Reduced Variation in *Drosophila simulans* Mitochondrial DNA

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ABSTRACT

We investigated the evolutionary dynamics of infection of a *Drosophila simulans* population by a maternally inherited insect bacterial parasite, Wolbachia, by analyzing nucleotide variability in three regions of the mitochondrial genome in four infected and 35 uninfected lines. Mitochondrial variability is significantly reduced compared to a noncoding region of a nuclear-encoded gene in both uninfected and pooled samples of flies, indicating a sweep of genetic variation. The selective sweep of mitochondrial DNA may have been generated by the fixation of an advantageous mitochondrial gene mutation in the mitochondrial genome. Alternatively, the dramatic reduction in mitochondrial diversity may be related to Wolbachia.

MITOCHONDRIAL genes have been used extensively in evolutionary studies because of their high rate of evolution, uniparental mode of inheritance, and the belief that most of the variation within a species is selectively neutral. However, mitochondrial DNA (mtDNA) variation and evolution may not obey the assumptions of the constant mutation rate equilibrium neutral model (reviewed in BALLARD and KREITMAN 1995). RAND *et al.* (1994) and BALLARD and KREITMAN (1994) compared levels of within- and between-species variation in mitochondrial and nuclear genes in sibling species of Drosophila. mtDNA diversity was significantly reduced compared to nuclear gene polymorphism in *D. simulans,* even after taking into account the fourfold reduction in variation expected from differences in mode of inheritance (haploid maternal *vs.* diploid biparental). With no recombination, the mitochondrial genome is particularly susceptible to genetic hitchhiking accompanying selection at linked sites. Selection need not even be acting on the mitochondrial genome itself: any maternally inherited factor could potentially influence haplotypic diversity. One such maternally inherited factor is the rickettsial microorganism *Wolbachia pipientis.*

Wolbachia has been found to elicit cytoplasmic incompatibility in 31 different species of insects, a crustacean and several species of mites (WERREN *et al.* 1995; JOHANOWICZ and HOY 1996). In California two incompatibility types of *D. simulans* have been identified: R (originally collected at Riverside), which is infected with Wolbachia, and W (originally collected at Watsonville), which is uninfected (HOFFMANN *et al.* 1986; HOFFMANN and TURELLI 1988; O'NEILL and KARR 1990). R and W types display unidirectional incompatibility with incompatible crosses producing hatch rates 30–70% lower than compatible crosses in the field (TURELLI and HOFFMANN 1995). The reciprocal cross and crosses within compatibility types all produce comparable numbers of adult progeny. As a consequence of incompatibility, offspring of W-type females experience significantly greater mortality rates in polymorphic populations than the offspring of R-type females. Although this is counterbalanced by a slight fecundity deficit for R-type females, the population frequency of the infected R type is generally expected to increase (HOFFMANN *et al.* 1990). This has been observed in several natural populations (TURELLI and HOFFMANN 1991; TURELLI *et al.* 1992). However, not all Wolbachia variants in *D. simulans* cause cytoplasmic incompatibility. Flies from Madagascar that harbor the *w*Ma variant do not exhibit incompatibility (ROUSSET and SOLIGNAC 1995). Furthermore, TURELLI and HOFFMAN (1995) found six of six lines from Tampa, Florida and eight of 18 lines from Ecuador were PCR-positive for Wolbachia but behaved in progeny tests as if they were uninfected. They termed this latter cytoplasmic incompatibility type A because it was first recorded in Australia. The *w*Ma and A variants may be related although they belong to distinct host mitochondrial haplotypes.

As an R-type infection spreads through a population, the frequency of the mtDNA variant(s) initially associated with a Wolbachia infection is also expected to increase. An infected population is expected to have a high frequency of one mitochondrial type and a loss of mitochondrial variation (TURELLI *et al.* 1992). However, the same Wolbachia infection would not be expected to severely affect autosomal variation of the diploid genome. In this study we investigate whether the level of mitochondrial variability in uninfected flies taken from a single population is compatible with a neutral model

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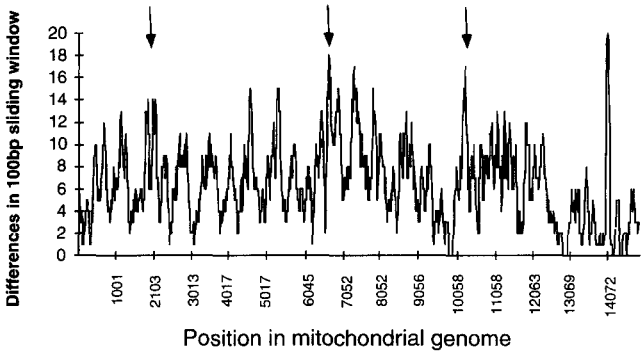

FIGURE 1.—Number of differences between the mitochondrial genomes of *D. melanogaster* and *D. yakuba* (excluding the A + T-rich region) in a 100-bp sliding-window. The arrows indicate the three regions chosen for further analysis.

