Diversity of *Wolbachia pipientis* Strain *w*Pip in a Genetically Admixtured, Above-Ground *Culex pipiens* (Diptera: Culicidae) Population: Association With Form Molestus Ancestry and Host Selection Patterns

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ABSTRACT Analysis of molecular genetic diversity in nine marker regions of five genes within the bacteriophage WO genomic region revealed high diversity of the *Wolbachia pipentis* strain *w*Pip in a population of *Culex pipiens* L. sampled in metropolitan Chicago, IL. From 166 blood fed females, 50 distinct genetic profiles of *w*Pip were identified. Rarefaction analysis suggested a maximum of 110 profiles out of a possible 512 predicted by combinations of the nine markers. A rank-abundance curve showed that few strains were common and most were rare. Multiple regression showed that markers associated with gene Gp2d, encoding a partial putative capsid protein, were significantly associated with ancestry of individuals either to form molestus or form pipiens, as determined by prior microsatellite allele frequency analysis. None of the other eight markers was associated with ancestry to either form, nor to ancestry to *Cx. quinquefasciatus* Say. Logistic regression of host choice (mammal vs. avian) as determined by bloodmeal analysis revealed that significantly fewer individuals that had fed on mammals had the Gp9a genetic marker (58.5%) compared with avian-fed individuals (88.1%). These data suggest that certain *w*Pip molecular genetic types are associated with genetic admixturing in the *Cx. pipiens* complex of metropolitan Chicago, IL, and that the association extends to phenotypic variation related to host preference.

KEY WORDS *Wolbachia*, *Culex pipiens*, bacteriophage WO, genetic substructuring, bloodmeal analysis

Many species of arthropods, as well as other invertebrates, harbor the endosymbiotic bacterium *Wolbachia pipientis*, a maternally transmitted alphaproteobacterium that exhibits strain-specific properties that interfere with reproductive processes (Werren et al. 1995, Werren 1997, Baldo and Werren 2007, Lo et al. 2007). In the mosquito host, *W. pipientis* typically induces sperm-egg cytoplasmic incompatibility (CI) when the mating partners harbor different strains or when the male is infected and the female is not (Laven 1951, 1967b; Yen and Barr 1971, 1973; Zabalou et al. 2008). Mosquitoes of the *Culex pipiens* L. species complex commonly harbor *W.* strain *w*Pip infections (Cornel et al. 2003, Klasson et al. 2008). This mosquito species complex is comprised of a group of closely related taxa categorized as species or forms with diverse biogeography, natural history, habitat associations, vertebrate host associations, and medical significance (Laven 1967a, Dahl 1988, Cornel et al. 2003, Fonseca et al. 2004). At least 17 CI phenotypes or cytotypes of *W. pipientis* strain *w*Pip have been observed in individuals of the *Cx. pipiens* species complex, resulting commonly in between-population infertile mating (Laven 1967a, Yen and Barr 1971, Guillemaud et al. 1997). Indeed, a population of *Culex quinquefasciatus* Say was eradicated from a village in Burma through intentional release of males showing full CI to the wild population, before the association of CI and *Wolbachia* infection was even known (Laven 1967b). It has been proposed that CI provides a mechanism for speciation in this species complex (Laven 1959, 1967a,7b).

Two members of the *Cx. pipiens* species complex, namely *Cx. pipiens* and *Cx. quinquefasciatus*, serve as important vectors of human and animal pathogens, including *Wuchereria bancrofti*, West Nile virus (WNV), St. Louis encephalitis virus, and some species of bird malaria (Foster and Walker 2009). However, blood host selection and expression of autogeny

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among the members of the species complex varies by taxon, geographic setting, habitat, and host availability (Spielman 1971; Hamer et al. 2009, 2011). Females of *Cx. pipiens* in North America, a member of the species complex found at northern latitudes, exhibit marked ornithophilia and even show behavioral preferences within the Passeriformes (Simpson et al. 2009), yet some amount of mammal feeding typically occurs in nearly all northern and mid-latitude populations (Apperson et al. 2004, Molaei et al. 2006, Savage et al. 2007). Hamer et al. (2008) suggested that *Cx. pipiens* could function both as enzootic, epizootic, and epidemic vector of WNV in the metropolitan Chicago, IL, because of a relatively high rate of feeding on humans, as well as the detection of a single WNV-positive female with a human bloodmeal (Hamer et al. 2009).

