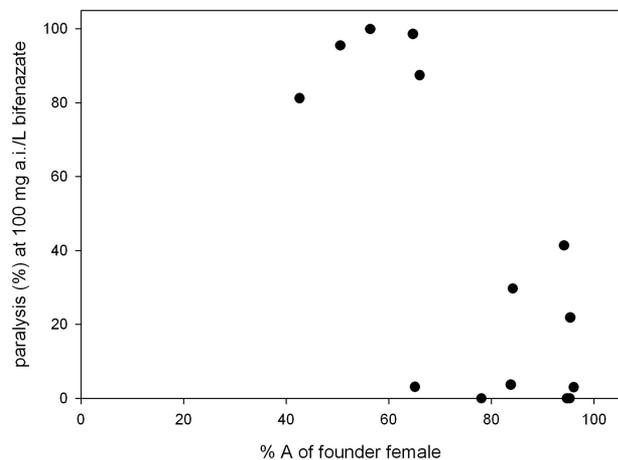
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Fig. 3. Establishing a link between phenotype and genotype. The relationship between frequency of the resistant haplotype in single founder females from the HOL3 strain of *T. urticae* and susceptibility of third-generation siblings to 100 mg of active ingredient (a.i.) per liter of bifenazate is shown.

for estimation is N_e, the effective number of mtDNAs sampled at each generation from the very large pool potentially available. Estimates of N_e based on examples of heteroplasmic inheritance shown in Fig. 2 (and assuming $k = 10$; see *Material and Methods*) ranged from 79 to 263, with a median value of 181 (Table 2). This figure is broadly in line with those from studies of mice and *Drosophila* (2, 9) and represents only a small proportion of the total number of mtDNA molecules likely to occur in mature oocytes (1). The influence of random drift on changes in mutation frequency between generations can be appreciated most clearly by examining the relationship between frequency of the resistant haplotype and expression of a bifenazate resistance phenotype. Although one would expect a continuous relationship between the frequency of mtDNA mutations and absolute levels of resistance, selection will only occur when the latter is sufficient to withstand concentrations of insecticide that mites encounter under field conditions. As described above, an approximate threshold of 60% mutated mtDNA is required to avoid symptoms of paralysis with the field application rate of bifenazate. It is notable that even the mother exhibiting lowest frequency of the resistant haplotype (37%; Fig. 2B) in the inheritance experiment produced progeny (one of eight) around this 60% criterion. However, this criterion for selection is undoubtedly too stringent. It equates to the highest concentration that mites are likely to encounter, but in practice they will also encounter insecticide concentrations far lower than the field rate as a result of uneven coverage or the decay of residues over time. Much lower mutation frequencies can therefore confer advantages and result in selection under field conditions. Thus, a combination of heteroplasmy, genetic bottlenecking, and sus-

tained selection can lead quickly to the emergence of a resistance phenotype and drive populations rapidly toward fixation of the advantageous trait, which is consistent with observations that all other bifenazate-resistant strains investigated so far have been homoplasmic for mutations conferring resistance.

Effects on Overall Fitness. As well as facilitating the selection of resistance mutations, processes operating during mitochondrial inheritance could also speed up their loss if they confer fitness costs in the absence of the selecting agent. Given the extent to which the sites containing the *cytb* mutations in *T. urticae* are strongly conserved in nature (Fig. 1*B*), a reasonable *a priori* expectation is that these mutations will have implications for the fitness of their carriers. This expectation is supported by studies investigating the effect of induced *cytb* mutations on respiratory function in model organisms such as the yeast *Saccharomyces cerevisiae*. Substitutions at the G137 site in *S. cerevisiae* (equivalent to G126 in *T. urticae*) were found to impair respiratory competence by uncoupling the complex and affecting FeS stability (10, 11). Substitutions at I147 (equivalent to I136 in *T. urticae*) were also shown to affect respiratory competence reflected by reduced cytochrome *c* reductase activity (12). We compared the overall biological fitness of the laboratory-selected BR-VL strain of *T. urticae* with that of the susceptible LS-VL strain by measuring a series of life history traits including fecundity, longevity, and development time, by calculating a composite demographic parameter (intrinsic rate of increase or r_m; Table 3). There were no significant differences in individual parameters or in estimates of r_m, implying that resistant mites can survive and reproduce as efficiently as susceptible ones in the absence of exposure to bifenazate.