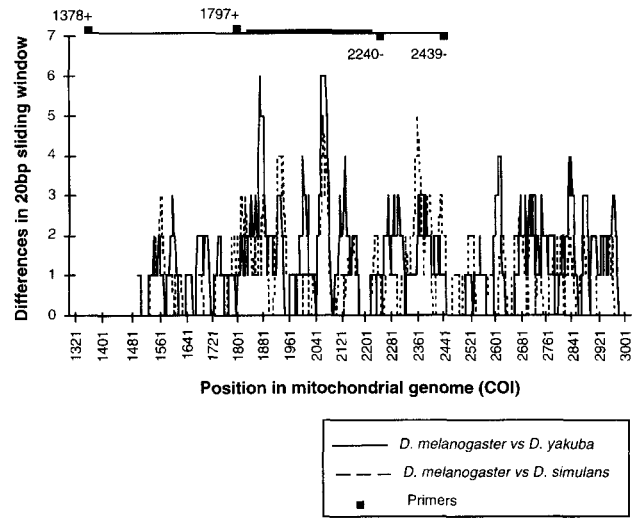

FIGURE 2.—Number of differences between the COI gene of *D. melanogaster* and *D. yakuba* and between *D. melanogaster* and *D. simulans* in a 20-bp sliding-window. The primer sites are indicated at the top of the figure. The region amplified is indicated by a thin black line and the region sequenced by a thick black line.

when compared to that of an autosomal locus. An alternate hypothesis, that mtDNA diversity of uninfected lines is significantly reduced relative to an appropriate autosomal locus, would suggest that the mitochondrial genome has been subjected to a selective sweep. The selective sweep of mtDNA may have been generated by the fixation of an advantageous mutation on the mitochondrial genome. Alternatively, a reduction in mitochondrial diversity may be related to Wolbachia. In the latter case the mtDNA is carried passively as the microorganism sweeps through the population, a parasite-induced selective sweep.

MATERIALS AND METHODS

Collection of flies and preparation of DNA: Adult Drosophila were collected by M. KREITMAN at a fruit packing plant in Lantana Florida on 1 May 1994 and reared in the laboratory on standard yeast/cornmeal food vials. We did not collect flies from California because extensive collecting by TURELLI and HOFFMANN (1991, 1995) has shown that Wolbachia has swept through most populations to near-fixation. Thus, we would not *a priori* expect neutral amounts of mitochondrial variation. Instead we focused on a geographical location, southern Florida, where Wolbachia was not known to have swept to a high frequency. Specifically, we identified a population from Lantana where the infection status was at intermediate frequency. A collection of 125 *D. simulans* isofemale lines was established from this population. After maintaining the flies at 25° for two generations DNA was prepared according to the method of EDWARDS and HOY (1993).

Wolbachia PCR assay: We tested each isofemale line multiple times to establish the infection status of each line. TURELLI and HOFFMANN (1995) found that PCR negative results of four single-fly preparations were consistent (>98%) with progeny tests for presence of Wolbachia. Two independent PCR reactions employing the 16S rDNA primers and conditions of O'NEILL *et al.* (1992) were employed to test for the presence or absence of Wolbachia microorganisms in two flies from each of the 125 isofemale lines. These primers amplify a wide range of rickettsial microorganisms and, therefore, within-species primer binding site variation is not likely to be a problem. A third individual from each of 35 randomly selected uninfected isofemale lines confirmed that these randomly selected lines were not infected. We paid careful attention to the problems of template quality and PCR reliability. As a positive control for each DNA preparation and PCR amplification, a 1200-bp fragment of the *D. simulans* cytochrome *b* gene was coamplified using the conditions and primers of BALLARD and KREITMAN (1994).

The Wolbachia 16S rDNA amplified from two lines was sequenced using both amplification primers and opposite strand internal primers (237 + s CACACTGGAACTGAGATACG and 366 − s CCGTCATTATCTTCCTCACT) with an ABI automated sequencer following BALLARD and KREITMAN (1994).

Drosophila mtDNA: Single individuals from each of 35 randomly selected uninfected isofemale lines and a single individual from each of four infected isofemale lines were used for mtDNA analysis. Three 300- to 400-bp regions of mtDNA were selected for sequencing based on their high rates of substitution. This ensured a conservative comparison with the autosomal locus. The divergence between *D. melanogaster* (CLARY *et al.* 1982; DE BRUIJN 1983; SATTA *et al.* 1987; BENKEL *et al.* 1988; GARESE 1988; SATTA and TAKAHATA 1990; BALLARD *et al.* 1992; KANEKO *et al.* 1993; LEWIS *et al.* 1996) and *D. yakuba* (CLARY and WOLSTENHOLME 1985), and between *D. melanogaster* and *D. simulans* (SATTA *et al.* 1987; SATTA and TAKAHATA 1990; KANEKO *et al.* 1993; BALLARD and KREITMAN 1994; RAND *et al.* 1994) was compared in a 100-bp sliding window. The A + T rich-region was excluded from the analysis due to the difficulty of alignment (LEWIS *et al.* 1994). From the sliding window analysis we identified the cytochrome oxidase subunit I (COI), NADH dehydrogenase subunit 5 (ND5) and NADH dehydrogenase subunit 6 (ND6) genes as regions of high divergence (Figure 1). We conducted a second sliding-window analysis on each of these regions at a higher resolution (20-bp window) to identify subregions for sequencing (Figures 2–4). DNA amplification and sequencing primers are presented in Table 1. The amplification and sequencing protocol follows BALLARD and KREITMAN (1994).

Drosophila autosomal DNA: To compare the variability observed in the mtDNA we selected the first intron of *Adh'* because the pattern of variability at this locus is compatible with a neutral equilibrium model in *D. simulans* (SUMNER 1991). *Adh'* is downstream of *Adh* and is related to that gene by an ancient tandem duplication. *Adh'* consists of three nonoverlapping open reading frames that are conserved between *D. pseudoobscura* and *D. simulans* (SCHAEFFER and AQUADRO

