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EVOLUTIONARY DYNAMICS OF A SPATIALLY STRUCTURED HOST-PARASITE ASSOCIATION: *DROSOPHILA INNUBILA* AND MALE-KILLING *WOLBACHIA*

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Abstract.—The mode and tempo of host-parasite evolution depend on population structure and history and the strength of selection that the species exert on each other. Here we genetically and epidemiologically characterize populations of the mycophagous fly *Drosophila innubila* and its male-killing *Wolbachia* endosymbiont, with the aim of integrating the local through global nature of this association. *Drosophila innubila* inhabit the forested ''sky island'' regions of the of the southwestern United States and northern Mexico, where its distribution is highly fragmented. We examine geographically isolated sky island populations of *D. innubila*, surveying the frequency and expression of *Wolbachia* infection as well as the distribution of genetic variation within and among populations of the host and parasite. In all populations, *Wolbachia* infection is associated with virtually complete male-killing, thus providing no evidence for the evolution of population-specific interaction phenotypes or local resistance. Although *Wolbachia* infection occurs in each of the main populations, there is variation among populations in the prevalence of infection and the resulting population-level sex ratio of *D. innubila*. Among these populations, the nuclear genes of *D. innubila* show moderate, though significant, differentiation. In contrast, the host mitochondrial DNA (mtDNA), which shares transmission with *Wolbachia*, exhibits substantially greater geographic differentiation, even after accounting for differences in transmission between nuclear and mitochondrial genes. We suggest that this pattern is caused by local *Wolbachia*—but not *D. innubila*—fluctuations in prevalence that increase the severity of drift experienced only by the mtDNA. Overall, our data suggest that the association between *D. innubila* and male-killing *Wolbachia* is ecologically dynamic within local populations, but evolutionarily coherent across the species as a whole.

Key words.—Endosymbiont, founder effect, gene flow, molecular polymorphism, population structure, sex ratio distortion.

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Recent theoretical and empirical studies have begun to explore how population structure affects the ecological and evolutionary dynamics of host-parasite interactions (e.g., Thrall and Antonovics 1995; Lively and Jokela 1996; Burdon and Thrall 2000). To date, however, virtually all studies relating population structure to variation in host-parasite interactions have focused on horizontally transmitted parasites (e.g., Thompson and Cunningham 2002; Fischer and Foitzik 2004; Forde et al. 2004). Meanwhile, parasites that are vertically transmitted from mother to offspring are being discovered at an accelerating pace (reviewed in Bandi et al. 2001), and may provide rich opportunities to explore the connection between population structure and the nature of host-parasite interactions. Compared to horizontally transmitted parasites, maternally transmitted symbionts exhibit an additional layer of population structure—at the level of the host individual—and may be evolutionarily more variable in their effects on host fitness, ranging from mutualistic to virulently parasitic (reviewed in Moran and Baumann 2000; Bandi et al. 2001).

Some maternally transmitted factors promote their own spread by skewing the offspring sex ratio of their host. These sex-ratio distorters include mitochondrial variants that cause cytoplasmic male sterility (CMS) in normally hermaphroditic plants (reviewed in Budar and Pelletier 2001) and a taxonomically diverse array of endosymbiotic microorganisms that cause embryonic male-killing in various arthropods (reviewed in Hurst et al. 2003). In theory, factors that sterilize or kill males can spread through a population if infected females produce more daughters (or seeds, in the case of CMS) than uninfected individuals. In the case of CMS this can result from reallocation of resources from pollen to seed production (Charlesworth and Charlesworth 1978); for male-killing endosymbionts, this results when the death of males benefits their surviving sisters via increased resource availability or decreased inbreeding.

Demographically, sex-ratio distorters can affect the population-level sex ratio of the host, which in turn can influence host population dynamics and potentially cause host extinction due to a paucity of males (Hatcher et al. 1999). Since both CMS and male-killing endosymbionts have been shown to vary among populations in presence and frequency (e.g., Taylor et al. 2001; Dyeson and Hurst 2004), their demographic effects may also vary among populations (e.g., Olson et al. 2005).

Sex-ratio distorters cause the death or sterility of a substantial fraction of the host population every generation, imposing strong selection on the host to evolve resistance. For instance, nuclear restorer loci have evolved repeatedly in plants harboring CMS factors, contributing to the variation among populations in the frequency of CMS (e.g., Manicacci et al. 1997; Taylor 1999). In contrast to the comparatively well-studied systems in plants, the evolution of resistance to the male-killing endosymbionts of arthropods has received very little empirical study.

In this paper, we use epidemiological and population genetic data to explore the broad-scale ecological and evolutionary interactions between the mushroom-feeding fly *Dro-*

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sophila innubila and a male-killing endosymbiont. About one-third of *D. innubila* females from the Chiricahua Mountains in southeastern Arizona are infected with a strain of *Wolbachia* that kills virtually all of their male offspring, resulting in a female bias in the population-level sex ratio (Dyer and Jaenike 2004). Two lines of evidence using patterns of polymorphism of the mitochondrial DNA (mtDNA; which is maternally co-transmitted with *Wolbachia*) indicate that the association between *D. innubila* and male-killing *Wolbachia* is evolutionarily old. First, all of the major mtDNA haplotypes are found in both infected and uninfected individuals, as would be expected if all individuals in the present population were descended from one originally infected female. Second, the level of mtDNA nucleotide diversity corresponds very closely with equilibrium expectations based on observed levels of infection prevalence (Dyer and Jaenike 2004). The achievement of this equilibrium depends on the long-term input of mutations subsequent to the initial fixation of the infected haplotype lineage.