Huang et al. (2009) showed that the population of *Cx. pipiens* in metropolitan Chicago consisted of a genetic admixture of *Cx. pipiens* form pipiens, *Cx. pipiens* form molestus Forskål, and *Cx. quinquefasciatus* taxa, based on certain taxon-diagnostic microsatellite alleles. Further, those females that had fed on mammals had a higher genetic ancestry for *Cx. pipiens* f. molestus while those that had fed on birds had a higher ancestry for *Cx. pipiens* f. pipiens. Microsatellite alleles indicative of a *Cx. quinquefasciatus* genetic background, by contrast, were randomly distributed with regard to bird or mammal host selection and there was relatively poor representation of this taxon in the samples. Of relevance is that a subterranean population of *Cx. pipiens* f. molestus was discovered in the Chicago metropolitan area recently, decades after its first discovery and characterization in the 1940s (Wray 1946, Mutebi and Savage 2009), suggesting the possibility of genetic introgression into the aboveground *Cx. pipiens* population from the below ground *Cx. pipiens* f. molestus population. Indeed, the taxon *Cx. pipiens* f. molestus has received considerable attention because of its unusual habitat associations, its highly disjunct population distribution geographically, its expression of autogeny in the first gonotrophic cycle, and the tendency of females to bite humans readily (Byrne and Nichols 1999, Fonseca et al. 2004, Kent et al. 2007, Kothera et al. 2010). However, the relationships between the population structure of the *Cx. pipiens* population in metropolitan Chicago and the diversity of *Wolbachia* in that population are completely unknown. One prediction is that variation in *Wolbachia* diversity should covary with the genotypic and phenotypic associations recently characterized in that population (Hamer et al. 2009, Huang et al. 2009). The prediction was examined in the current study, using a *Wolbachia* prophage marker system as the genetic assessment tool (Duron et al. 2011).

Materials and Methods

Mosquito Samples. Extracted DNA from individual mosquitoes used in previous studies of blood host choice and *Cx. pipiens* ancestry (Hamer et al. 2009, Huang et al. 2009), were used in this study. Briefly, mosquitoes were collected from 26 sites within a 10 km radius of southwestern suburban Chicago, IL. Sampling was conducted from May to October, 2005–2006, using Center for Disease Control (CDC) light traps, CDC gravid traps, and backpack aspirators. Mosquitoes were identified to species morphologically, and DNA was extracted from the abdomens of blood fed female *Cx. pipiens* using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Extracted DNA was used as template to confirm identification of *Cx. pipiens* by polymerase chain reaction (Crabtree et al. 1995), as well as for bloodmeal analysis (Hamer et al. 2009) and microsatellite genotyping (Huang et al. 2008, 2009). Previous results showed an association between genotype and host choice, such that those *Cx. pipiens* that fed on mammals had a significantly higher *Cx. pipiens* f. molestus genetic ancestry than did those that fed on birds, which were genotyped as *Cx. pipiens* f. pipiens (Huang et al. 2009).

Wolbachia Prophage WO Genetic Profiling. Presence of *Wolbachia* DNA in samples was confirmed by polymerase chain reaction (PCR) using primers Wsp81 F and Wsp691R (Zhou et al. 1998). The genetic profiling system used here was based on hypervariable open reading frames in genes of the WO prophage, developed by Fujii et al. (2004) and elaborated by Duron et al. (2006, 2011). The WO prophage is a complex of phage that exists in duplicate copies in the *W. pipientis* strain *w*Pip genome (Klasson et al. 2008). Nine markers spanning five genes from this mobile element were selected, namely: Gp1b, Gp2a, Gp2d, Gp2e, Gp3a, Gp3b, Gp3c, Gp7d, and Gp9a. PCR was accomplished using the Epicenter Failsafe PCR kit (Epicenter, Madison, WI). One microliter of DNA sample suspensions was used per reaction (total reaction volume, 50 μl). Primers used are listed in Table 1. Reaction conditions were as in Duron et al. (2006), briefly: initial denaturation of 1 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C, followed by a final elongation step of 7 min at 72°C. Amplicons were separated on 2% agarose gels (E-gel System, Invitrogen, Carlsbad, CA) and visualized with an AlphaImager 2200 system (Alpha Innotech/Cell Biosciences, Santa Clara, CA). The genetic profiles were characterized as presence or absence of an amplicon for each marker, following Duron et al. (2006). All reactions were run in duplicate for purposes of confirmation.