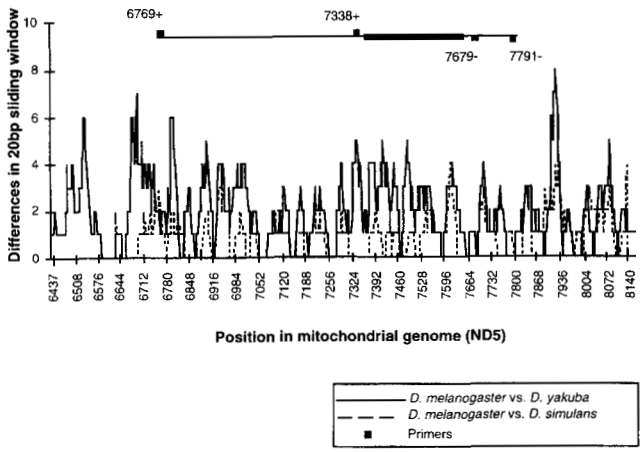

FIGURE 3.—Number of differences between the NADH dehydrogenase subunit 5 (ND5) gene of *D. melanogaster* and *D. yakuba* and between *D. melanogaster* and *D. simulans* in a 20-bp sliding-window. The primer sites are indicated at the top of the figure. The region amplified is indicated by a thin black line and the region sequenced by a thick black line.

1987). To ensure that one copy of the locus was sequenced, we crossed males of *D. simulans* with virgin females of *D. melanogaster* containing an *Adh* deficiency (Df(2L)A178, b rd[s] pr cn/In(2LR)O, Cy dp[lvI] pr cn[2]). The genotype of F_1 hybrids was easily scored by wing phenotype. Straight-winged F_1 females were chosen for sequencing as these females are hemizygous for the *D. simulans Adh'* locus. We obtained *Adh'* sequence data for 27 of the 35 uninfected lines. DNA amplification and sequencing primers are presented in Table 1. The amplification and sequencing protocol follows BALLARD and KREITMAN (1994) but the annealing temperature for both PCR and cycle sequencing was 56°.

The genealogical relationship of alleles was analyzed by parsimony using PAUP 3.2d (SWOFFORD 1993). Each insertion and deletion was scored as an additional character. Permutation tail probability (PTP) testing was employed to investigate phylogenetic structure (ARCHIE 1989; FAITH and CRANSTON 1991). Topological permutation tail probability (T-PTP) testing (FAITH 1991) and bootstrapping (EFRON 1982; FELSENSTEIN 1985) were used to test monophyly of the Lantana lines.

Statistical analysis: To compare the levels of variation of

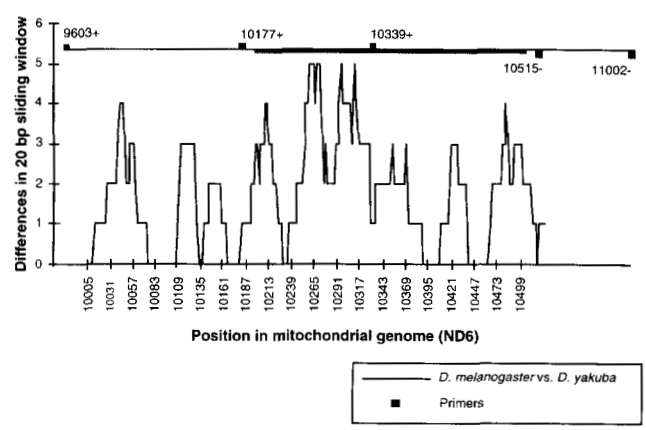

FIGURE 4.—Number of differences between the NADH dehydrogenase subunit 6 (ND6) genes of *D. melanogaster* and *D. yakuba* in a 20-bp sliding-window. The primer sites are indicated at the top of the figure. The region amplified is indicated by a thin black line and the region sequenced by a thick black line.

TABLE 1

Primers for the amplification and sequencing of the cytochrome oxidase I (COI), NADH dehydrogenase subunit 5 (ND5), NADH dehydrogenase subunit 6 (ND6) and intron 1 of alcohol dehydrogenase-duplicated (*Adh'*) loci of *D. simulans*

Locus	5′	3′
COI		
1378+a	CAGTTTGATATCATTATTGA	
1797+s	TACTACCCCCTGCTCTTTCT	
2240−s	TTTTCCTGATTCTTGTCTAA	
2439−s	GAGTTCCATGTAAAGTAGCT	
ND5		
6769+a	CCCCCACAATACATACAAAT	
7338+s	AAAGTAGATAAAGCAATAAT	
7679−s	GCTTTTCTTCTTGCTATTGC	
7791−a	AGGGTGAGATGGATTAGGAC	
ND6		
9603+a	TAATCATTACCATGAGTACG	
10177+s	TTTTTAGGAGGAATACTTGT	
10339+s	ATAAACAATGACATACAATC	
10515−s	TTTCGTAAAGGTTTATTCAT	
11002−a	GGCAAACCCACCTCATAATC	
Adh'		
2359+a	TGACCGTCACCTTCTACCCC	
3385+s	GGAGACCAGCAAGGTTCTCA	
3514+s	GTGACCGTGCCCATTGCCGA	
3690−s	TCCGTTCTGGTTCAGCTCGA	
3909−s	GTCACGTCGTAGGTCCAGAA	
3977−a	GACATCGATGTAGTCCATTT	

The numbering of mitochondrial primers corresponds to the 3′ position in *D. yakuba* mtDNA (CLARY and WOLSTENHOLME 1985). The numbering of *Adh'* primers corresponds to the 3′ position in the *D. simulans* pCAS clone sequence (COHN and MOORE 1988). An a denotes an amplification primer and s a sequencing primer. The +/− denotes the DNA strand.

mitochondrial and autosomal genes we modified Equation 5 of the HKA test, (HUDSON *et al.* 1987) so that the effective population size of mitochondrial genes was one-quarter that of autosomal genes. The HKA test is a conservative test of an equilibrium neutral model's prediction that polymorphism within species and divergence between species will be positively correlated. The mtDNA polymorphism in *D. simulans* Lantana was compared to the corresponding level in *Adh'*. Divergence was estimated by comparing a single sequence of each gene with a sequence from *D. melanogaster*.