The Chiricahua population of *D. innubila* exhibits no evidence of resistance to the male-killing effects of these *Wolbachia*, even though the infection has been resident in the population for a substantial period of time and DNA polymorphism data indicate that *D. innubila* has a very large effective population size (Dyer and Jaenike 2004; J. Jaenike, unpubl. data). In addition, there is no evidence for parthenogenetic reproduction in this system (Dyer et al. 2005). Because the distribution of *D. innubila* is highly fragmented within its range in Arizona and New Mexico, it is possible that the Chiricahua population has an atypical association with these *Wolbachia*. *Drosophila innubila* is restricted to mesic habitats, and in southern Arizona it is found in oak-juniper riparian woodlands at 1500 m elevation up through coniferous forests at 3000 m (Patterson and Wagner 1943; Heed et al. 1962). These mesic mountain habitats form an archipelago of ''sky islands,'' each of which is surrounded by much more xeric lowland desert and grassland habitats (Brown 1994). Several other organisms with sky island distributions in Arizona exhibit marked differentiation among populations (Maddison and McMahon 2000; Masta 2000; Boyd 2002; K. R. Zamudio and H. W. Greene, unpubl. ms.), indicating a significant degree of evolutionary independence among the different populations. Because *D. innubila* is also restricted to sky islands, this species and its associated *Wolbachia* may provide an excellent opportunity to explore the connection between local and global processes in host-parasite interactions.

In this study, we address the following questions: (1) Are all populations of *D. innubila* infected with *Wolbachia*, thus providing multiple opportunities for evolution of host-parasite interactions? (2) Do sky island populations of *Wolbachia* and/or *D. innubila* exhibit significant genetic differentiation, thus providing an opportunity for evolutionary divergence among sky islands in the nature of this interaction? (3) Do the phenotypic effects of *Wolbachia* vary among isolated populations, as would be expected if populations of *D. innubila* differed in their levels of resistance to male-killing? (4) Do the genetic and demographic structures of the host and parasite populations suggest the occurrence of extinction-colonization dynamics or local bottlenecks in either species? In sum, we ask whether the evolution of the *D. innubila–Wolbachia* association results from the integration of divergent local processes, or whether dynamics in different local populations proceed largely in parallel and are thus independent of the spatial structure of these populations.

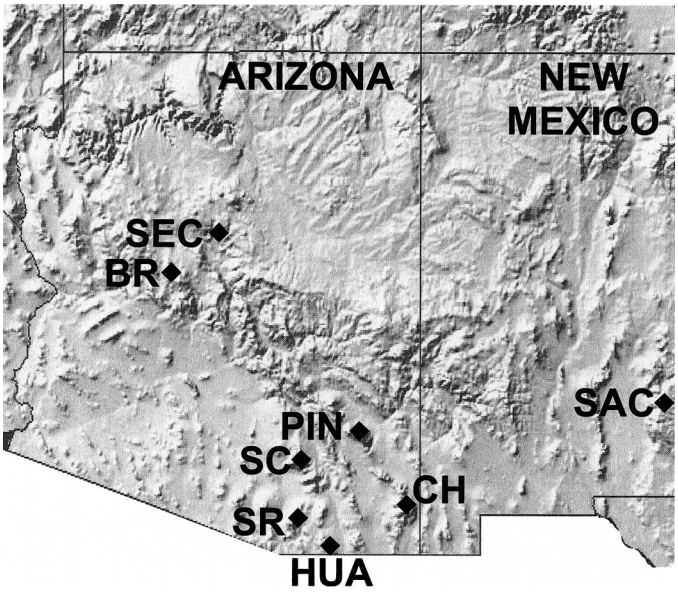

FIG. 1. Sky island populations surveyed in this study. The black diamonds indicate collection localities. See Table 1 for full names of abbreviations.

MATERIALS AND METHODS

Sampling and Screening for Wolbachia

Drosophila innubila were collected in woodland habitats from geographically isolated sky-island populations in southern Arizona and New Mexico (Fig. 1; Table 1). We chose our collecting sites based on the known range (Heed et al. 1962; Patterson and Wagner 1943) and on knowledge of local habitats. Each population was surveyed one to three times during August–September of 2001–2004, during which flies were collected by sweep-netting over baits of decaying mushrooms. All collection and population genetic data from the Chiricahua population are from Dyer and Jaenike (2004), and were gathered in the same manner as for the other populations. Wild-caught *D. innubila* females were placed individually into culture vials and the number of male and female offspring determined. All fly cultures were maintained at 22°C on Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC) supplemented with commercial mushroom (*Agaricus bisporus*). Females producing at least 10 offspring were used to determine the correlation between *Wolbachia* infection and offspring sex ratio.

To ascertain infection by *Wolbachia*, we extracted DNA from each wild-caught fly using Gentra's Puregene kit (Gentra Systems, Minneapolis, MN), and simultaneously amplified the *Wolbachia* genes *wsp* (using the primers wsp81F and wsp691R from Zhou et al. 1998) and *ftsZ* (using ftsZBr and ftsZBf from Werren et al. 1995). Although the focus of the present study is on the male-killing strain that belongs to the

TABLE 1. Collecting sites and summary of the population-level data from *Drosophila innubila*.

Sky island population	Code	Collection site	Elevation (m)	Years sampled	Fraction female (total flies collected)	Infection frequency (total tested)	
						Females	Males
Chiricahuas	CH	Cave Creek	1520	2001–2003	0.671 (1772)	0.334 (1087)	0.021 (517)
Santa Catalinas	SC	Cypress	1770	2002–2003	0.625 (32)	0.35 (20)	0.091 (11)
Santa Ritas	SR	Madera Canyon	1615	2002	0.525 (101)	0.283 (53)	0 (48)
Huachucas	HUA	Huachuca Canyon	1740	2003	0.462 (132)	0.119 (59)	0 (61)
Bradshaws	BR	White Spar	1710	2002–2003	1.0 (1)	1.0 (1)	0 (0)
Pinalenos	PIN	Wet Creek	1890	2002	1.0 (2)	0 (2)	0 (0)
Sacramentos	SAC	Fresnal	2040	2002	(0.5) (2)	0 (1)	0 (1)
Secret Mountain Wilderness	SEC	Oak Creek Canyon	1675	2002	0 (0)	0	0

A group of *Wolbachia*, *D. innubila* is also infected at very low frequency with a non-sex-ratio-distorting B group infection (Dyer and Jaenike 2004). This assay distinguishes flies infected with each strain: the *wsp* primers amplify any strain of *Wolbachia*, whereas these *ftsZ* primers are specific to the B group. Outside the Chiricahuas, a group B *Wolbachia* infection was found in one female from the Santa Ritas, though it was not transmitted to any of her offspring (data not shown). Therefore, we do not consider the B strain infection further here.

