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# Decreased Diversity but Increased Substitution Rate in Host mtDNA as a Consequence of Wolbachia Endosymbiont Infection

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ABSTRACT

A substantial fraction of insects and other terrestrial arthropods are infected with parasitic, maternally transmitted endosymbiotic bacteria that manipulate host reproduction. In addition to imposing direct selection on the host to resist these effects, endosymbionts may also have indirect effects on the evolution of the mtDNA with which they are cotransmitted. Patterns of mtDNA diversity and evolution were examined in *Drosophila recens*, which is infected with the endosymbiont Wolbachia, and its uninfected sister species *D. subquinaria*. The level of mitochondrial, but not nuclear, DNA diversity is much lower in *D. recens* than in *D. subquinaria*, consistent with the hypothesized diversity-purging effects of an evolutionarily recent Wolbachia sweep. The $d_N/d_S$ ratio in mtDNA is significantly greater in *D. recens*, suggesting that Muller's ratchet has brought about an increased rate of substitution of slightly deleterious mutations. The data also reveal elevated rates of synonymous substitutions in *D. recens*, suggesting that these sites may experience weak selection. These findings show that maternally transmitted endosymbionts can severely depress levels of mtDNA diversity within an infected host species, while accelerating the rate of divergence among mtDNA lineages in different species.

INNUMERABLE species of insects and other arthropods are infected with maternally transmitted endosymbionts. Among the most prevalent of these are bacteria that manipulate host reproduction in a variety of ways to increase the relative transmission rates of infected lineages (Stouthamer *et al.* 1999). The two most widely reported modes of reproductive manipulation are cytoplasmic incompatibility (CI), in which matings between infected males and uninfected females result in high levels of offspring mortality, and male killing, in which the male offspring of infected females suffer high rates of embryonic mortality (Stouthamer *et al.* 1999). The substantial mortality caused by these infections is likely to favor the evolution of countermeasures by the host species. These endosymbionts may also affect the evolution of their hosts in more subtle ways, having specific effects on the population genetics and molecular evolution of host mitochondrial DNA (Turelli and Hoffmann 1991; Turelli *et al.* 1992; Johnstone and Hurst 1996). Because of the maternal transmission of both elements, patterns of molecular evolution of endosymbionts and mtDNA are more strongly correlated with each other than with nuclear alleles within a species.

The spread of a maternally transmitted microorganism will result in the hitchhiking of all maternally inherited organelles, including mitochondria, associated with the initially infected female (Turelli and Hoffmann 1991; Solignac *et al.* 1994; Turelli 1994; Turelli and Hoffmann 1995; Hoffmann *et al.* 1998). The mtDNA will be forced through a bottleneck of one host female, from which all mtDNA haplotypes in the population will be descended. Consequently, infected populations may have lower mtDNA diversity (Caspari and Watson 1959; Fine 1978; Turelli 1994). Because the expected coalescent time of mtDNA in an uninfected species is $2N_f$, where $N_f$ is the effective population size of females (Avise 2000), a decrease in mtDNA diversity is expected to be evident for $\sim 2N_f$ generations following a Wolbachia sweep or strain replacement. Given this expectation and the large population sizes of many insect species, mtDNA diversity may be reduced for millions of generations.

Another possible effect of these infections on mtDNA arises from the small effective population size that organelles pass through as a consequence of recurrent endosymbiont sweeps. Several species of asexual endosymbionts have experienced accelerated rates of molecular evolution relative to their free-living relatives (Moran 1996; Brynnel *et al.* 1998; Peek *et al.* 1998; Clark *et al.*

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1999; Wernegreen and Moran 1999; Thao *et al.* 2000; Abbot and Moran 2002; Woolfit and Bromham 2003). This has been interpreted as a result of Muller's ratchet—the increased probability of fixation of slightly deleterious mutations in species such as endosymbionts with small effective population sizes. Consider an endosymbiont that undergoes a series of variant replacements within a host species, due perhaps to the evolution of new CI interaction types (Turelli 1994; Hurst and McVean 1996) or simply as a result of adaptation to the intracellular environment of the host. For each such turnover within the endosymbiont population, the associated mtDNA in a host species will be taken through an effective population size of one host female. Thus, Muller's ratchet is expected to affect the mtDNA of an endosymbiont-infected species, and such effects may be evident in elevated rates of substitution of slightly deleterious mutations.