Cytoplasmic incompatibility assay: Two randomly selected strains PCR-positive for Wolbachia infection (lines 28 and 34) and one randomly selected PCR-negative strain (line 82) were used in the incompatibility assays described below. We were unable to test additional infected lines because of an incubator failure. The infection status of these three lines was also determined by confocal microscopy examination of eggs as described by KOSE and KARR (1995). Bacteria were visualized using either the DNA-specific fluorochrome Chromomycin A_3, or with a Wolbachia-specific monoclonal antibody, Wol-1 (KOSE and KARR 1995).

Incompatibility assays were performed as described by BOYLE *et al.* (1993). Briefly, strains were crossed by mating single virgin females with two males. Cytoplasmic incompatibility crosses were performed using 5- to 7-day-old virgin males and virgin females. Females were collected within 6 hr after eclosion and stored in vials for 5–7 days to allow deposition

of unfertilized eggs and virginity was assured by discarding females from vials that contained larvae. Lines 28 and 34 were cured of Wolbachia infection by tetracycline treatment as previously described (O'NEILL and KARR 1990). Cytoplasmic incompatibility as determined by percentage mortality was compared using ANOVA with Bonferroni/Dunn correction following arcsin square root transformation. Fitness effects as determined by egg counts were examined by ANOVA following a natural logarithm transformation. To investigate the significant effects in the ANOVA we employed Fisher's Protected Least Significant Difference (PLSD) test.

RESULTS

Wolbachia PCR assay: Fifty of 125 isofemale lines collected in Lantana were positive for Wolbachia as determined by PCR assay. Two of these amplicons were sequenced from position 102 to 821 of the *Escherichia coli* 16S rRNA genome. This sequence corresponds to positions 81–662 of the previously published *Wolbachia* sequences isolated from *D. simulans* Riverside (ROUSSET *et al.* 1992; O'NEILL *et al.* 1992) without any substitutions, insertions or deletions. These data imply that only one strain of Wolbachia infected these two lines and suggest the Wolbachia isolated from *D. simulans* Lantana is very closely related to the Wolbachia isolated from *D. simulans* Riverside. Sixteen variable positions have been observed in 803 bp of the four Wolbachia 16S rDNA sequences isolated from *D. simulans* (ROUSSET *et al.* 1992; O'NEILL *et al.* 1992; ROUSSET and SOLIGNAC 1995) indicating that this locus has the potential to distinguish strains of Wolbachia.

Drosophila mtDNA: One synonymous substitution was observed at the COI locus in one of the 35 uninfected lines. This was a substitution from CGG to CGA (Arg) at position 1983 in the upper strand of CLARY and WOLSTENHOLME (1985). The ND5 locus in all *D. simulans* Lantana (uninfected and infected) had a fixed replacement at position 7409 of CLARY and WOLSTENHOLME (1985) and position 715 of RAND *et al.* (1994). The substitution replaces an ATA (Met) with a TTA (Leu). No substitutions were observed at the ND6 locus. No differences were observed at the COI or the ND6 loci in the four infected lines.

Drosophila autosomal DNA: We obtained *Adh*^{r} sequence data for 27 of the 35 uninfected lines. Nineteen substitutions, four deletions and three insertions were observed in a 446-bp region of *Adh*^{r} from *D. simulans* (Table 2). Two standardized estimators of nucleotide polymorphism, nucleotide diversity (π = 0.0134) and the neutral parameter ($\hat{\theta}$ = 0.0146 per site) indicate a high level of nucleotide polymorphism. This is larger than the neutral parameter estimate of polymorphism for introns 1 + 2 of *D. melanogaster Adh*^{r} ($\hat{\theta}$ = 0.007) and is consistent with the general observation of greater nucleotide polymorphism in *D. simulans* than in *D. melanogaster* (AQUADRO 1992).

Phylogenetic analysis of this region using the *D. melanogaster* sequence as the outgroup shows little resolution (Figure 5), though there is significant structure in the dataset (*PTP* ≤ 0.01). The *D. simulans* lines from Lantana are not significantly nonmonophyletic (*T-PTP* = 0.13, four steps) relative to the previously published sequence of COHN and MOORE (1988). This result is consistent with the tenet that there is little population substructure in North American *D. simulans*.

TABLE 2

Summary of variation in a 446-bp region of the first intron of *Adh*^{r} from *D. simulans*

Position[a]	Substitution (consensus to variant)	Frequency
19	G → A	4/27
21	T → G	1/27
45	A → G	9/27
90	T → G	1/27
92	A → – (deletion)	1/27
113	A → T	3/27
125	A → C	7/27
194	C → T	1/27
198	A → T	5/27
207	G → T	11/27
210	C → G	3/27
240	T → A	1/27
247	T → –(deletion)	9/27
248	→ T (insertion)	9/27
249	→ T (insertion)	4/27
272	A → T	1/27
321	G → A	2/27
335 + 336	AT → –(deletion)	1/27
337 + 338	AT → –(deletion)	8/27
343	T → C	27/27[b]
372	T → A	1/27
389	G → T	4/27
403	A → G	3/27
407	C → T	5/27
431 + 432	→ GC (insertion)	2/27
439	C → T	6/27

[a] Position 1 in this table refers to position 3403 of COHN and MOORE (1988).

[b] All Lantana lines differ from COHN and MOORE (1988).