Polymerase Chain Reaction and DNA Sequencing

To survey *Wolbachia* polymorphism and differentiation among populations, we sequenced *wsp*, the most rapidly evolving gene known in *Wolbachia*, from 1 to 31 randomly chosen infected females from each population using the conserved primers 81F and 691R from Zhou et al. (1998). For this and all sequencing we purified amplicons with QIAquick columns (Qiagen Inc., Valencia, CA) or Exosap-IT (USB Corporation, Cleveland, OH) and directly sequenced both strands using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA). Chromatograms were verified using Sequencher (GeneCodes, Ann Arbor, MI) and aligned manually.

Mitochondrial DNA is maternally co-transmitted with *Wolbachia* and evolves at a faster rate than *Wolbachia*, thus it is often a more sensitive indicator of the evolutionary history of an endosymbiont infection (e.g., Shoemaker et al. 2004). We used patterns of host mtDNA variation and association with *Wolbachia* infection to infer the age of the *D. innubila–Wolbachia* association within each sky island population and to quantify levels of genetic differentiation among populations. We surveyed variation at the *cytochrome oxidase I* (*COI*) gene: the primers for polymerase chain reaction (PCR) and sequencing of the entire *COI* gene are described in Dyer and Jaenike (2004), and the sample sizes of infected and uninfected individuals from each population are shown in Table 2.

To infer host population genetic parameters not associated with *Wolbachia*, we sequenced four nuclear gene regions from a random sample of wild-caught *D. innubila* individuals (n = 4–46 chromosomes per locus per population). These

TABLE 2. Summary of *COI* polymorphism data by population and infection with male-killing *Wolbachia*. *COI* includes 1473 base pairs, of which 347 are silent sites. *n*, number of samples; *h*, number of haplotypes; Hd, haplotype diversity; *S*, total number of segregating sites; *s*, total number of singleton sites. Asterisk indicates significance at the $P < 0.05$ level.

									Silent sites only		
		n	*h*	Hd	M_n[1]	M_s[1]	*S*	*s*	π[2]	θ_W[2]	D_T
CH	total	114	17	0.623	3	15	18	13	0.37	0.81	−1.47*
	infected	38	7	0.550	1	5	6	3	0.01	0.34	−0.23
	uninfected	62	12	0.672	1	12	13	8	0.19	0.73	−1.27
SR	total	70	6	0.555	0	6	6	2	0.20	0.36	−1.09
	infected	13	2	0.538	0	1	1	0	0.156	0.09	1.48
	uninfected	56	6	0.565	0	6	6	2	0.21	0.38	−1.19
SC	total	32	4	0.236	1	4	5	2	0.15	0.29	−1.17
	infected	8	1	0.000	0	0	0	0	0.00	0.00	—
	uninfected	24	4	0.308	1	4	5	2	0.05	0.31	−0.95
HUA	total	48	12	0.608	1	11	11	7	0.26	0.71	−1.73*
	infected	7	2	0.571	0	1	1	0	0.16	0.12	1.34
	uninfected	41	12	0.591	1	11	11	7	0.26	0.74	−1.80*
PIN	uninfected	2	2	1.000	0	3	3	3	0.86	0.86	—
SAC	uninfected	2	2	1.000	0	3	3	3	0.86	0.86	—
BR	infected	1	1	—	—	—	—	—	—	—	—
ALL	total	270	28	0.728	4	24	27	15	0.44	1.12	−1.57*
	infected	67	9	0.686	1	6	7	7	0.42	0.36	0.42
	uninfected	187	24	0.728	3	21	23	12	0.43	1.04	−1.53*

[1] Number of nonsynonymous (M_n) and silent (M_s) segregating mutations.
[2] Estimates of nucleotide diversity. All values are expressed as percentages.

loci include the autosomal *alcohol dehydrogenase-related* (*Adhr*) protein and *triose phosphate isomerase* (*tpi*), and the X-linked *period* (*per*) and *vermilion* (*v*); the primers for PCR and sequencing are described in Dyer and Jaenike (2004). The *per* and *tpi* fragments are all coding sequence, while the primers for *Adhr* and *v* are anchored in exons but amplify across an intron. We primarily used males to survey polymorphism in the X-linked loci *per* and *v*; otherwise, we inferred heterozygous sites by manually examining chromatograms according to the restrictions of Hare and Palumbi (1999). Haplotypes were reconstructed for each locus using the program PHASE version 2.0.2 (Stephens et al. 2001; Stephens and Donnelly 2003); we excluded singletons and ran the program four times to ensure convergence, each with a different starting seed and allowing for recombination.

Data Analyses

Sites with alignment gaps were abundant in noncoding regions of fly nuclear loci, and were excluded from all analyses. The program DnaSP version 4.0 (Rozas et al. 2003) was used to quantify levels of intra- and interpopulation genetic variability. Polymorphism was measured at silent sites using π (Tajima 1983) and θ_W (Watterson 1975) statistics. The frequency spectrum of silent-site polymorphisms was tested for departure from neutrality using D_T (Tajima 1989), with significance determined using 10^4 coalescent simulations and assuming no recombination.

To visualize relationships and relative abundances of mtDNA haplotypes within *D. innubila*, a minimum-spanning network was created in Arlequin version 2.000 (Schneider et al. 2000), with a *D. falleni* individual used to root the network (Genbank accession number AY541122). At *COI* we estimated the level of differentiation between infected and uninfected individuals within each sky island population. At *COI* and the nuclear loci we measured differentiation among sky island populations, pooling infected and uninfected flies within each population. We tested for differentiation using several tests based on genetic distance and haplotype frequencies (described in Wright 1951; Nei 1987; Lynch and Crease 1990; Hudson et al. 1992; Hudson 2000), as implemented in DnaSP, and also partitioned variation within and among sky island populations using an analysis of molecular variance (AMOVA), as implemented in Arlequin. F_{ST} was used as modified for DNA sequence data (Lynch and Crease 1990). When comparing F_{ST} among autosomal, X-linked, and mitochondrial loci we corrected for differences in effective population size due to differences in transmission patterns (see Hartl and Clark 1989) and standardized these values to the autosomes. Estimates of *Nm* were calculated using Wright's (1951) approximations for both males and females (nuclear loci) and for females only (mtDNA). We used permutation-based statistics to test whether there was significant differentiation among populations. These included H_{ST} (Hudson et al. 1992) for the mtDNA, which is an estimator of Nei's G_{ST} (Nei 1973) and is a frequency-based haplotype statistic appropriate for nonrecombining loci, and S_{nn} (Hudson 2000) and K^*_{ST} (Hudson et al. 1992) for the nuclear loci, which are appropriate for loci experiencing recombination.