Statistical Analysis. Multiple regression with categorical predictor variables (Allison 1999, Eberly 2007) was used to associate genotype as determined by the microsatellite method (Huang et al. 2009) with the prophage genetic profiles obtained in this study. The dependent variable was the estimated proportion of each individual's genetic ancestry (*Cx. pipiens* f. pipiens, *Cx. quinquefasciatus*, and *Cx. pipiens* f. molestus), derived from microsatellite genotyping (Huang et al. 2008) and previously reported in Huang et al. (2009). Separate regression analyses were performed for each of these taxa. The independent variables (nine in total) were dichotomous and represented the presence or absence of an amplicon

Table 1. Primers used in WO genetic profile analysis of *Cx. pipiens* samples

Name	Forward	Name	Reverse	Amplicon size
Gp1b-F	AAGTGGCTGGAAAATGTATAAC	Gp1b-R	TGAGTTTGCTATTTACTGCTAG	307
Gp2a-F	GCAAATATTTTAGGTGAGGCGC	Gp2abd-R	ACGGAGTTCTCCACAAAGTACT	363
Gp2d-F	AGAACACCCTGGTGAAAATACC	Gp2abd-R	ACGGAGTTCTCCACAAAGTACT	586
Gp2e-F	TTCTACAACAGATGATCAAACG	Gp2e-R	CATCATCGGCCTACATAGCCA	306
Gp3a-F	AAGTGGGTTTGATGAAAAATGT	Gp3a-R	TACATCATCATGCGGAATGTGC	1339
Gp3bc-F	CAGAGGTCTTTCAATTGAAAAG	Gp3b-R	GCGGTTATAAAATTTAAATGCA	428
Gp3bc-F	CAGAGGTCTTTCAATTGAAAAG	Gp3cd-R	AAGAACTTCAGTACGATACTTG	196
Gp7d-F	AAAAGGTTCTACAAGATTTTTGAA	Gp7d-R	CCTTTATAACCTCTTGGCATTGT	423
Gp9a-F	TTTTGCCATTGCAGAGTTACAG	Gp9a-R	TGATAACTCTCCCAATGGT	220

(each representing a genetic marker). Logistic regression was also used to associate the host choice phenotype (avian or mammal) with particular WO genetic markers. The proportion of genetic ancestry was transformed using the arcsine of the square root. Regression analysis was carried out using SAS/STAT 9.2 software (SAS Institute Inc, 2009). Rarefaction analysis was carried out using EstimateS software (Colwell 2009).

Results

In total, 166 female mosquitoes were analyzed. Out of 2^9 or 512 possible genetic profiles based on all combinations of the nine markers used here, samples were distributed into a much more limited set of 50 genetic profiles (Fig. 1). The first five profiles encompassed 51.2% (85/166) of individuals, with the remaining 48.8% distributed among the other 45 profiles; that is, few profiles were common and most were rare, including many singletons (Fig. 2A). The most common profile contained 35 individuals, that is, 21% of the total.

A profile accumulation chart was produced using the genetic profiles as a prelude to rarefaction analysis (Fig. 2B). Briefly, data were randomized and sampled without replacement. The first incidence of each unique profile was noted and added to the total profile count. A rarefaction curve was plotted using the Coleman Rarefaction Curves feature of the EstimateS software. The plot suggests a plateau in unique profiles as it approached the observed sample of 50. Based on this trend, the nonparametric Chao1 estimator was calculated using the EstimateS software and yielded an estimate of 110 profiles likely to be present in the study population (95% CI [72.1, 213.32]).