Statistical analysis: To determine whether the lack of mtDNA polymorphism in uninfected lines (a total of one segregating site in 35 sequences) is significantly lower than the corresponding level in *Adh*^{r} (19 segregating sites in 27 sequences), we conducted an HKA test (HUDSON *et al.* 1987). HKA segregating sites test is significant for both Wolbachia uninfected lines (χ^2 = 4.9, P = 0.03) and for Wolbachia uninfected and infected pooled lines (χ^2 = 5.4, P = 0.02), indicating a departure from neutrality (Table 3). Unless the polymorphism level in *D. simulans Adh*^{r} has been elevated by balancing selection, which we deem unlikely, the mtDNA polymorphism level in uninfected flies must be suppressed below its neutral equilibrium value. The *D. simulans* Lantana lines must have been recently swept of variation by genetic hitchhiking.

Cytoplasmic incompatibility assay: Concordant with the results of the PCR assay, confocal microscopy examination of eggs showed that *D. simulans* Lantana lines

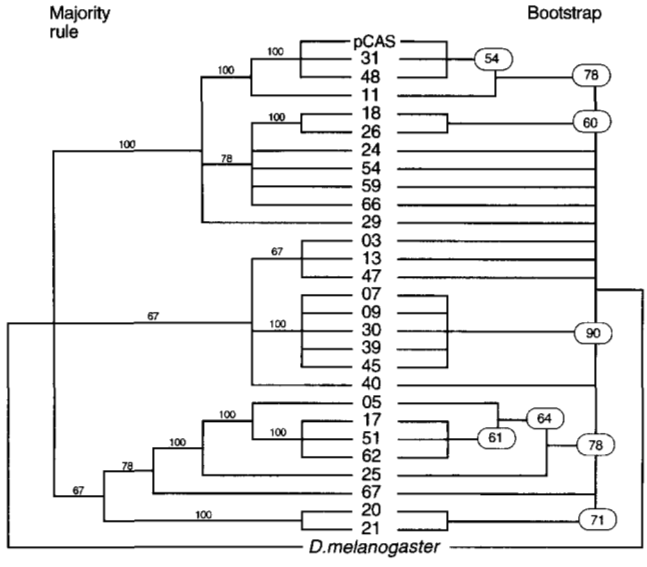

FIGURE 5.—Fifty percent majority rule and 50% bootstrap consensus derived from parsimony analysis of 446 sites in intron 1 of *Adh*^{r}. Each insertion/deletion event was scored once. In the initial parsimony analysis 216 trees of length 57 steps (CI = 0.823, RI = 0.867) were generated. On the left hand side of the figure the 50% majority rule consensus of the 216 shortest trees is presented (percentage shown above each line). On the right hand side of the figure the 50% consensus bootstrap from 1000 iterations is shown (percentage in circles at each node). The "pCAS" refers to the *D. simulans* pCAS clone sequence (COHN and MOORE 1988). The numbers correspond to isofemale lines of *D. simulans* Lantana not infected with Wolbachia. The defined outgroup was *D. melanogaster* (KREITMAN and HUDSON 1991).

28 and 34 were infected with Wolbachia while line 82 was not infected (Figure 6). Moreover, eggs from the Lantana lines and the R line that were tested harbor different densities of Wolbachia (Figure 6). Preliminary estimates indicate that bacterial numbers in 28 and 34 are approximately twofold lower than that observed in *D. simulans* from Riverside California (DSR) (T. L. KARR, unpublished observations). Bacterial density has been correlated to levels of incompatibility (BOYLE *et al.* 1993; BREEUWER and WERREN 1993, SINKINS *et al.* 1995). It will be interesting to make similar comparisons between all strains.

In lines 28 and 34 Wolbachia infection in both males and females caused a significant reduction in the number of eggs laid. For line 28 treatment with tetracycline significantly increased the number of eggs laid. ANOVA shows that there is significant heterogeneity between rows *k*, *l* and *n* of Table 4 ($F_{2,34} = 4.13$, $P = 0.02$). Fisher's PLSD test shows a significant difference only between rows *k* and *l*, and between *k* and *n* ($P = 0.02$ for each). For line 34, ANOVA shows that there is significant heterogeneity between rows *r*, *s* and *u* of Table 4 ($F_{2,38} = 3.2$, $P = 0.05$). Fisher's PLSD test shows a significant difference only between rows *r* and *u* ($P = 0.02$) but not between *r* and *s* ($P = 0.08$). Reduction in egg laying in DSR is between 8 and 18% (HOFFMANN *et al.* 1990). The reduction in egg laying in lines 28 and 34 is higher, ranging from approximately 22 to 46%. It remains to be determined if these differences are due to the strain of Wolbachia, the lines of *D. simulans*, or both.

The presence of Wolbachia infection suggested that lines 28 and 34 would express cytoplasmic incompatibility. To determine levels of incompatibility, egg mortality was measured in lines 28 and 34 and in lines that had been treated with tetracycline to remove any microorganisms. In all cases reported, infection status of infected and uninfected lines was independently determined by

TABLE 3

Single species HKA test comparing mitochondrial diversity and autosomal variability in a population of *D. simulans* collected in Lantana

Region	Infection status	No.[a]	Starting position[b]	Length[c]	Polymorphism[d]	Divergence[e]	χ^2 deviation[f]	Probability
Adh^r	−	27	3403	446	25	26		
COI	−	35	1801	386	1	27		
ND5	−	35	7368	328	0	13		
ND6	−	35	10189	321	0	12		
Total uninfected	−	35		1035	1	52	4.90	0.03
COI	+	4	1801	386	0	27		
ND5	+	4	7368	328	0	13		
ND6	+	4	10189	321	0	12		
Total infected	+	4		1035	0	52	2.16	0.142
Total mtDNA	−/+	39		1035	1	52	5.09	0.02

[a] Number of lines sequenced.

[b] The mitochondrial positions correspond to CLARY and WOLSTENHOLME (1985). The *Adh*^{r} sequence position corresponds to COHN and MOORE (1988).