To test for a correlation between geographic and genetic distance among populations, we implemented Mantel's test (Mantel 1967) using the program Isolation by Distance (Bohonak 2002). Geographic distances were calculated in kilometers using GPS coordinates taken at collection localities, and were log-transformed for the analyses (Slatkin 1993). Genetic distance was estimated by pairwise F_{ST}. We completed the analyses separately for each gene.

RESULTS

Frequency and Population-Level Effects of Wolbachia

Table 1 shows the number of *D. innubila* collected from each population, the population sex ratio, and the frequency of group A *Wolbachia* in males and females. The data are summed over all years the populations were sampled. The number of *D. innubila* collected from each population varied, so we limit our discussion to the Chiricahua (CH), Santa Catalina (SC), Santa Rita (SR), and Huachuca (HUA) populations, because these have sufficient sample sizes for statistical comparison.

In every population, the uninfected females produced offspring sex ratios that centered at 1:1, whereas almost all infected females produced sex ratios that were very strongly female biased (Fig. 2). In addition, the single female collected from the Prescott population was infected and produced only female offspring. The overall offspring sex ratio did not vary significantly among populations for either uninfected females ($\chi^2 = 4.076$, $P = 0.25$) or infected females ($\chi^2 = 5.87$, $P = 0.12$). Thus, this *Wolbachia* strain causes a very high level of male-killing in all populations of *D. innubila* examined.

The prevalence of *Wolbachia* infection in females varied significantly among the CH, SC, SR, and HUA populations ($\chi^2 = 14.5$, $P = 0.0023$). The proportion of females among all wild-caught flies also varied significantly among these four populations ($\chi^2 = 29.6$, $P < 0.0001$). Across populations, there is a positive and marginally significant correlation between the proportion of females in our field collections and the prevalence of *Wolbachia* infection among females (Fig. 3; r^2 adjusted = 0.78; $P = 0.07$).

Wolbachia and mtDNA Variation and Differentiation

All *wsp* sequences from flies infected with group A *Wolbachia* were identical ($n = 52$), consistent with there being a single origin of male-killing *Wolbachia* in *D. innubila*. Because of this lack of polymorphism in *wsp*, all inferences of *Wolbachia* gene flow were made using the host's mtDNA. We sequenced the mtDNA *COI* gene from a total of 270 individuals, which included both infected and uninfected individuals from the seven populations in which *D. innubila* were collected. The polymorphism levels within each infection class and population are shown in Table 2. Among the 1473 base pairs we identified 27 segregating sites and 28 haplotypes. Figure 4 is a specieswide haplotype network that shows the distribution of haplotypes among sky islands, and Figure 5 shows these data broken down by population and infection status.

The proportion of infected and uninfected females is approximately the same for all of the major mtDNA haplotypes within each population (Fig. 5). This is the pattern expected

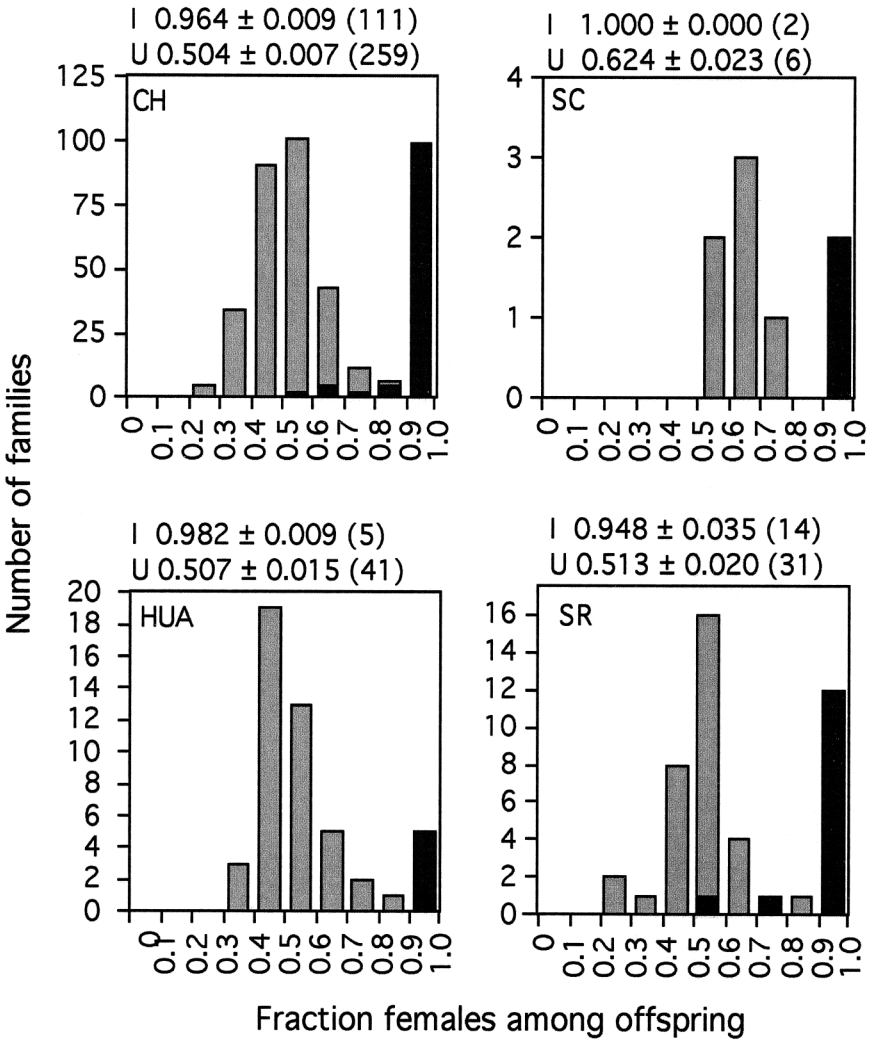

FIG. 2. Fraction of daughters produced by wild-caught females from each population. Only females producing ≥10 offspring are included. Dark shading indicates females infected with group A *Wolbachia* and light shading indicates uninfected females, as determined by polymerase chain reaction. Above each graph is the mean fraction of daughters ± SE for infected (I) and uninfected (U) females, with the sample size in parentheses. Abbreviations as in Table 1.