Discussion

Resolution of the mitochondrial target site of bifenazate and confirmation that resistance is mitochondrially encoded and inherited has provided rare experimental insights into the different evolutionary dynamics of mtDNA genes and those in the nuclear genome. There are interesting parallels with cases of Q_o site inhibitor resistance reported in various crop and human pathogens. Resistance to strobilurin fungicides, which block electron transfer at the Q_o site, in field isolates of fungal pathogens is correlated with principally two mutations: G143A and F129L in the cd1 helix of *cytb* (13). G143A is the more common mutation, and isolates carrying this mutation express very strong resistance to strobilurins. Cases of strobilurin resistance, as in *Mycosphaerella graminicola* (the cause of septoria leaf blotch disease in wheat), have also emerged extremely rapidly, and examples of isolates showing heteroplasmy have proved extremely rare in the field (13–15). As is the case with *T. urticae*, mutations conferring strobilurin resistance have generally had no adverse effects on overall fitness (13). Atovaquone, a well known Q_o inhibitor, was recently introduced as a clinical antimalarial in combination with proguanil. Where atovaquone

Table 2. Distribution of mitochondrial genotypes in the progeny of four founder heteroplasmic female mites and the effective number of segregating mtDNA units

					N_e				
$p_o \times 10^2$	N	$p_n \times 10^2$	Range	$V_n \times 10^4$	$k = 8$	$k = 10$	$k = 12$	$k = 14$	$k = 16$
37	8	36	(5–58)	296	63	79	95	1,104	126
53	8	53	(29–69)	166	117	146	175	204	233
68	8	71	(51–80)	90	190	237	285	332	379
81	8	73	(62–87)	58	210	263	315	367	420

p_o, frequency of the resistant haplotype in the founder female; n, number of offspring tested; p_n, average frequency of the resistant haplotype in offspring; V_n, variance of frequency in offspring; N_e, number of effective segregating units for mtDNA; k, number of cell divisions.

Table 3. Life table parameters for the susceptible LS-VL and laboratory-selected bifenazate-resistant BR-VL strains

Parameters	LS-VL	BR-VL
Female longevity, days	17.51 ± 1.65a	18.95 ± 1.58a
Total fecundity, no. of eggs	105.8 ± 14.21a	125.5 ± 11.53a
Average daily fecundity, eggs per female per day	4.44 ± 0.58a	5.03 ± 0.52a
Generation time T, days	18.72 ± 0.01a	18.72 ± 0.005a
Intrinsic rate of increase, r_m, females per female per day	0.209 ± 0.005a	0.205 ± 0.004a

Means (±SEM) within rows followed by the same letter are not significantly different (t test, $P > 0.05$).

has been used in monotherapy in clinical trials, resistance also emerged extremely rapidly linked to point mutations affecting Y268 in the ef helix of *Plasmodium falciparum* (16).

More speculatively, the rate at which Q_o inhibitor resistance develops may also be accelerated by an increased mutation rate. It is known that the lack of histones and repair mechanisms in mtDNA, together with its close proximity to the electron transport chain, leads to an elevated rate of mtDNA damage. Damage and thus mutagenesis of mtDNA are affected by the oxidation of nucleotides by reactive oxygen species (ROS), leading to a mispairing event during replication (17). The concentration of ROS is known to increase under the action of Q_o site inhibitors, and this may in turn increase the rate of mutagenic oxidation (18). Potentially increased mutation rates in response to inhibitor action conform to the concept of "adaptive mutations" (19, 20), rather than supporting the model of constant rare mutations leading to preexisting phenotypes expressing resistance.

Mitochondrial control of resistance, coupled with a lack of fitness costs, challenges much of the conventional theory of resistance management, based on Mendelian principles and exploiting features such as recombination and segregation, the dominance of resistance alleles, and their dilution through immigration (21). The speed at which *cytb*-based resistance has developed in mites is, in hindsight, explicable through the idiosyncrasies of mitochondrial evolution and inheritance. It is likely that Q_o site fungicide resistance has arisen in the same manner. Although mitochondria are considered attractive targets for unique pesticides, the development and deployment of such agents without specific information on the target site evidently require great care.

Materials and Methods

Mite Strains. The original susceptible strain (LS-VL) of *T. urticae* was collected in October 2000 from roses near Ghent, Belgium, where pesticides had not been used for at least 10 years (22). The BR-VL resistant strain was selected from LS-VL by successively applying bifenazate at concentrations that killed 90% of individuals (7). Other field strains were collected from glasshouses at different locations in The Netherlands (Table 1). In the laboratory, mites (at least 500 adult females) were transferred to bean plants (*Phaseolus vulgaris* L.) and allowed to reproduce for two to four generations before analysis. All strains were maintained at 25 ± 0.5°C with 60% relative humidity and a 16/8 h (light/dark) photoperiod.