Coupling of Wolbachia and mtDNA dynamics has been demonstrated in a variety of species, including *Drosophila simulans* (Turelli and Hoffmann 1991; Hoffmann *et al.* 1994; Solignac *et al.* 1994; Ballard 2000; Ballard *et al.* 2002), *D. recens* (Shoemaker *et al.* 1999), the mosquitoes *Aedes albopictus* (Kambhampati *et al.* 1992) and *Culex pipiens* (Guillemaud *et al.* 1997), the fire ant *Solenopsis invicta* (Shoemaker *et al.* 2000, 2003), the oak gallwasp *Biorhiza pallida* (Rokas *et al.* 2001), and the isopods *Porcellionides pruinosus* (Marcade *et al.* 1999) and *Armadillidium vulgare* (Rigaud *et al.* 1999). By far, the most thorough studies have been conducted on *D. simulans*. Indeed, the first study documenting the spread of a Wolbachia infection (and associated mtDNA haplotype) in nature was conducted in *D. simulans* (Turelli and Hoffmann 1991). Subsequent studies have demonstrated that this species is infected with four genetically distinct strains of Wolbachia, presumably representing four independent invasions across three distinct clades of mitochondrial haplotypes (James and Ballard 2000; Ballard 2004). There is very little sequence variation within each of the three defined haplotype clades, but substantial differences among them (Ballard 2000). Furthermore, each mitochondrial lineage is characterized by an excess of nonsynonymous relative to synonymous substitutions, consistent with a possible effect of Muller's ratchet. Much of the groundbreaking work on the effects of Wolbachia on mtDNA dynamics has focused on *D. simulans*, including studies demonstrating reduced mtDNA diversity within lineages (Turelli and Hoffmann 1991, 1995; Turelli *et al.* 1992; Ballard *et al.* 1996, 2002; Ballard 2000, 2004; James and Ballard 2000; James *et al.* 2002; Dean *et al.* 2003). However, because *D. simulans* and all of its close relatives are infected with Wolbachia, this has precluded a comparative analysis of rates of molecular evolution in Wolbachia-infected and uninfected sister taxa. Thus, the specific hypothesis that a Wolbachia infection leads to an accelerated rate of molecular evolution of the mtDNA genome remains to be tested.

In this article, we address three questions:

1. Does a Wolbachia-infected species harbor lower levels of mtDNA diversity than a closely related but uninfected species?
2. Does the mtDNA within an infected species show evidence of having experienced a greater rate of nucleotide substitution than that within an uninfected species?
3. If the infected species exhibits an elevated rate of mtDNA substitution, are the patterns of substitution consistent with expected effects of Muller's ratchet?

To address these issues, we exploit a trio of Drosophila species. *D. recens* is a mycophagous member of the quinaria species group whose range encompasses cool forested regions of eastern North America and is thought to have high rates of gene flow among populations (Shoemaker and Jaenike 1997). All populations are infected at a very high frequency (98–99%) with CI-causing Wolbachia, with the rare uninfected individuals (1–2%) resulting from incomplete maternal transmission of the endosymbiont, as they harbor mitochondrial haplotypes identical to those found in infected individuals. *D. subquinaria* is a mycophagous species found in forests of western North America and is the closest known relative of *D. recens*. Of several hundred wild-caught individuals of *D. subquinaria* surveyed from throughout the range of this species, none have been found to be infected with Wolbachia. *D. quinaria* is a closely related outgroup species that breeds in decaying vegetation and is found in eastern North America (Perlman *et al.* 2003).

## MATERIALS AND METHODS

**Samples:** *D. recens* were collected from Rochester, New York ($n = 9$), Big Moose, New York (10), Brunswick, Maine (10), Great Smoky Mountains National Park, Tennessee (17), and Edmonton, Alberta (12). *D. subquinaria* were collected from Deary, Idaho (3), Seattle, Washington (4), Port Hardy, British Columbia (9), and Peachland, British Columbia (10). One *D. quinaria* was used as an outgroup for molecular evolutionary analyses.

A previous study showed that neither *D. quinaria* nor *D. palustris* was infected with Wolbachia (Werren and Jaenike 1995). These two species represent two closely related, successive outgroups to the *recens-subquinaria* clade (Perlman *et al.* 2003). More extensive surveys of *D. quinaria* and *D. palustris* lead to the conclusion that the common ancestor of these four species was uninfected with Wolbachia (K. A. Dyer and J. Jaenike, unpublished data). Therefore, the Wolbachia infection in *D. recens* is a derived state within this species.

**DNA sequencing:** Wolbachia variation within *D. recens* was surveyed by sequencing a portion of the *wsp* gene (~590 bp) from 33 Wolbachia-infected *D. recens* using the primers *wsp* 81F and *wsp* 691R (Zhou *et al.* 1998; Shoemaker *et al.* 2003). For all sequencing, PCR amplicons were cleaned using Agencourt magnetic beads or QIAGEN (Chatsworth, CA) columns and sequenced directly using ABI Prism Big Dye terminator chemistry.

To contrast patterns of molecular evolution between *D. recens* and *D. subquinaria*, the entire mtDNA *cytochrome oxidase I* (COI) gene was amplified and sequenced from 58 individuals of *D. recens*, 26 of *D. subquinaria*, and one of the outgroup *D. quinaria*. PCR was carried out using the primers TY-J-1460 and TL2-N-3014 (Simon *et al.* 1994). These as well as two internal primers (DR-CO I internal forward, 5′-AATAATATCTACAGATGAGT TAG-3′; DR-CO I internal reverse, 5′-AGCAATTTTTTCTTTA CATTTAG-3′) were used to sequence both strands. In addition to sequencing COI, we generated sequences for a much larger portion of the mtDNA genome for 12 *D. recens*, one *D. subquinaria*, and one *D. quinaria*. This included a total 7248–8757 bp representing portions of 12 of the 14 mitochondrial protein-coding genes (primers available upon request).