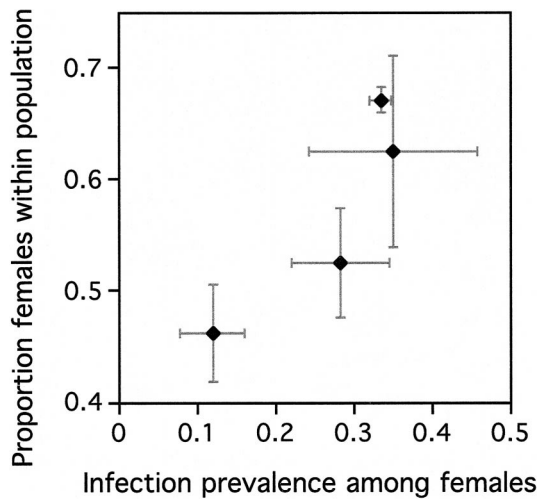

FIG. 3. Population-level sex ratio as a function of female infection prevalence. Gray lines indicate ± SE for each sample.

for a male-killing infection maintained at equilibrium by a balance between cytoplasmic fitness advantage of infection and incomplete transmission (Dyer and Jaenike 2004). A suite of statistical tests corroborates this lack of differentiation between mtDNA haplotypes carried by infected and uninfected individuals (Wright 1951; Nei 1987; Lynch and Crease 1990; Hudson et al. 1992; Hudson 2000), both within each population and for the species sample as a whole ($P > 0.1$ for all statistics for all comparisons). In addition, for the four most common haplotypes and the four main populations, there is no within-population effect of haplotype on probability of infection (logistic regression; $\chi^2 = 4.5$, df = 8, $P = 0.81$).

Within populations, the level of *COI* polymorphism found in infected versus uninfected individuals is roughly the same, though there is a slight excess of low frequency substitutions in the uninfected class of individuals, causing D_T to be more negative for the uninfected class (Table 2). Mutations that arise in uninfected individuals lack the transmission advan-

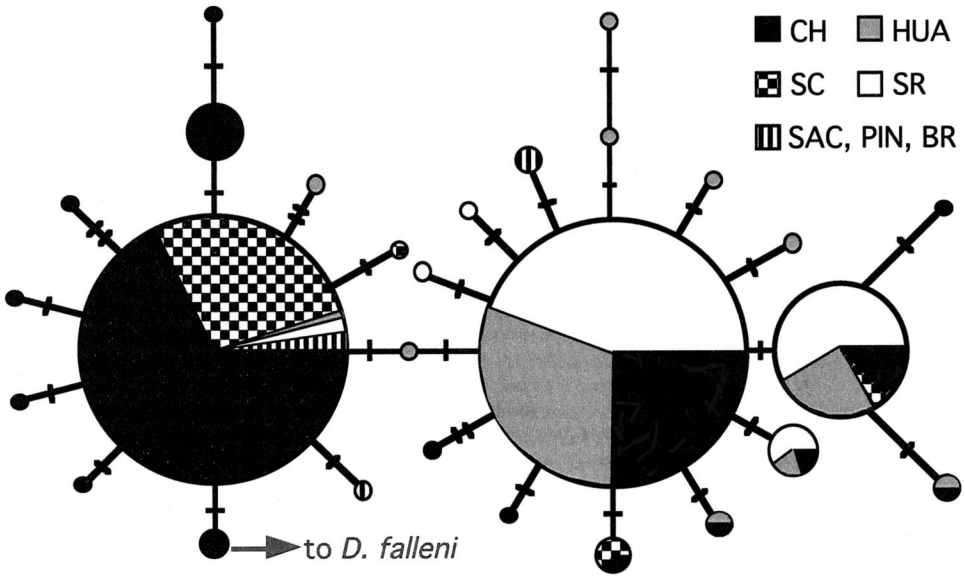

FIG. 4. Specieswide *Drosophila innubila COI* minimum-spanning network. The areas of the circles are proportional to the abundances of the different haplotypes, with samples pooled across populations. Slash marks on lines indicate one nucleotide substitution. Abbreviations defined in Table 1.

tage associated with the infection, and thus selection is expected to prevent these mutations from becoming common. Consistent with this, Fay and Wu's *H*, which can indicate an excess of high-frequency derived mutations (Fay and Wu 2000), is not significantly different from neutral expectation for *COI* in either the infected or uninfected class of individuals (infected: $H = -2.22$, $P = 0.14$; uninfected: $H = -0.203$, $P = 0.29$).

Sky island populations of *D. innubila* differ greatly in mtDNA haplotype frequencies (Figs. 4 and 5). These differences result in highly significant H_{ST} values among five of the six population comparisons (Table 3), with an analysis of molecular variance (AMOVA; Table 4) revealing that a large fraction of the mtDNA variation is distributed among populations. No significant pattern of isolation by distance was found at *COI* ($r = -0.05$, $P = 0.65$).