Bioassays. Bioassays with commercially formulated bifenazate (Floramite, 240 SC) entailed transferring 10–25 young adult female mites to 9-cm² bean leaf disks sprayed with 0.6 ml of serial insecticide dilutions (22). Disks were kept at 25 ± 0.5°C, 60% relative humidity, 16/8 h (light/dark) photoperiod on wet cotton. Mortality was scored after 24 h, and LC_{50} values and their 95% fiducial limits were calculated by probit regression by using POLO-PC software (LeOra Software).

Amplification and Sequencing of Mitochondrial Genomes. Mites were chilled at 4°C for 30 min and gently homogenized in a prechilled (4°C) mortar after which 10 ml of ice-cold buffer [30 mM Tris·HCl, 300 mM sucrose, 10 mM EDTA, 1% BSA (fatty acid-free), pH 7.5] was added. The suspension was centrifuged for 5 min at 1,000 × g at 4°C, and the resulting pellet was homogenized on ice in 10 volumes of buffer with a Heidolph RZR2020 motorized Teflon pestle for 30 s at 2,000 rpm. The homogenate was filtered through eight layers of

cheesecloth. Mitochondria were isolated by differential centrifugation (3 times 500 × g for 5 min, 3 times 1,000 × g for 5 min, and 90 s at 1,600 × g). Each time the supernatant was collected. Mitochondria were subsequently pelleted by centrifugation at 15,000 × g for 30 min at 4°C. The crude mitochondrial pellet was purified on a discontinuous sucrose density gradient (1.5–1.0 M sucrose in 10 mM Tris·HCl, 10 mM EDTA at pH 7.5) by centrifugation for 1 h at 100,000 × g at 4°C in a Beckman ultracentrifuge with a SW55i rotor. Purified mitochondria were lysed in 500 μl of SDS buffer (200 mM Tris·HCl, 400 mM NaCl, 10 mM EDTA, 2% SDS at pH 8.2) followed by chloroform–phenol extraction (23).

The purified circular mtDNA was subsequently used as template for rolling circle amplification (RCA). RCA was performed with phi29 DNA polymerase (Fermentas). Reactions were started by mixing 10 μl of reaction buffer, 10 μl of 500 μM exo-resistant random primers (with two 3′-terminal phosphorothioate modifications) (Fermentas), 70 μl of sterile double-distilled water, and 10 μl of template (1–50 ng of mtDNA). This mixture was denatured for 3 min at 95°C followed by slowly cooling down to 20°C, allowing primer binding. A mix containing 10 μl of dNTPs (2 mM each; Invitrogen), 10 μl of reaction buffer, 3 μl of yeast pyrophosphatase (0.1 unit/μl; Sigma–Aldrich), 5 μl of phi29 (5 units), and 72 μl of sterile double-distilled water was subsequently added. The mixture was briefly vortexed and spun down after which it was incubated for 18 h at 37°C. Reactions were stopped by heating (5 min, 65°C). Large DNA fragments were selectively precipitated with 2 volumes of 10% PEG in 150 mM NaCl (pH 8.0) and resolved in 0.1 M Tris·HCl (pH 8.0). RCA-amplified material was partially sequenced by shotgun sequencing (Agowa sequencing service). Based on this shotgun library, PCR primers were designed in the *cox1* and *cytb* gene sequences (C2F, 5′-CCAGCTATAATTGGAGGATT-TGG; C2R, 5′-TGTCCTCAAGGTAAAACATACCC; C3F, 5′-TGGGTCTCCTCCTC-CTCTTG; C3R, 5′-CCGCTTTTATCGGGTATGTTTTAC) for the amplification of the complete mitochondrial genome of susceptible and resistant mites in two overlapping pieces with the Expand Long Range PCR kit (Roche). Resulting PCR fragments (≈8 and 6 kb) were sequenced by primer walking on both strands (Agowa sequencing service).

The complete genomic sequence was assembled and annotated by using VectorNTI (Invitrogen) according to Masta and Boore (24). ORFs were identified with the program Getorf from the EMBOSS package. Translated sequences were used for Blastp searches against the nonredundant protein database. Two large non-protein-coding regions were candidates for the large and small rRNA (16S and 12S, respectively). The boundaries of the rRNA genes were identified based on alignments and secondary structures of rRNA genes of other mite species.