To distinguish effects specific to mtDNA from factors that affect the entire genome, we sequenced three nuclear gene regions: *period, adhr* (alcohol-dehydrogenase-related protein), and *tpi* (triose phosphate isomerase). All three genes have been used to investigate patterns of divergence and polymorphism in other Drosophila, and the patterns for at least synonymous sites are consistent with the standard neutral model (Wang and Hey 1996; Gleason and Powell 1997; Andolfatto and Przeworski 2000; Rand *et al.* 2000; Weinrich and Rand 2000; Begun 2001; Przeworski *et al.* 2001; Machado *et al.* 2002). *period* is an X-linked gene in *D. recens* and in other species of Drosophila. A 642-bp coding portion was amplified and sequenced on both strands using two primers that we designed (5′-GAACGTCAACCCCAGGCGGAAGG-3′ and 5′-ACAAGGAGAAGTCCAGGAAGAAG-3′) from 36 of *D. recens*, 11 of *D. subquinaria*, and one individual of *D. quinaria*. Only male flies were used because they carry a single X chromosome and thus only one *period* allele. *adhr* and *tpi* are autosomal, with the latter likely occurring on the second chromosome in *D. recens* (Patterson and Stone 1952). A 516-bp portion of *adhr* was amplified and sequenced from 7 *D. recens*, 8 *D. subquinaria*, and one individual of *D. quinaria*, using the two previously published primers D1 and D4 (Betran and Ashburner 2000). A 381-bp coding portion of *tpi* was amplified and sequenced using two primers that we designed (5′-CAAC TGGAAGATGAAYGGIGACC-3′ and 5′-TTCTTGGCATAGGC GCACATYTG-3′) from 13 *D. recens*, 12 *D. subquinaria*, and one individual of *D. quinaria*. For both *adhr* and *tpi* we inferred heterozygous sites by manually examining the chromatograms for double peaks; we did not attempt to infer haplotypes. We sequenced a total of 14 and 26 alleles from *D. recens* for *adhr* and *tpi*, respectively.

**Levels and patterns of diversity:** To visualize relationships and relative abundances of mtDNA haplotypes within *D. recens*, a median-joining network was generated using the program Network (Figure 1; http://www.fluxus-engineering.com/sharenet.htm). One individual of *D. quinaria* was included to root the network.

Levels of polymorphism estimated included nucleotide diversity ($\pi$; Tajima 1983; Nei 1987), number of segregating sites ($\theta_w$; Watterson 1975; Nei 1987), haplotype number, and haplotype diversity (Rozas and Rozas 1999). Unless otherwise noted, the program DnaSP v.3.53 (26) was used to estimate parameters and to perform statistical analyses. We then asked whether the mtDNA has a disproportionate reduction in diversity in *D. recens*; thus, we used an HKA test to contrast levels of polymorphism and sequence divergence in 7248 bp of mtDNA with the three nuclear genes in *D. recens* and *D. subquinaria* (Hudson *et al.* 1987). For mtDNA, we included either all sites or silent sites only and for *period, adhr*, and *tpi* we used only silent sites. The difference in effective population sizes among the loci was taken into account in assessing statistical significance (Hudson *et al.* 1987).

Neutrality tests were performed on all the genes to determine if the frequency spectrum of polymorphisms conformed to the predictions of the neutral model of molecular evolution. At equilibrium with selective neutrality, both Tajima's $D$ (32) and Fu and Li's $D$ (33) are expected to be zero. A value significantly less than zero indicates a higher-than-expected number of low-frequency variants and can result from a recent selective sweep or population expansion (Tajima 1989; Aris-Brosou and Excoffier 1996).

**Rates of molecular evolution:** Rates of molecular evolution of mtDNA in *D. recens* and *D. subquinaria* were compared using a relative-rates test, as implemented in the program RRTree (Robinson-Rechavi and Huchon 2000). We estimated substitution rates for synonymous and nonsynonymous sites within the 7248-bp mtDNA sequences of the two species. We used a closely related species, *D. quinaria*, as the outgroup to increase the statistical power of the relative-rates test (Muse and Weir 1992).

To test whether differences in substitution rates were limited to mtDNA or were genome-wide, relative-rate tests were also carried out on four nuclear genes, *period, adhr, tpi*, and *R1*, the latter being a retrotransposable element that inserts into the rDNA locus. *R1* sequences, representing 781 bp of the 3′-untranslated region of the element, were obtained from one individual each of *D. recens* (GenBank accession no. AF248076), *D. subquinaria* (W. Burke and T. Eickbush, unpublished data), and *D. quinaria* (AF24874). Previous analyses have demonstrated that *R1* elements are passed vertically within species and evolve at rates similar to those of nuclear genes (Gentile *et al.* 2001).