Variation and Differentiation at Nuclear Loci

All four nuclear loci exhibit high levels of polymorphism in all populations (Table 5). θ_W was calculated using the number of segregating sites, yielding conservative estimates for these loci, which all have more mutations than segregating sites. *Adhr* was characterized by a positive D_T in three of four populations, whereas D_T was negative for *per*, *tpi*, and *v* within each population, indicating an excess of low-frequency mutations at these three loci. To determine an overall mean D_T for the nuclear loci, we used J. Hey's program HKA (available via http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm), with 10^4 coalescent simulations and adjusting for differences in transmission of the X chromosome and autosomes. For all populations, the observed mean D_T was lower than the mean of the simulated D_T (CH: $D_{T(obs)} = -0.909$, $D_{T(exp)} = -0.096$, $P = 0.032$; SC: $D_{T(obs)} = -0.437$, $D_{T(exp)} = -0.094$, $P = 0.229$; SR: $D_{T(obs)} = -0.778$, $D_{T(exp)} = -0.095$, $P = 0.060$; HUA: $D_{T(obs)} = -0.826$, $D_{T(exp)} = -0.099$, $P = 0.050$). Because the HKA program does not incorporate recombination, these *P*-values are conservative.

The four main *D. innubila* populations exhibit significant differentiation at the nuclear loci (Table 4). The AMOVA, S_{nn}, and K^*_{ST} tests are largely but not completely concordant: whereas differentiation at *Adhr* is significant for all three tests and *tpi* shows no differentiation by any test, *per* and *v* show significant differentiation for two of three tests. Across populations, genetic differentiation is not correlated with geographic distance (*Adhr*: $r = -0.049$, $P = 0.65$; *per*: $r = 0.145$, $P = 0.34$; *tpi*: $r = 0.100$, $P = 0.49$; *v*: $r = -0.244$, $P = 0.57$).

DISCUSSION

Three lines of evidence show that the *D. innubila–Wolbachia* association is appropriate for analysis of evolutionary divergence among populations. First, all of the *Wolbachia wsp* sequences were identical and distinct from all other known *Wolbachia* (this study; Dyer and Jaenike 2004). Since *wsp* sequences almost invariably differ among *Wolbachia* from different insect species (Jiggins et al. 2002a), this indicates a single infection of *D. innubila* by male-killing *Wolbachia*. Thus, any differences among populations in the nature of the host-parasite interaction must have arisen subsequent to the initial infection. Second, phylogenetic analysis shows that the male-killing phenotype has evolved multiple times within *Wolbachia* (Jiggins et al. 2002a), with the male-killing *Wolbachia* infecting *D. innubila* most closely related to other *Wolbachia* strains with very different phenotypic effects (Dyer and Jaenike 2004). Thus, there is no evidence that the clade of *Wolbachia* to which the *D. innubila*–infecting strain belongs is evolutionarily locked into one particular phenotype.

Finally, the significant differentiation among populations

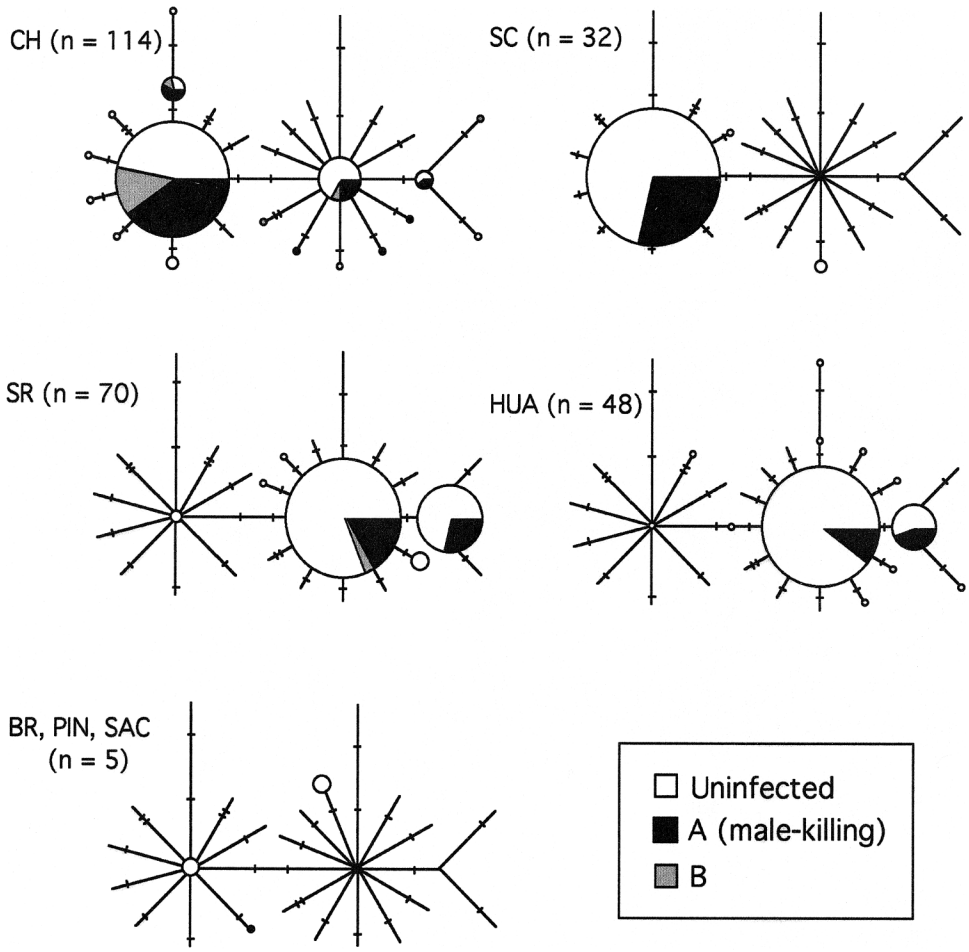

FIG. 5. *COI* minimum-spanning network separated by population, using the same specieswide outline for each. Abbreviations defined in Table 1. For CH, HUA, SC, and SR, the relative abundance of each haplotype within in each population is indicated by the size of each circle. Slash marks on lines indicate one nucleotide substitution. Association of each haplotype with infection status (male-killing, B group, or uninfected) is indicated by the shading within each circle.

of *D. innubila* (Table 4) for both nuclear genes and mitochondrial haplotypes indicates that gene flow is restricted among the sky island populations. If we assume that F_{ST} reflects an equilibrium between drift and migration in an island model, the inferred level of *Nm* for the nuclear genes is on the order of 20. However, because the effective population size of *D. innubila* inferred from levels of DNA sequence variation is very large ($>10^6$; Dyer and Jaenike 2004), the actual level of gene flow (*m*) among populations is likely to be very small. Thus, gene flow is unlikely to impede selectively based divergence among these sky island populations in the nature of the *Drosophila—Wolbachia* interaction. Adaptive differentiation in the face of gene flow has been documented in other species, including the acorn barnacle *Semibalanus balanoides* (Brown et al. 2001) and the pocket mouse *Chaetodipis intermedius* (Hoekstra et al. 2005).