Multiple regression revealed a statistically significant relationship between the WO prophage genetic markers and *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus ancestry. For *Cx. pipiens* ancestry, the regression model was significant ($F_{9,156} = 1.98$; $P = 0.04$) and varied significantly with marker Gp2d (Gene product 2d: partial putative capsid protein (Fujii et al. 2004, Duron et al. 2006) (Coefficient = 8.5; $t = 2.51$; $P = 0.013$). When marker Gp2d was absent ($n = 29$), *Cx. pipiens* f. pipiens ancestry was 90.3%, whereas when the marker was present ($n = 137$), *Cx. pipiens* f. pipiens ancestry was 97.8% (Fig. 3). None of the other eight markers was associated with *Cx. pipiens* f. pipiens

Fig. 1. Bacteriophage WO genetic profiles based on combinations of nine genetic markers of five genes, identified in *Cx. pipiens* sampled in metropolitan Chicago, IL. Fifty profiles are depicted. Marker patterns are represented as follows: dark boxes indicate positive results (PCR amplification of the gene segment as visualized by bands on gel), and white boxes indicate negative results. Profiles are organized from most to least common.

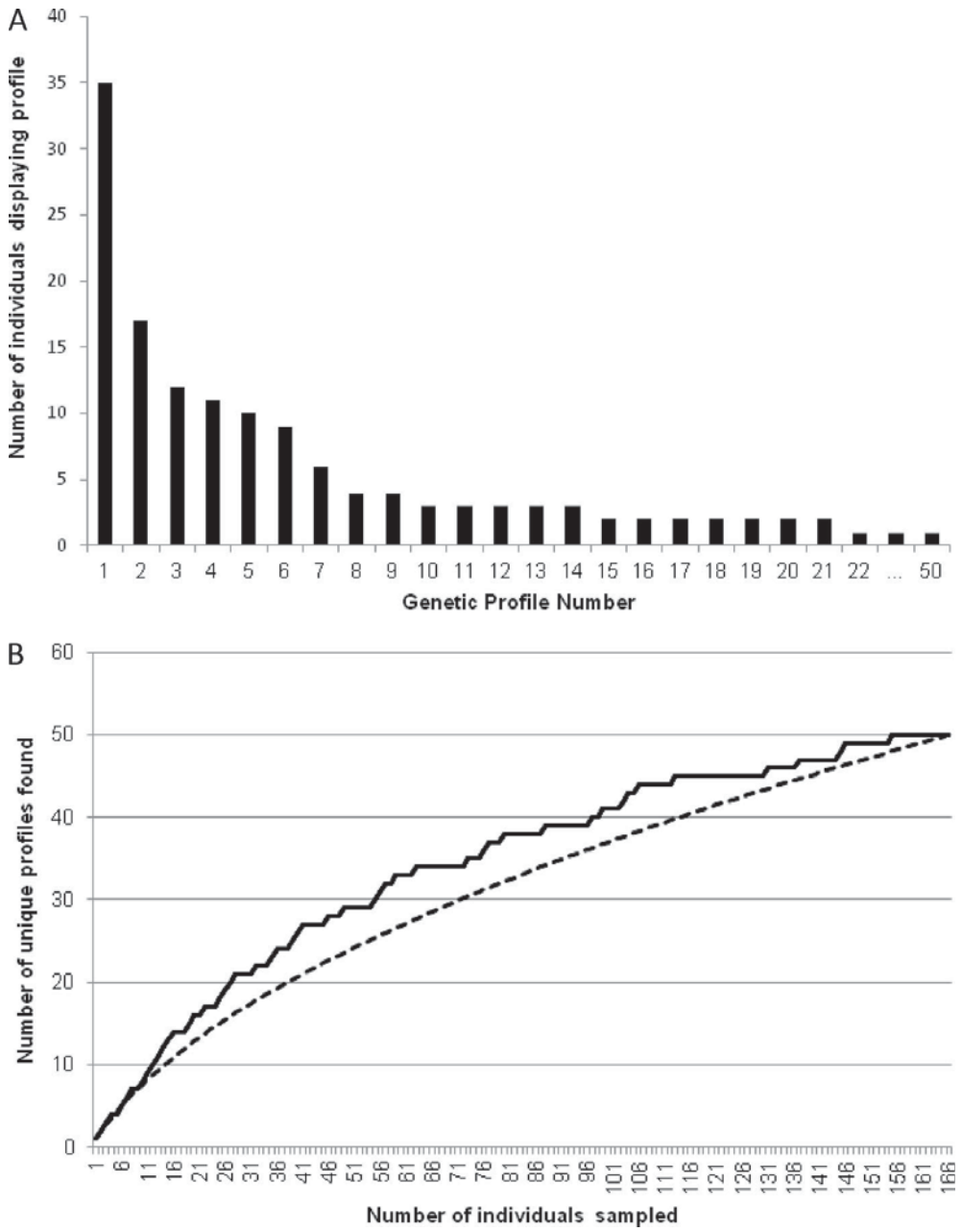

Fig. 2. (A) Rank-abundance curve showing distribution of common and rare genetic profiles of the WO bacteriophage in *Cx. pipiens* females sampled in metropolitan Chicago, IL. The number of individuals displaying each profile from Fig. 1 is indicated by bar height. (B) Genetic profile accumulation chart showing the number of unique profiles detected during analysis. The solid line indicates a sample profile accumulation pattern, and the broken line indicates the rarefaction curve generated in EstimateS.