**Characterization of substitution patterns:** To address whether Muller's ratchet has affected mtDNA evolution in *D. recens*, we asked whether the $d_N/d_S$ ratio ($\omega$) of the 7248 bp of mtDNA is elevated in this species relative to *D. subquinaria* and *D. quinaria*. The parameter $\omega$ was estimated in three ways: (1) constraining all branches in the phylogeny to have the same value, (2) allowing $\omega$ to differ between the *D. recens* and the *D. subquinaria* + *D. quinaria* branches of the tree, and (3) allowing $\omega$ to vary among all three species. Likelihood-ratio tests were used to determine if a *D. recens*-specific value of $\omega$ significantly improved the fit to the data. These analyses were performed using the codeml program in the PAML package, with expected codon frequencies estimated from the average nucleotide frequencies in the three positions and one ratio for $d_N/d_S$ among codons (Yang 1997, 2000). The data set comprises 12 mtDNA sequences from *D. recens* and thus includes both fixed and polymorphic mutations. Because only one sequence per species is available for *D. subquinaria* and *D. quinaria*, all mutations identified in *D. recens* (some of which may be polymorphic) were used to estimate its $d_N/d_S$ ratio.

We also determined whether mutational changes in these mtDNA sequences were from either G or C to either A or T or in the other direction. In *D. recens*, both polymorphic and apparently fixed mutations were identified. Because only one comparable *D. subquinaria* sequence has been obtained, polymorphic and fixed mutations in this lineage are not distinguished.

## RESULTS AND DISCUSSION

**Levels and patterns of diversity:** All 33 isolates of Wolbachia from wild-caught *D. recens* had identical *wsp* sequences, including the hypervariable regions (GenBank AY154399). Consistent with earlier studies, the Wolbachia strain present in *D. recens* falls within the A clade of Wolbachia and is closely related to the Wolbachia strains found in *D. simulans* and *D. melanogaster* (Dyer and Jaenike 2004). Because *wsp* is the most rap-

idly evolving gene known in Wolbachia (Zhou *et al.* 1998) and because Wolbachia are vertically transmitted, this lack of sequence variation indicates that all extant Wolbachia and mtDNA haplotypes within *D. recens* are descended from a single ancestral infected female. The *D. recens* mtDNA haplotype network (Figure 1) shows that this particular infection has been in the species long enough for a considerable number of mutations to accumulate. The discrepancy between the number of polymorphisms within the mtDNA and *wsp* presumably is due to a higher mutation rate in the mtDNA. While we cannot pinpoint the ancestral mtDNA haplotype, it is not the one that is currently at highest frequency (H1), as this is a derived state (Shimodaira-Hasegawa test, $P = 0.021$, rejecting an alternative tree with H1 placed ancestral to all other *D. recens* haplotypes).

Does this pattern of mtDNA variation within *D. recens* differ from its uninfected sister species *D. subquinaria*, and more importantly, from the rest of the *D. recens* genome? Analyses of polymorphism (Table 1) show that mitochondrial DNA variation is much lower in *D. recens* than in *D. subquinaria* by all measures, including the number of segregating sites ($\theta_w$), nucleotide diversity ($\pi$), intraspecific sequence divergence, and haplotype diversity. The numbers of segregating sites and nucleotide diversity at the COI gene were, respectively, 4-fold and 10-fold lower in *D. recens* than in *D. subquinaria*, whether all sites or only silent sites were considered. In contrast, the nuclear genes *period*, *adhr*, and *tpi* have higher levels of diversity in *D. recens* than in *D. subquinaria* (Table 1).

HKA tests were used to contrast patterns of polymorphism and divergence among the mtDNA sequences encompassing 12 protein-coding genes and the nuclear sequences encoding *per*, *adhr*, and *tpi* in *D. recens* and *D. subquinaria* (Hudson *et al.* 1987). In the contrasts comparing silent sites among mtDNA and the three nuclear genes, the results were significant or nearly so for all three comparisons (mtDNA-*per*, $\chi^2 = 6.06$ and $P = 0.014$; mtDNA-*adhr*, $\chi^2 = 3.78$ and $P = 0.052$; mtDNA-*tpi*, $\chi^2 = 5.82$ and $P = 0.016$). However, none of the comparisons among the nuclear genes was significant ($P > 0.5$ in every case). Again, these data support an mtDNA-specific reduction in variation within *D. recens*.

Comparing *D. recens* with another ecologically and phylogenetically related species bolsters the conclusion that this species has disproportionately low levels of mtDNA variation. *D. falleni* has a geographical range size and population density similar to those of *D. recens* yet it is not infected with Wolbachia (Shoemaker *et al.* 1999). Average heterozygosity across 18 allozyme loci is very similar between the two species [0.191 for *D. recens* and 0.185 for *D. falleni* (Lacy 1982)]. Furthermore, two autosomal loci (*tpi* and *Adhr*), whose patterns of variation are consistent with neutral expectations, exhibit very similar levels of silent-site nucleotide diversity, whether measured by $\pi$ (mean = 0.043 for both *D. recens* and *D. falleni*) or $\theta$ (mean = 0.042 for *D. recens* and 0.055 for *D. falleni*). The data for *D. falleni* were obtained from Table 4 of Dyer and Jaenike (2004). In striking contrast, mtDNA nucleotide diversity, estimated from RFLP variation, is five times greater in *D. falleni* than in *D. recens* (Shoemaker *et al.* 1999).