Despite low levels of gene flow, the phenotypic effect of *Wolbachia* infection is identical in all of the populations we surveyed, with virtually all infected females producing highly female-biased offspring sex ratios. Surprisingly, even in the face of strong selection to resist the male-killing effects of *Wolbachia*, no populations of *D. innubila* appear to have evolved any significant resistance. It is interesting to note that although most known CMS systems are polymorphic for

TABLE 3. Pairwise differentiation among the four main populations at the mtDNA *COI* gene. Above the diagonal is the uncorrected F_{ST} with $N_f m$ in parentheses, and below the diagonal is H_{ST}, with the significance value from permutation tests shown in parentheses. Abbreviations defined in Table 1.

	CH	SC	SR	HUA
CH		0.074 (6.09)	0.490 (0.53)	0.433 (0.66)
SC	0.033 ($P = 0.003$)		0.716 (0.20)	0.658 (0.26)
SR	0.172 ($P < 0.0001$)	0.349 ($P < 0.0001$)		−0.002 (∞)
HUA	0.141 ($P < 0.0001$)	0.367 ($P < 0.0001$)	−0.001 ($P = 0.401$)	

TABLE 4. Measures of global differentiation among the four main sky island populations of *Drosophila innubila*. For the analysis of molecular variance (AMOVA), corrected Φ_{ST} adjusts for different transmission patterns to make all loci comparable to the autosomes (see text for details).

		mtDNA	Autosomal		X-linked	
			Adhr	*tpi*	*per*	*v*
AMOVA	Variation among populations (%)	42.06	2.86	−0.68	0.78	3.91
	Variation within populations (%)	57.94	97.14	100.68	99.22	96.09
	Φ_{ST}	0.421 ($P < 0.0001$)	0.026 ($P = 0.033$)	−0.007 ($P = 0.859$)	0.008 ($P = 0.136$)	0.039 ($P = 0.0002$)
	Corrected Φ_{ST}	0.154	0.026	−0.007	0.006	0.030
F_{ST} variants	S_{nn}	0.453 ($P < 0.0001$)	0.363 ($P = 0.0001$)	0.234 ($P = 0.729$)	0.464 ($P = 0.0001$)	0.266 ($P = 0.361$)
	K_{ST}*	0.313 ($P < 0.0001$)	0.027 ($P = 0.003$)	−0.002 ($P = 0.664$)	0.012 ($P = 0.0017$)	0.022 ($P < 0.0001$)

TABLE 5. Silent site polymorphism in four nuclear loci of *Drosophila innubila*. *n*, number of chromosomes sampled; *S*, total number of silent segregating sites; M_s, total number of silent segregating mutations; *s*, total number of silent singleton segregating sites. Asterisk indicates significance at the $P < 0.05$ level.

Population		Autosomal		X-linked	
		Adhr	*tpi*	*per*	*v*
CH	*n*	26	46	22	21
	S	25	24	28	128
	π^1	4.46	3.15	2.56	6.90
	$\theta_W{}^1$	3.90	5.92	4.50	9.24
	D_T	0.53	−1.63*	−1.50*	−1.03
SC	*n*	32	32	30	15
	S	29	16	26	143
	π^1	4.33	2.61	2.96	6.37
	$\theta_W{}^1$	4.87	4.30	3.85	7.15
	D_T	−0.16	−1.32	−0.76	0.48
SR	*n*	26	30	25	24
	S	18	22	32	129
	π^1	3.11	3.49	2.88	7.09
	$\theta_W{}^1$	2.81	6.02	4.97	8.93
	D_T	0.55	−1.48	−1.36	−0.82
HUA	*n*	30	32	23	24
	S	24	23	30	122
	π^1	4.27	3.02	2.73	6.67
	$\theta_W{}^1$	3.61	6.19	4.77	8.32
	D_T	0.76	−1.79*	−1.42*	−0.79
PIN	*n*	2	4	2	—
	S	8	8	0	—
SAC	*n*	4	4	—	—
	S	9	6	—	—
BR	*n*	2	2	2	—
	S	5	4	1	—
All	*n*	122	150	104	84
	$L_{ALL}{}^2$	522	383	704	338
	$L_{SIL}{}^2$	168	92	171	299
	M_s	37	41	64	193
	S	32	38	56	157
	s	4	11	17	46
	π^1	4.27	3.10	3.02	6.59
	$\theta_W{}^1$	3.54	7.37	6.30	10.50
	D_T	0.61	−1.73*	−1.66*	−1.26

[1] Estimates of silent site nucleotide diversity. All values are expressed as percentages.
[2] Length of all sites and silent sites only, excluding alignment gaps.

nuclear restorer factors (reviewed in Budar and Pelletier 2001), polymorphism for resistance has not yet been discovered in any insect infected a with male-killing endosymbiont (but see Cavalcanti et al. 1957), including *Acraea encedon* (Jiggins et al. 2002b), *Drosophila bifasciata* (Veneti et al. 2004), and *D. innubila* (this study).

Though *Wolbachia* is present and causes strong male-killing in all the populations from which we collected two or more *D. innubila* females, our data suggest that the frequency of *Wolbachia* infection is highly variable, both among and within populations (Table 1 and Fig. 3; Dyer and Jaenike 2004). Variation among sky island populations in *Wolbachia* prevalence and sex ratio suggests that these populations are epidemiologically and demographically largely independent. Because our sampling was limited, the levels of temporal and spatial variation in *Wolbachia* infection prevalence are likely to be greater than revealed in our data, although we do not yet know whether *Wolbachia* prevalence increases to high enough frequencies to cause the extinction of local popula-

tions of *D. innubila*. The consistently high levels of polymorphism within *D. innubila* populations (Table 5) suggest long-term persistence rather than repeated extinction-colonization events.