[c] Includes insertions.

[d] Polymorphisms within *D. simulans* Lantana.

[e] COI divergence between line 13 and DE BRUIJN (1983). ND5 divergence between line 31 and GARESSE (1988). ND6 divergence between line 17 and GARESSE (1988). *Adh*^{r} divergence between line 3 and *D. melanogaster* (KREITMAN and HUDSON 1991).

[f] We modified the HKA test, Equation 5 of HUDSON *et al.* (1987) so the effective population size of mitochondrial genes was one-quarter that of autosomal genes.

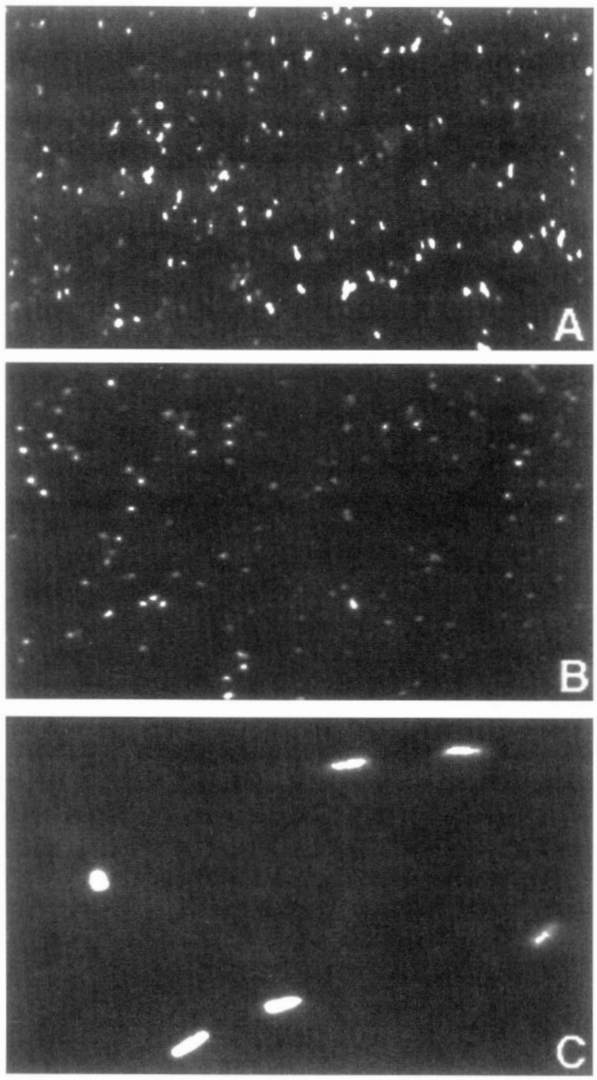

FIGURE 6.—Microscopic analysis of *D. simulans* eggs. Eggs were fixed and stained with a DNA-specific dye as described in MATERIALS AND METHODS. Stained eggs were examined for the presence of Wolbachia by confocal microscopy. (A) Numerous small punctate dots were observed in eggs laid by females of *D. simulans* Riverside. (B) Fewer punctate dots were observed in eggs laid by females of *D. simulans* Lantana line 28. (C) Only ovoid nuclei of the host egg were observed in eggs from line 82.

DAPI staining and immunofluorescence examination of eggs as described in MATERIALS AND METHODS (KOSE and KARR 1995). For comparison, egg mortality was also measured in DSR and a tetracycline-treated uninfected line of DSR (DSRT) (HOFFMANN and TURELLI 1986). As shown in Table 4 crosses between similarly infected or uninfected lines of 28 and 34 displayed relatively low egg mortality (crosses *k, n, r* and *u*; range 1.8–10.7%). These values were not significantly different from egg mortality measured within either DSR or DSRT (crosses *a, f*, respectively, Table 5). Egg mortality was higher in crosses between uninfected females and infected males of lines 28 and 34 (crosses *l, s*). Statistical analysis indicated that the increased egg mortality was significantly different compared to the parental lines. However, the levels of egg mortality in lines 28 and 34 were considerably lower compared to egg mortality in DSR (cross *e*). ANOVA with Bonferroni/Dunn correction comparing egg mortality between these lines clearly show they are statistically significantly different (*e vs. g*; *e vs. s*, respectively, Table 5).

The cytoplasmic incompatibility in lines 28 and 34 also appears to be distinct and separate from the recently described A-type. The A-type lines are infected with Wolbachia but are compatible with uninfected lines (TURELLI and HOFFMANN 1995). These data indicate that a spectrum of incompatibility levels may exist in nature. Further work will be needed to identify the relevant symbiont and/or host factors that mediate these differences.

In this study, lines 28 and 34 have been used to characterize incompatibility. However, it is not clear whether these lines represent a new crossing type (or types), or are a small sample from a continuous distribution of cytoplasmic incompatibility levels in the Lantana population. As a result we provisionally call this "L-trait" incompatibility to indicate its geographical origin (Lantana) and to distinguish it from other crossing types. We do not introduce the term "trait" into the literature to add additional unnecessary nomenclature but rather to denote some uncertainty over the cytoplasmic incompatibility status of the strain of Wolbachia parasitizing flies collected from Lantana.

DISCUSSION

The lack of mitochondrial variation compared to the autosomal locus, Adh^r, provides the best evidence yet for the loss of variation by genetic hitchhiking in one population of *D. simulans*. Two previous studies found evidence for reduced nucleotide diversity in mitochondrial DNA in this species (BALLARD and KREITMAN 1994; RAND *et al.* 1994). However, neither study quantified nuclear gene variability in the same lines, rather both relied on published estimates of nuclear gene diversity in other populations. Here, we have conclusive evidence for a nonneutral pattern of nucleotide variability in mitochondrial DNA.