Next, we ask whether this reduction in mtDNA diversity in *D. recens* is the result of a Wolbachia-driven selective sweep or is, instead, consistent with demographic effects that shape both mtDNA and nuclear genes. Statistical tests of departure from neutral expectations are presented in Table 1. Both Tajima's *D* and Fu and Li's *D* were significantly less than zero for mtDNA of *D. recens*, indicating an excess of rare variants. In contrast, the nuclear genes in *D. recens* did not deviate significantly from neutral expectations, nor did either the COI or the nuclear genes of *D. subquinaria*. Thus, an excess of rare variants is specific to the mtDNA of *D. recens*, consistent with a recent selective spread of a particular mtDNA haplotype.

Population genetic data provide little evidence that an mtDNA sweep is currently occurring in *D. recens*. First, the most abundant haplotype, H1, is not at a significantly higher frequency than would be expected under neutrality [Hudson's haplotype test (31), $P = 0.06$, $P = 0.50$ if H1 and haplotypes one derived mutational step away are considered]. Furthermore, Fay and Wu's *H* (Fay and Wu 2000), which tests for an ongoing sweep by examining the frequency of derived mutations, is not significantly greater than zero for the mitochondrial COI gene of *D. recens* ($H = 0.619$; $P > 0.10$, accounting for backmutations and using *D. quinaria* as the outgroup; see Figure 1). Thus, the most recent mtDNA sweep in *D. recens* appears to be in a recovery phase that is expected to last up to $2N_f$ generations or until the next sweep occurs (Avise 2000).

In *D. recens*, decreased diversity and departure from neutrality of the mtDNA are most likely the result of hitchhiking with a Wolbachia infection during CI-driven sweep. This is consistent with the observed pattern of decreased diversity of the mtDNA relative to the rest of the genome shown in other Wolbachia-infected organisms (Ballard *et al.* 2002; Jiggins 2003). Given the high incidence of endosymbiont infection among insects and other arthropods, our results support the notion that low levels of mtDNA diversity within a species may not indicate a small effective population size overall.

**Rates of molecular evolution:** Because the mtDNA in an infected host species will be taken through an effective population size of one host female with each Wolbachia sweep, Muller's ratchet is expected to affect the mtDNA of an endosymbiont-infected species. Such effects may be evident in elevated rates of substitution. To compare rates of evolution between *D. recens* and *D. subquinaria*, we used a relative-rates test. This test requires identification of an appropriate outgroup to the taxa being compared. All regions sequenced, including

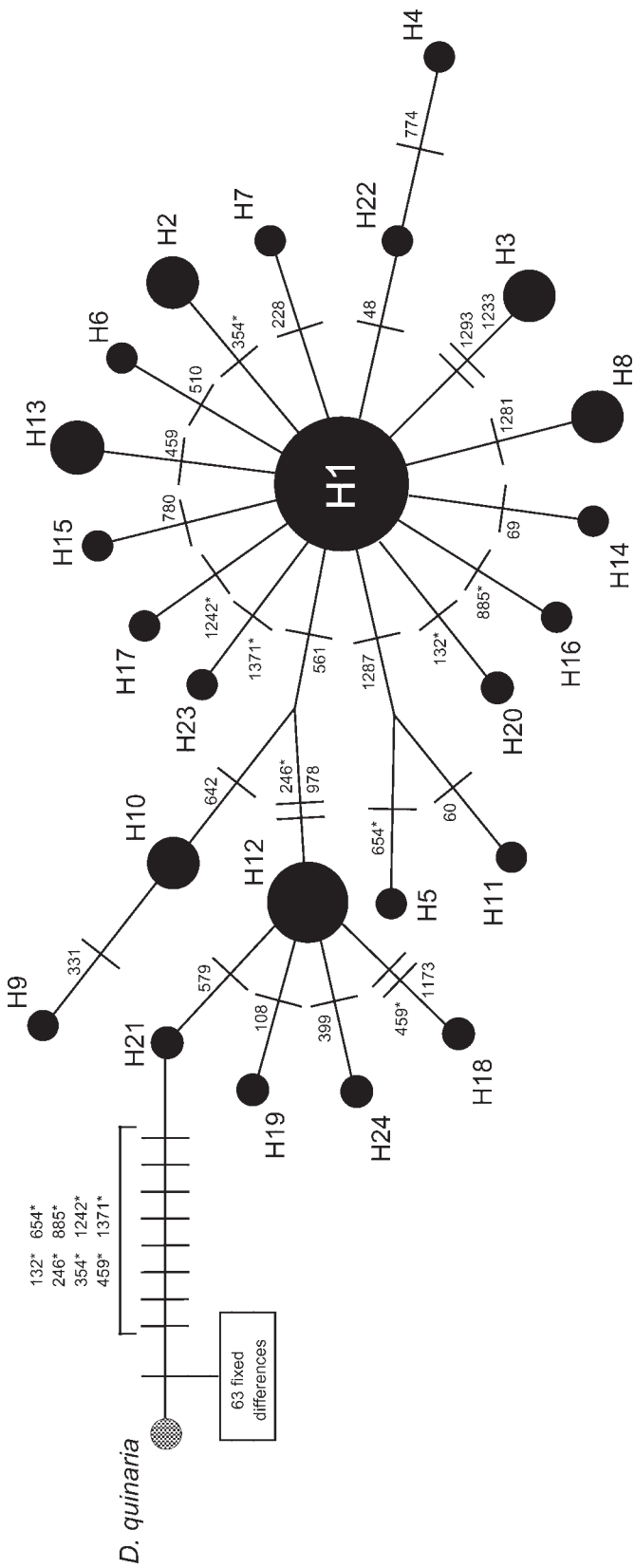


FIGURE 1.—Median-joining network of mtDNA haplotypes within *D. recens*. The frequency of each haplotype is proportional to its area. The predominant haplotype is indicated as H1. *D. quinaria* is included as an outgroup. Numbers on the network indicate the base-pair positions of mutations within the mtDNA sequences. Asterisks indicate the eight inferred back mutations within *D. recens*.