Amplification and Sequencing of *cytb* of Single Mites and Quantification of Heteroplasmy. Based on the annotated complete mtDNA sequence, primers in *cytb*-flanking regions were designed to amplify the complete *cytb* gene with Long Range PCR (Cytbdia2F, 5′-ATTTGGTTAGATGAGCCTTTTTC; Cytbdia2R, 5′-TCATGAAAAAGGCTCATCTAACC) to allow screening for *cytb* polymorphisms in individual mites. Mites were homogenized in 10 μl of buffer (10 mM Tris·HCl, 100 mM NaCl, 1 mM EDTA), 2 μl of proteinase K (10 mg/ml) was added, and the mixture was incubated for 30 min at 37°C. After proteinase K activity was destroyed by heating (5 min at 95°C), 1 μl was used as a template for PCR. The amplified products were purified with a QIAquick PCR purification kit (Qiagen) and used directly for sequencing using the original PCR primers and two internal primers (cytbWTF, 5′-CGGAATAATTTTACAAATAACTCATGC; cytbWTR, 5′-TGGTACAGATCGTAGAATTGCG) so that four times coverage was obtained for each nucleotide.

For the quantification of heteroplasmy, two primers flanking the 784 position on *cytb* at equal distance of 200 bp from position 784 were designed (PEWYF, 5′-AAAGGCTCATCTAACCAAATAGG; PEWYR, 5′-AATGAAATTTCTG-TAAAAGGGTATTC). The degree of heteroplasmy was quantified by analyzing the heights of fluorescence peaks from the electropherogram (Bioedit). The

proportion A on the forward strand at position 784 in the mixture (%A) was expressed as the percentage of the A peak height (HA) to the total of the two peak heights (HA and HB) (%A = HA/(HA + HB) × 100). The same procedure was repeated for T784 on the reverse strand. Average frequencies (estimates based on both strands) were used for calculations in the model and presentation in Table 2. This way of proportional sequencing gives a high correlation with proportional data derived from real-time PCR (25).

Determination of Fitness Parameters. Thirty adult females from the susceptible (LS-VL) and resistant (BR-VL) stock colonies were allowed to lay one egg on a bean leaf arena (3 cm × 3 cm). The arenas were checked every 12 h to track the development of individual life stages. Mites that died or escaped during the experiment were omitted from the dataset. For life table analyses, 36 single-pair matings were established by placing a female in the teleiochrysalis stage of each strain with a male on a leaf arena. Male and females were transferred daily to a new arena, and the following parameters were recorded: interval between the present and previous observation, number of dead or escaped mites, and number of eggs laid per female. All eggs laid were followed until reaching adulthood. Experiments were performed at 25 ± 0.5°C, 60% relative humidity, 16/8 h (light/dark) photoperiod. Based on these data, mean longevity, duration of the oviposition period, and fecundity (expressed as the number of eggs over the oviposition period) of females were calculated. The intrinsic rate of increase r_m was calculated from the equation $\Sigma e^{-r_m x} l_x m_x = 1$, 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. Mean generation time was calculated from the equation $[T = \ln(\Sigma l_x m_x)/r_m]$. Variance distributions for r_m were calculated by jack-knifing pseudovalues of r_m and transferring them to SPSS 12.0 (SPSS, Inc.) in which independent t tests were used to evaluated differences between means (26–27).

Modeling the Inheritance of Heteroplasmy. We used a mathematical model taken from population genetics (2, 9) to investigate the influence of heteroplasmy on resistance development in *T. urticae*. This model uses stochastic theory to estimate the effective number of mtDNAs (N_e) that are sampled from a large pool in successive generations, assuming that segregation occurs by genetic drift. The variance of mtDNA frequencies (V_n) at the nth generation is given by the following equation:

$$V_n = p_0(1 - p_0)\left[1 - \left(1 - \frac{1}{N_e}\right)^{kn}\right] \qquad [1]$$

in which p_o is the frequency of the mutated *cytb* haplotype, N_e is the number of segregating units taken from a large pool, and k is the number of random samplings (i.e., cell division number) for every generation. If the variability of transmission of resistant haplotypes is only dependent on random partitioning, the number of segregating units can be inferred from the equation by the experimentally determined variances, if the number of cell divisions per generation is known (or can be inferred with sufficient accuracy). However, the embryology of *T. urticae* is poorly studied. Approximately 2 h after oviposition, the embryo undergoes the first total cleavage followed by two rounds of synchronous divisions leading to the formation of eight equally sized blastomeres. After this stage, however, cleavages become asynchronous, and cells migrate to the periphery of the embryo (28). In *Drosophila*, ≈8–10 divisions take place from egg to stem cells, and assuming $k = 10$ is probably a good estimate (9). Taking a conservative approach and allowing k to vary between 8 and 16, and by using data on mutation frequencies and variances, the median effective number of segregating units (N_e) was calculated.

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