ancestry. For *Cx. pipiens* f. molestus ancestry, the regression model was significant ($F_{9,156} = 2.34$; $P = 0.017$) and also varied significantly only with marker Gp2d (Coefficient = −7.9; $t = 2.49$; $P = 0.014$). *Cx. pipiens* f. molestus ancestry when marker Gp2d was absent ($n = 29$) was 8.3%, whereas *Cx. pipiens* f. molestus ancestry when marker Gp2d was present ($n = 137$) was 1.6% (Fig. 3). The overall multiple regression model for *Cx. quinquefasciatus* ancestry was not significant ($F_{9,156} = 1.25$; $P = 0.27$). Logistic regression of host choice (mammal vs. avian) on genetic marker ($n = 9$, binomial: presence or absence) revealed that fewer mammal-fed *Cx. pipiens* had the Gp9a genetic marker (58.5%) than did avian-fed *Cx. pipiens* (88.1%) (Likelihood Ratio Test: $\chi^2 = 19.4$; df = 1,8; $P < 0.0001$). There were no statistically significant relationships between any other markers and the host choice phenotype.

Discussion

Wolbachia in mosquito hosts nearly ubiquitously contain WO-B prophage and other mobile genetic elements (Masui et al. 2000, 2001; Sanogo and Dobson

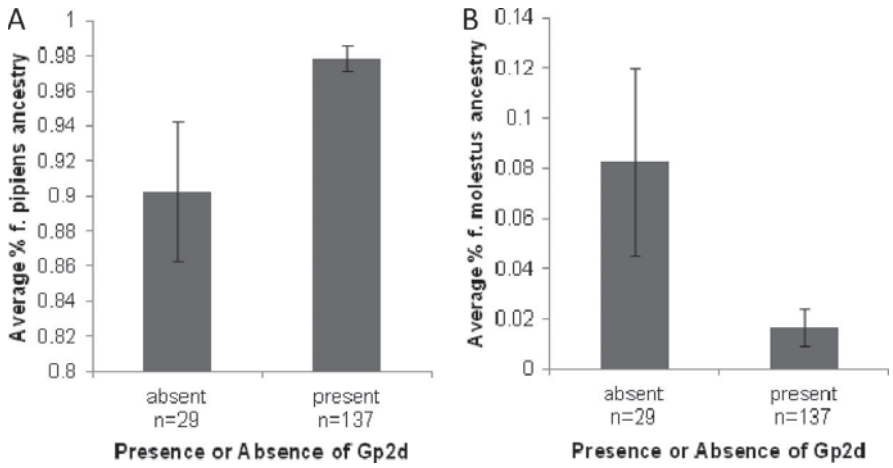

Fig. 3. Association of marker Gp2d with greater *Cx. pipiens* f. pipiens ancestry and lower *Cx. pipiens* f. molestus ancestry. (A) Average *Cx. pipiens* f. pipiens ancestry. (B) Average *Cx. pipiens* f. molestus ancestry. Error bars indicate standard error. Note: Y-axes have different scales.