Population genetic data can also be used to infer the duration of local *D. innubila–Wolbachia* associations. Within populations, each major mtDNA haplotype is found in similar proportions of infected and uninfected flies (Fig. 5). Such similarity is the expected outcome of a balance between the cytoplasmic fitness advantage of *Wolbachia* infection and incomplete maternal transmission of *Wolbachia*, a balance that is expected to take several hundred generations to achieve (Dyer and Jaenike 2004).

The mtDNA of *D. innubila* shows extremely strong differentiation among populations, with an overall Φ_{ST} of 0.42 in contrast to the much lower differentiation at nuclear loci (Table 4). For completely isolated populations, the degree of differentiation due to drift depends on both the number of generations that have elapsed since isolation and the local effective population sizes (Kimura 1955; Crow and Kimura 1970). Paleoclimatic, fossil pollen, and pack rat midden data indicate that the low-elevation woodland communities on these sky islands were more expansive and thus less isolated in the cooler and more mesic climates during the last glacial maximum 18,000 years ago (Van Devender 1990; Thompson and Anderson 2000). Suppose that *D. innubila* was panmictic until 18,000 years ago, after which its populations became completely isolated due to elevational contraction of suitable habitat. At any time during the differentiation process, the among-population variance for mtDNA variants is expected to be four times greater than among nuclear genes (Crow and Kimura 1970). Alternatively, suppose that levels of differentiation are governed by an equilibrium between migration and drift in an unstructured island model. In this case, the ratio of mtDNA to nuclear differentiation is expected to be $(4Nm + 1):(Nm + 1)$; that is, in the range of one to four depending on the magnitude of Nm. Empirically, however, the ratio of Φ_{ST} for mtDNA to Φ_{ST} for nuclear genes is closer to 30. Thus, whether we assume ongoing divergence among completely isolated populations or migration-drift balance, the level of mtDNA differentiation is much greater than expected. Further evidence of mitochondrial differentiation is provided by the 21 singleton mtDNA haplotypes in our samples, which tend to be most closely related to *locally* common haplotypes, not haplotypes common in other sky island populations (Fig. 4).

Excessively high mtDNA differentiation could be caused by male-specific migration among sky islands. We think this is unlikely, because the X-linked and autosomal loci exhibit similar levels of differentiation (with male-specific migration, one would expect greater differentiation at X-linked loci) and because this species is not sexually dimorphic for body size or wing length. Among *Drosophila* there is little evidence for differential dispersal of the sexes (reviewed in Powell 1997; Markow and Castrezana 2000), particularly for passive dispersal, the likely method of movement among sky islands.

We propose that the amplified geographic differentiation of mtDNA in *D. innubila* results from its association with male-killing *Wolbachia*. Under selection-transmission balance for *Wolbachia* dynamics, all individuals in a population carry mtDNA haplotypes descended from infected females. Therefore, the effective population size of the mtDNA is very nearly proportional to the prevalence of *Wolbachia* infection in the host population (Johnstone and Hurst 1996). Because the observed Φ_{ST} was 30 times greater for mtDNA than for the nuclear genes, in contrast to the expected fourfold difference, this suggests an effective species-wide infection prevalence I_e of $4/30 \approx 13\%$. This is at the lower end of our empirically estimated infection frequencies, which ranged from 12% to 46% in our collections (this study; Dyer and Jaenike 2004).

The difference between the observed and effective infection frequencies may be due to fluctuations in *Wolbachia* frequency. In particular, occasional periods of low infection frequency would drive the mtDNA through population bottlenecks. In the long run, all individuals will carry mtDNA descended from the infected females that survived the most recent low point in infection prevalence. Such fluctuations in *Wolbachia* infection prevalence will thus amplify the genetic drift experienced by mtDNA and cause elevated differentiation of mtDNA relative to nuclear genes. Even within three years of collecting in the Chiricahuas we observed considerable temporal fluctuation in prevalence, with the proportion of infected females in this population dropping from 46% in 2001 to 25% in 2003 (Dyer and Jaenike 2004). Furthermore, in the Santa Catalina (SC) population all of the infected and most of uninfected *D. innubila* carry a single mtDNA haplotype. Because the SC population harbors normal levels of nuclear diversity (Table 5), this pattern may have resulted from *Wolbachia* extinction or a period of low frequency in the Santa Catalinas, followed by mtDNA reestablishment by infected female(s) carrying a single mitochondrial haplotype.

Patterns similar to those in *D. innubila* have been found in plants harboring CMS factors, which can exhibit significant variation among local populations in the frequency of female versus hermaphroditic plants (reviewed in Olson et al. 2005). Cytoplasmic loci in such species show greater population structure than the nuclear genome (e.g., McCauley 1998), as expected from local rather than large-scale CMS bottlenecks (Taylor et al. 2001; Ingvarsson and Taylor 2002).

In conclusion, despite genetic differentiation among *D. innubila* populations and strong selection on the host for resistance to *Wolbachia*, the phenotypic manifestation of *Wolbachia* infection—strong embryonic male-killing—is the same in all sky island populations of these flies. Thus, the *D. innubila–Wolbachia* interaction phenotype shows no evidence of having undergone evolutionary divergence among local populations, either in the overall phenotype or the level of resistance. This contrasts with several other host-parasite systems that can exhibit local matching or mismatching between host and parasite genotypes (e.g., Lively 2001; Thrall et al. 2002; Gasnier et al. 2000).

Although male-killing *Wolbachia* affect the sex ratio of *D. innubila* populations (Fig. 3), we found no genetic evidence that these populations have low effective population sizes, as would result from repeated extinction-colonization events. In contrast, our demographic data suggest that the prevalence of the *Wolbachia* infection is dynamic both within and among

populations. This observation is strengthened by the high level of mtDNA differentiation among *D. innubila* populations, which indicates the occurrence of substantial fluctuations in the prevalence of *Wolbachia* infection. Overall, the association between *D. innubila* and male-killing *Wolbachia* is ecologically dynamic within local populations, but evolutionarily coherent across the species as a whole.

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