If there has been no prior selection or hitchhiking in the mitochondrial genome, uninfected isofemale lines are expected to exhibit neutral levels of variation. In this study we observed a significant reduction in the mtDNA diversity based on the HKA test in both the Wolbachia infected and the pooled sample of Wolbachia uninfected and infected flies. The selective sweep of mitochondrial DNA may have been generated by the fixation of an advantageous mutation in the mitochondrial genome. Alternatively, the dramatic reduction in mitochondrial diversity may be related to a Wolbachia induced selective sweep. For the latter to be true, infected females must have become largely cured of infection or produced uninfected offspring.

In the laboratory, infected flies may be cured by antibiotics or by high temperature. Eight out of 10 males

TABLE 4

Expression of cytoplasmic incompatibility in strains 28 and 34 of *D. simulans* Lantana

	Cross						
	Female × male		No. eggs	No. unhatched eggs	No. females	No. eggs/female[a]	Egg mortality (%)[a]
a	DSR[b]	DSR	935	62	11	85 ± 9.3	6.7 ± 0.9
b	DSR	DSRT[c]	550	46	11	50.0 ± 5.1	9.6 ± 2.6
c	DSR	28[d]	1255	176	30	41.8 ± 2.2	17.3 ± 3.5
d	DSR	34[e]	796	93	27	29.5 ± 4.0	13.9 ± 3.3
e	DSRT	DSR	1203	1078	12	100.2 ± 4.6	89.0 ± 3.2
f	DSRT	DSRT	888	58	11	80.7 ± 3.8	4.5 ± 0.17
g	DSRT	28	804	47	19	42.3 ± 4.3	9.4 ± 3.2
h	DSRT	34	899	107	22	40.9 ± 5.1	15 ± 4.2
i	28	DSR	1314	678	19	69.2 ± 6.5	50.3 ± 7.8
j	28T	DSR	1065	1009	19	56.1 ± 5.7	94.4 ± 1.0
k	28	28	284	29	10	28.4 ± 3.1	10.7 ± 3.6
l	28T	28	828	192	17	48.7 ± 4.6	25.8 ± 7.1
m	28	28T	939	47	18	52.2 ± 4.4	5.0 ± 1.1
n	28T	28T	471	21	9	52.3 ± 4.6	4.4 ± 1.0
o	28	DSRT	261	85	9	29 ± 3.7	36.3 ± 7.6
p	34	DSR	1557	1380	19	81.9 ± 7.8	88.2 ± 5.3
q	34T	DSR	1215	1078	19	63.9 ± 3.8	87.3 ± 4.6
r	34	34	391	7	11	35.5 ± 3.8	1.9 ± 0.8
s	34T	34	1125	240	20	56.2 ± 2.6	19.8 ± 4.7
t	34	34T	827	26	20	41.4 ± 3.2	3.9 ± 1.5
u	34T	34T	460	18	10	46.0 ± 3.4	1.8 ± 0.5
v	34	DSRT	250	28	12	20.8 ± 2.7	11.4 ± 2.9

[a] Values are means ± SE.
[b] *D. simulans* Riverside (infected with Wolbachia).
[c] *D. simulans* Riverside treated with tetracycline (not infected with Wolbachia).
[d] *D. simulans* Lantana line 28 (infected with Wolbachia); 28T, tetracycline-treated line (not infected).
[e] *D. simulans* Lantana line 34 (infected with Wolbachia); 34T, tetracycline-treated line (not infected).

and nine of 10 females of *Tribolium confusum,* for example, were cured of Wolbachia when they were raised in the laboratory on wheat grain molded with *Streptomyces aureofaciens* (STEVENS and WICKLOW 1992). *S. aureofaciens* is a common soil-inhabiting, tetracycline-producing actionomycete bacterium. It is not known whether an environmental factor, such as antibiotic-producing bacteria or high temperature has reduced the population infection rate to the low level observed in the Lantana population. However, unless this population's infection is in the process of sweeping to fixation, it is unlikely that the 40% infection rate is stable in the absence of an infection-reducing mechanism (TURELLI and HOFFMAN 1995).

Confocal microscopy shows that the density of Wolbachia in the Lantana lines is lower than that in the *D. simulans* R-type (Figure 6). The lower infection density may also contribute to a higher cure rate. It has been proposed that density variation of Wolbachia may directly effect the numbers of infected progeny that are produced (HOFFMANN *et al.* 1990; TURELLI and HOFFMANN 1995). In support of this view, TURELLI and HOFFMANN (1995) showed that on average infected females from nature produce 3–4% uninfected ova, with a small number of females contributing disproportionately to the number of uninfected males.

The density of infection also affects the expression of cytoplasmic incompatibility. Density effects on the expression of cytoplasmic incompatibility in the wasp Nasonia (BREEUWER and WERREN 1993), *D. melanogaster* (BOYLE *et al.* 1993) and possibly *Aedes albopictus* (SINKINS *et al.* 1995) have been demonstrated in the laboratory. A lowering of the absolute numbers of Wolbachia either by administration of antibiotics (BREEUWER and WERREN 1993) or by horizontal transfer into a related species (BOYLE *et al.* 1993) resulted in a significant reduction in the levels of incompatibility. There is also a well documented decrease in cytoplasmic incompatibility with male age in *Culex pipiens* (SINGH *et al.* 1976; KRISHNAMURTHY *et al.* 1977) and Drosophila spp. (HOFFMAN *et al.* 1986, 1994; HOFFMAN 1988). These data in other species raise the possibility that the observed cytoplasmic incompatibility in the Lantana flies also may be related to Wolbachia density.