2004; Chauvatcharin et al. 2006; Klasson et al. 2008). Hypervariable genes within the genome of the WO prophage have been developed as effective population genetic markers for *Wolbachia* strain characterizations (Duron et al. 2006, 2011); other systems include multilocus sequence typing (Baldo et al. 2006, Atyame et al. 2011) and nucleotide sequence variation of the *wsp* gene (Zhou et al. 1998). We selected the WO prophage system because of its successful application in revealing substantial within and among population variation in *Cx. pipiens* hosts (Duron et al. 2006, 2011) as well as for its repeatability (Sanogo and Dobson 2004, Chauvatcharin et al. 2006). Several genes have been examined in attempts to correlate variation in CI phenotype, including *ftsZ* (Guillemaud et al. 1997), 16S rRNA (Rousset et al. 1992), and *wsp* (Duron et al. 2005). In one study, there was no association between MLST strains of *w*Pip and the taxa comprising the *Cx. pipiens* species complex (Atyame et al. 2011). Further, a remarkably high degree of cytoplasmic compatibility exists among genetically diverse *Wolbachia* strains (Duron et al. 2011), suggesting that diversification of *W. pipientis* strain *w*Pip is not always associated with variation in the CI phenotype.

The current study documents a high level of variation in *W. pipientis* strain *w*Pip in the *Cx. pipiens* population in suburban Chicago, IL, based on phage WO genetic profiling. The central hypothesis of the current study is that this variation associates with both genotypic and phenotypic variation in the native *Cx. pipiens* population under study. A previous study by Duron et al. (2006) revealed a total of 66 *w*Pip variants in a survey of 208 mosquitoes from 12 laboratory strains and 19 field populations, spanning 12 countries and four continents, and surveyed at 15 genetic markers. Later, Duron et al. (2011) estimated an average of 11 *w*Pip variants per population. The mosquitoes used in the current study all came from one study area within a 10 km radius in the Chicago, IL, area, easily comprising a single above-ground population given the geographic setting, and were only tested for nine genetic markers because of limitations of available DNA in the extractions. As such, it was expected that variation in *w*Pip profiles would be relatively limited. However, a total of 50 unique variants were found in the study area, many more than predicted based on observations from Duron et al. (2006). However, the within-site sample size in that study was modest ($n \leq 20$) compared with ours ($n = 166$), so it is possible that had they sampled more intensively in a single area, a pattern more like the one found in Chicago may have emerged. In fact, in a subsequent study, the same group found 37 distinct strains in 178 individuals from four sites in southern France, which showed a distribution similar to that found here, in which few strains were common and many strains were rare (Duron et al. 2011).

Genetic profile one likely represents the dominant *w*Pip profile present in the study population. Many of the other profiles are probably minor variants of this dominant strain. Inspection of Fig. 1 supports this conclusion. The prophage sequence used here is known to be hypervariable because of high rates of recombination (Bordenstein and Wernegreen 2004, Klasson et al. 2008). The hypervariability can also be attributed to numerous single nucleotide polymorphisms found in these markers (Duron et al. 2006). Profiles 2–4 differ from profile one by the presence or absence of only one amplicon (see Fig. 1). In profiles two and three this difference lies within Gp3, an *orf7*gene described by Bordenstein and Wernegreen (2004) as the most rapidly recombining gene in the genome of not just the prophage itself, but the endosymbiont. Taken together, the above discussion suggests that the presence or absence of marker amplicons likely reflects mutation in the primer region, rather than presence or absence of the entire gene. This conclusion is further supported by the fact that while some genes studied contained more than one marker region, absence of an amplicon for one marker did not preclude presence of another amplicon in the same gene. Our study assumes that each profile rep-

resents a single *Wolbachia* strain in each mosquito, and not two or more strains. If the latter were the case, then some of the profiles we observed would represent combinations of the strains, but the method we used would not provide a means to differentiate them.