**TABLE 1**

**Nucleotide diversity estimates and tests for departure from neutrality for mtDNA and nuclear genes from *D. recens* and *D. subquinaria***

| Gene species | *N* | *L* | *h* | *S* | $\theta_w$ (total) | $\theta_w$ (silent) | $\pi$ (total) | $\pi$ (silent) | Tajima's *D* | Fu and Li's *D* |
|---|---|---|---|---|---|---|---|---|---|---|
| COI | | | | | | | | | | |
| *D. recens* | 58 | 1411/329 | 23 | 25/24 | 0.0038 | 0.0158 | 0.0014 | 0.0058 | −2.040* | −3.422* |
| *D. subquinaria* | 26 | 1411/329 | 25 | 83/81 | 0.0149 | 0.0630 | 0.0140 | 0.0592 | −0.297 | −0.390 |
| mtDNA[a] | | | | | | | | | | |
| *D. recens* | 12 | 8757/1827 | 12 | 63/49 | 0.0024 | 0.0087 | 0.0013 | 0.0047 | −2.120** | −2.470* |
| *period* | | | | | | | | | | |
| *D. recens* | 36 | 624/138 | 34 | 66/44 | 0.0254 | 0.0735 | 0.0250 | 0.0717 | −0.308 | −0.901 |
| *D. subquinaria* | 11 | 624/138 | 8 | 20/13 | 0.0110 | 0.0307 | 0.0119 | 0.0385 | 0.158 | 0.300 |
| *adhr* | | | | | | | | | | |
| *D. recens* | 14 | 516/114 | — | 56/18 | 0.0305 | 0.0367 | 0.0266 | 0.0376 | −0.938 | −0.880 |
| *D. subquinaria* | 18 | 516/114 | — | 39/14 | 0.0197 | 0.0356 | 0.0216 | 0.0271 | 0.189 | 0.322 |
| *tpi* | | | | | | | | | | |
| *D. recens* | 26 | 381/95 | — | 28/26 | 0.0192 | 0.0477 | 0.0120 | 0.0467 | −1.682 | −2.218 |
| *D. subquinaria* | 24 | 381/95 | — | 8/8 | 0.0056 | 0.0224 | 0.0047 | 0.0187 | −0.533 | −1.0527 |

*N*, the number of sequences; *L*, average length of sequences from each species (entire sequence/synonymous sites only); *h*, number of different haplotypes; and *S*, the number of polymorphic (segregating) sites for all sites/synonymous sites only. $*P < 0.05$, $**P < 0.01$.

[a] Represents portions of 12 mitochondrial protein-encoding genes.

four nuclear gene regions and the ~8-kb mtDNA sequence encompassing 12 protein coding genes within the mtDNA genome, are consistent with an earlier study by Perlman *et al.* (2003) that showed that *D. recens* and *D. subquinaria* are the most similar and that *D. quinaria* is a very closely related outgroup (Table 2).

The relative-rates tests for mtDNA indicate that *D. recens* has experienced a significantly greater rate of substitution than *D. subquinaria* at synonymous sites and an elevated, but not significantly greater, rate at nonsynonymous sites (Table 3). In contrast, the rates of molecular evolution for the four nuclear genes were very similar between *D. recens* and *D. subquinaria*, with the exception of an accelerated rate of nonsynonymous evolution at the *period* gene in *D. recens* (Table 3). This latter finding is consistent with earlier studies suggesting that nonsynonymous sites at this gene are subject to selection and evolve at rates that vary among lineages (Wang and Hey 1996; Gleason and Powell 1997; Rand *et al.* 2000; Machado *et al.* 2002).

How might Wolbachia infection cause an increase in the rate of mtDNA evolution in *D. recens*? If this effect were due to Muller's ratchet, one would expect to see evidence of a greater rate of fixation of slightly deleterious mutations, one signature of which would be an elevated rate of nonsynonymous relative to synonymous substitutions. A maximum-likelihood estimate of $d_N/d_S$ (Yang 1997) is a more sensitive test than the relative-rates test for nonsynonymous changes, as the former test can decompose rate variation into species-specific lineages, rather than contrasting *recens* + *quinaria* to *subquinaria* + *quinaria* lineages as does the relative-rates test. Across ~8 kb of the mtDNA genome, the $d_N/d_S$ ratio ($\omega$) in *D. recens* is over twice as great as in the branches leading to *D. subquinaria* and *D. quinaria* (Table 4). A likelihood-ratio test showed that models with

**TABLE 2**

**Average pairwise sequence divergence between *D. recens*, *D. subquinaria*, and *D. quinaria*, showing that for all genes *D. recens* and *D. subquinaria* are the most similar**

| | Species pair | | |
|---|---|---|---|
| Gene | *D. recens-D. subquinaria* | *D. recens-D. quinaria* | *D. subquinaria-D. quinaria* |
| mtDNA (~8 kb) | 0.029 (0.114)[a] | 0.039 (0.156) | 0.031 (0.128) |
| *period* | 0.041 (0.184) | 0.123 (0.348) | 0.114 (0.336) |
| *adhr* | 0.011 (0.047) | 0.033 (0.113) | 0.039 (0.105) |
| *tpi* | 0.003 (0.046) | 0.029 (0.128) | 0.033 (0.130) |
| *R1* (untranslated region) | 0.046 | 0.077 | 0.08 |

[a] Values in parentheses represent divergence estimates at synonymous sites only.