In this study 40% of lines collected in Lantana were positive for Wolbachia as determined by PCR assay. CASPARATI and WATSON (1959) show that unless a fecundity deficit or imperfect transmission is found in nature any level of compatibility will suffice to cause a sweep. In this study we show that the L-trait of Wolbachia causes reduced fecundity in the laboratory (Table 4). Reduced fecundity based on laboratory crosses, however, may not be a good indicator of cytoplasmic incompatibility in nature. HOFFMANN *et al.* (1990) ana-

TABLE 5

Analyses of cytoplasmic incompatibility in *D. simulans* strains from California and Florida

Comparison		
Cross	*p*	Comments
a vs. e	*	Standard R crossing type
a vs. c	NS	28 males crossed to DSR females do not express CI[a]
a vs. d	NS	34 males crossed to DSR females do not express CI
a vs. k	NS	DSR and 28 have indistinguishable basal egg hatch rates
a vs. r	NS	DSR and 34 have indistinguishable basal egg hatch rates
e vs. j	NS	DSR males mated to uninfected 28 females are indistinguishable from R-type CI
e vs i	*	DSR males mated to infected 28 females are not R-type
e vs. l	*	R and 28 CI levels are statistically different
e vs. s	*	R and 34 CI levels are statistically different
f vs. n	NS	28T is indistinguishable from W-type
f vs. u	NS	34T is indistinguishable from W-type
i vs. j	*	Incompatibility type of line 28 is different from R-type. CI levels of infected 28 females crossed to DSR males are different from CI levels of uninfected 28 females crossed to DSR males.
i vs. l	*	CI in line 28 is lower than CI expression elicited by DSR males mated to 28 females
k vs. r	NS	Lines 28 and 34 have similar egg hatch rates
j vs. q	NS	28T and 34T hatch rates are indistinguishable (and both express CI at the R-type levels when in matings with DSR males)
l vs. s	NS	CI expression in 28 and 34 are indistinguishable
l vs. n	*	CI in line 28
p vs. q	NS	Unlike line 28, both line 34 and 34T females mated to DSR males express R-type CI
s vs. u	*	CI in line 34

* Significant to 95% confidence interval by Bonferroni/Dunn ANOVA; NS, not significant.
[a] Cytoplasmic incompatibility.

lyzed several fitness-related traits of R-strain infected and uninfected flies and found that laboratory infected females were 8–18% less fecund than uninfected females. NIGRO and PROUT (1990) compared the net "productivity" (including viability and fecundity effects) in strains from Italy and found 6–11% lower productivity for their infected strain. Similar comparisons between untreated and treated lines in Table 10 of HOFFMANN and TURELLI (1988) imply reduction of 10–31%. However, the fecundity deficit caused by the R-strain of Wolbachia was shown to be small in experimental crosses carried out in a "natural" setting (HOFFMANN *et al.* 1990; TURELLI and HOFFMANN 1995) and, in general, R-strains of Wolbachia sweep to near-fixation.

The mtDNA sequences from the uninfected and infected lines share a fixed difference in the ND5 locus compared to *D. simulans* lines from several other natural populations (GARESE 1988; RAND *et al.* 1994) indicating that the sweep equally affected all lines tested and was geographically restricted and/or very recent. It is not clear that the L-trait of Wolbachia has the potential to sweep through a population or that the loss in mitochondrial diversity in all 35 uninfected lines can be solely explained by the loss of the current parasite. Thus, it is possible that the previous selective sweep may have predated the current L-trait incompatibility. Again, a previous selective sweep may have been generated by the fixation of an advantageous mitochondrial gene or may be related to a previous unrelated Wolbachia induced selective sweep. If a previous selective sweep was related to Wolbachia, the previous Wolbachia strain may have been lost from the population or the evolutionary dynamic of the strain may have changed. In this latter case mutation in either the parasite or the host may have affected the density of Wolbachia and cytoplasmic incompatibility.

If a reduction in cytoplasmic incompatibility results from mutation in the parasite or the host it may be predicted that reduced cytoplasmic incompatibility will be observed independently in other populations. *D. simulans* cytoplasmic incompatibility type A populations behave in progeny tests as if they were uninfected and have been recorded in Australia, Ecuador and in Tampa, USA (TURELLI and HOFFMANN 1995). If a reduction in incompatibility is due to relatively recent substitution(s), the DNA sequence of Wolbachia types L and A is expected to be similar to Wolbachia type R. Consistent with this prediction, we did not observe any substitutions, insertions or deletions in the 16S rRNA of Wolbachia isolated from two *D. simulans* lines from Lantana compared to the Wolbachia isolated from *D. simulans* Riverside (O'NEILL *et al.* 1992; ROUSSET *et al.* 1992).

The hypothesis that Wolbachia may be lost from a host due to environmental or genetic factors has implications for the study of mitochondrial DNA in other species. SOLIGNAC *et al.* (1994) provided evidence of Wolbachia infection of *D. melanogaster* early in the evolu-

tion of the species, with subsequent cycles of loss and gain of the microorganism that now causes an A type of incompatibility (HOLDEN *et al.* 1993). Consistent with this view, BALLARD and KREITMAN (1994) found evidence of excess homozygosity in cytochrome *b* haplotypic diversity of a world-wide collection of 16 isofemale *D. melanogaster* lines. Such a pattern may be expected in the recovery phase following a selective sweep.

While genetic drift may be the prevailing force in mitochondrial molecular evolution, the mitochondrial genome is particularly susceptible to genetic hitchhiking at linked sites and the allele frequencies may rarely be at the stationary neutral distribution. Indeed any maternally inherited factor could influence haplotypic diversity. This study raises the possibility that the presence of Wolbachia has not only caused the loss of mtDNA variability among infected individuals but also among individuals that no longer harbor the parasite.

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