Logistic regression showed that the presence of marker Gp9a, which is a partial putative baseplate assembly protein (Fujii et al. 2004, Duron et al. 2006), was associated with the avian host selection phenotype, and absence of the marker with the mammal host selection phenotype. Although we do not infer a functional relationship underlying this association, marking it as noncausal but positive, other studies have documented *Wolbachia*-associated behavioral variations in invertebrates (Fleury et al. 2000, Panteleev et al. 2007, Peng et al. 2008), and specifically in blood feeding behaviors of *Aedes aegypti* (L.) (Moreira et al. 2009, Turley et al. 2009). *Wolbachia*-infected, older *Ae. aegypti* mosquitoes required more probing to pierce the skin to take a bloodmeal, and were less successful in doing so compared with uninfected *Ae. aegypti* of the same age, resulting in fewer and smaller bloodmeals, although *Ae. aegypti* is an aberrant host for *Wolbachia* thus these observations may be because of pathologic effects not observed in natural *Wolbachia* hosts such as *Cx. pipiens.* The reduced feeding efficiency was associated with the strain of *Wolbachia* present, rather than merely presence or absence of *Wolbachia,* and so may help to explain the association found between *Wolbachia* genetic profile and host choice in the current study. Additionally, there is evidence that *Wolbachia* infection also affects the ability of *Ae. aegypti* mosquitoes to successfully process bloodmeals during egg development (McMeniman et al. 2011).

The species accumulation curves and rarefaction analysis suggest a limit in the natural population variation that is well below the possible number of combinations ($2^9 = 512$) based on the nine markers used. Graphical analysis showed a distinct tapering of the number of genetic profiles toward an asymptotic limit. This finding also aligns with the nonparametric Chao1 analysis that predicts ≈110 profiles, slightly more than twice the number observed. This prediction necessarily includes mostly rare profiles, such as singletons in the projected sample. The likely reason for a limit to the natural variation is simply that many genetic changes in functional genes such as those used in the current study result in detrimental mutations, and are unlikely to be neutral. The majority of the markers used in this study fall within a putative capsid protein gene (Fujii et al. 2004), so variation from standard sequences could easily cause a lethal condition.

When data were analyzed by individual markers rather than whole genetic profiles, one pair of significant associations emerged. Based on multiple regression, the presence of marker Gp2d, which is a partial putative capsid protein (Fujii et al. 2004, Duron et al. 2006), was associated with greater *Cx. pipiens* f. pipiens ancestry and lesser *Cx. pipiens* f. molestus ancestry. Huang et al. (2009) showed widespread *Cx. pipiens* f. molestus ancestry in the native *Cx. pipiens* population. The fact that a population of *Cx. pipiens* f. molestus was discovered underground very near the study site in 1946 (Wray 1946) and a cryptic *Cx. pipiens* f. molestus population was recently discovered or possibly rediscovered within the study area (Mutebi and Savage 2009, Kothera et al. 2010) suggests a source of the genes that could explain the presence of *Cx. pipiens* f. molestus ancestry in the above ground *Cx. pipiens* population under study (Huang et al. 2009), and the one responsible for epizootic and epidemic transmission of WNV (Hamer et al. 2009). Two hypotheses regarding origin of *Cx. pipiens* f. molestus populations are that they have a Mediterranean center of evolution (Fonseca et al. 2004), or that they evolved locally from above ground *Cx. pipiens* f. pipiens populations (Kothera et al. 2010). The *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus populations in Chicago were shown to be divergent from one another (Kothera et al. 2010), but with some evidence for hybridization (and see Huang et al. 2010). Our data suggest an association between genetic variation and substructuring in *W. pipientis* strain *w*Pip and *Cx. pipiens* relative to the molestus form, indicating the likelihood of local interactions such as mating and introgression between the two mosquito taxa. Atyame et al. (2011) conclude that *W. pipientis* strain *w*Pip has a monophyletic origin and five distinct variant groups, and that "a considerable degree of *Wolbachia* diversity can evolve within a single host species over short evolutionary periods." Clearly a high degree of diversification is present in the *W. pipientis* strain *w*Pip population associated with the above-ground *Cx. pipiens* population we studied, a finding consistent with their observation. We did not sample the below-ground, wild f. molestus population described by Mutebi and Savage (2009) to investigate the range of genetic variants of *W. pipientis* strain *w*Pip associated with it and to discover if the variants are similar to, or different than, those associated with the above ground *Cx. pipiens* population. It would make a sensible follow up study to this one.

Acknowledgments

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