**TABLE 3**

**Relative rates test for silent and replacement sites for mtDNA, *period*, *adhr*, *tpi*, and *R1* sequences in *D. recens vs. D. subquinaria*, using *D. quinaria* as an outgroup**

| Gene | Species | $N$ | $L$ | No. of substitutions | | Probability of rate similarity | |
|---|---|---|---|---|---|---|---|
| | | | | Synonymous | Nonsynonymous | $\Pr(K_s)$ | $\Pr(K_a)$ |
| mtDNA[a] | *D. recens* | 11 | 7248 | 326 | 25 | 0.002 | 0.354 |
| | *D. subquinaria* | 1 | 7248 | 260 | 21 | | |
| *period* | *D. recens* | 36 | 633 | 83 | 32 | 0.664 | 0.007 |
| | *D. subquinaria* | 11 | 633 | 78 | 26 | | |
| *adhr* | *D. recens* | 14 | 516 | —[b] | 10 | —[b] | 0.388 |
| | *D. subquinaria* | 18 | 516 | —[b] | 12 | | |
| *tpi* | *D. recens* | 26 | 381 | 16 | 3 | 0.477 | 0.161 |
| | *D. subquinaria* | 24 | 381 | 18 | 3 | | |
| *R1* | *D. recens* | 1 | 781 | 64[c] | — | 0.74 | — |
| | *D. subquinaria* | 1 | 781 | 66[c] | — | | |

$N$, the number of *D. subquinaria* and *D. recens* sequences for each gene; $L$, the length of the gene region analyzed. $\Pr(K_s)$ and $\Pr(K_a)$ represent the probabilities that the rates of evolution of silent and replacement sites, respectively, do not differ between the two lineages compared.

[a] Represents data from portions of 12 protein-coding genes.

[b] $\Pr(K_s)$ not calculated for *adhr* due to saturation of silent sites.

[c] Noncoding region.

ω varying between the *D. recens* and the *D. subquinaria* + *D. quinaria* branches fit the data significantly better than a model with ω the same in all branches ($P = 0.004$). Allowing ω to vary between *D. quinaria* and *D. subquinaria* did not significantly improve the fit ($P = 0.63$). Thus, the significant heterogeneity in $d_N/d_S$ ratios among these species is attributable to the elevated value in *D. recens*. These findings are consistent with the hypothesis that Muller's ratchet has affected the molecular evolution of mtDNA in *D. recens*. Further, because it is highly unlikely that a mitochondrial haplotype containing multiple nonsynonymous substitutions would spread as a consequence of a single Wolbachia sweep, the data suggest that there has been a series of Wolbachia variant replacements within *D. recens*.

Not only is the mitochondrial $d_N/d_S$ ratio significantly greater in *D. recens*, but also the $d_S$ is, being equal to 0.125 in *D. recens* and 0.078 in *D. subquinaria* in the model in which ω is allowed to vary among lineages. The significance of this difference is evident in the relative-rates test of synonymous-site evolution of the mtDNA from *D. recens vs. D. subquinaria* ($P = 0.002$; Table 3). If synonymous sites are indeed neutral, this finding suggests that the mtDNA of *D. recens* has experienced a greater mutation rate than that of *D. subquinaria*, as the long-term rate of neutral substitutions is expected to equal the neutral mutation rate (Kimura 1968, 1987).

Itoh *et al.* (2002) argued that the greater rate of molecular evolution in some endosymbiotic bacteria is due to an elevated mutation rate, caused by the loss of DNA repair enzymes, which in turn is due to massive genome reduction in obligate endosymbionts. This explanation is very unlikely to account for the higher rate of substitution in the mtDNA of *D. recens*, because (1) there are no known repair pathways for Drosophila mtDNA, and (2) our sequences of ~50% of the mtDNA from both species have revealed no deletions from the mitochondrial genome of *D. recens*. Alternatively, it is possible that Wolbachia affect the intracellular environment in a way that increases the mutation rate of mitochondrial, but not nuclear, DNA.

An elevated rate of synonymous substitution could

**TABLE 4**

**Maximum-likelihood estimates of ω ($d_N/d_S$ ratio) of mtDNA genes in *D. recens*, *D. subquinaria*, and *D. quinaria* lineages, using model F3X4 of PAML (Yang 1997)**

| Model | Parameter estimates | ln $L$ | $2(\Delta \ln L)$ |
|---|---|---|---|
| One ratio | $\omega = 0.020$ | −13328.47 | — |
| Two ratios[a] | $\omega = 0.012_{\text{quin+subquin}}$, $\omega = 0.033_{\text{recens}}$ | −13324.29 | 8.36 $P = 0.004$, d.f. = 1 |
| Three ratios[a] | $\omega = 0.011_{\text{quinaria}}$, $\omega = 0.032_{\text{recens}}$, $\omega = 0.015_{\text{subquinaria}}$ | −13324.17 | 0.24 $P = 0.63$, d.f. = 1 |

[a] In the one-ratio model all lineages have the same ω-ratio. In the two-ratio model the non-*D. recens* and *D. recens* lineages have separate ω-ratios ($\omega_{\text{quin+subquin}}$ and $\omega_{\text{recens}}$, respectively). For the three-ratio model, each species has a separate ω-ratio.

**TABLE 5**

**Frequencies of A/T → G/C and G/C → A/T mutations within *D. recens* and *D. subquinaria* lineages across ~8 kb of mtDNA**

| | Mutation type | |
|---|---|---|
| Species | A/T → G/C[a] | G/C → A/T[a] |
| *D. recens* | 117 | 40 |
| *D. subquinaria* | 45 | 29 |

[a] An mtDNA sequence from *D. quinaria* was used to infer direction of change. Wald $\chi^2 = 4.45$; $P = 0.035$.

also arise if these mutations are not selectively equivalent. In this case, Muller's ratchet, which is driven by recurrent Wolbachia sweeps, could increase the rate of substitution of slightly deleterious synonymous mutations, such as those that result in unpreferred codons. In fact, many of the synonymous substitutions in the mtDNA of *D. recens* represent changes to codons that are generally relatively underutilized in Drosophila, specifically, those that end in either G or C (Clary and Wolstenholme 1985). For the ~8-kb sequence of mtDNA, we categorized all mutations that occurred in the *D. recens* and *D. subquinaria* lineages as either A/T → G/C or G/C → A/T. On the basis of our 12 sequences from *D. recens*, the mutations were further categorized as polymorphic or apparently fixed within the species. Because the frequencies of A/T → G/C and G/C → A/T mutations were virtually identical between the polymorphic and fixed categories within *D. recens* (Wald $\chi^2 = 0.035$; $P = 0.85$), we combined the fixed and polymorphic mutations found within *D. recens* to contrast with the mutations found in *D. subquinaria*. The contrast between the two species is shown in Table 5. The results indicate that *D. recens* has experienced a significant excess of A/T → G/C mutations relative to *D. subquinaria* (Wald $\chi^2 = 4.45$; $P = 0.035$). To the extent that the low GC content of Drosophila mtDNA is a result of selection (*e.g.*, preference for AT-ending codons), the excess of A/T → G/C mutations in *D. recens* may reflect the weakened effect of selection on mtDNA in this species. Alternatively, this excess may reflect mutational bias operating via an as-yet-unknown mechanism.

With respect to Muller's ratchet, the dynamics of a newly arisen, favorable Wolbachia mutation will be governed by a selection coefficient that reflects the positive effects of this mutation discounted by the negative effects of any deleterious mutations in the mitochondria with which the Wolbachia are cotransmitted. The net selection coefficient for the Wolbachia mutation will be greater—and thus its spread to fixation variant more likely—if it is associated with slightly deleterious synonymous mitochondrial mutations than with more deleterious nonsynonymous mutations. The recent findings that the spontaneous mutation rates of *Caenorhabditis elegans* and human mtDNA are 1–2 orders of magnitude greater than the substitution rate among phylogenetic lineages (Denver *et al.* 2000; Howell *et al.* 2003) indicate that most mitochondrial mutations in these species, including those that are synonymous, are deleterious (see also Ballard and Whitlock 2004). Our finding of an elevated synonymous substitution rate in *D. recens* is consistent with there being a similar mutational spectrum in Drosophila and with the fact that Wolbachia evolution, through a series of variant turnovers, can accelerate the rate of fixation of slightly deleterious synonymous mutations.

In conclusion, our findings show that Wolbachia, and probably other maternally transmitted endosymbionts, can severely depress levels of mtDNA diversity within an infected host species. In contrast, such infections may increase the rate of substitution in mtDNA. The significantly elevated $d_N/d_S$ ratio in *D. recens* implicates the operation of Muller's ratchet and suggests that endosymbiont infection may contribute to mutational degradation of a host species' mtDNA. The greater rate of molecular evolution of mtDNA in endosymbiont-infected insect species, if general, has important consequences for the use of mtDNA as a molecular clock in insects. Finally, a positive correlation between polymorphism and divergence, as expected under the standard neutral theory, may not hold for vast numbers of endosymbiont-infected insects and other arthropods. Given these dramatic yet unappreciated effects and the widespread distribution of endosymbionts in arthropods, additional comparative studies similar to our study clearly are warranted to test the generality of these predictions. The discovery of appropriate species pairs for future comparisons rests on the reliable determination that a species in fact is not infected with Wolbachia or is infected with only a single strain, which in turn depends on more extensive within-species sampling than has been done in surveys to date (*e.g.*, Werren *et al.* 1995; Jeyaprakash and Hoy 2000; Werren and Windsor 2000).

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