SYMPOSIUM

Defense Heterogeneity in Host Populations Gives Rise to Pathogen Diversity*

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ABSTRACT: Host organisms can harbor microbial symbionts that defend them from pathogen infection in addition to the resistance encoded by the host genome. Here, we investigated how variation in defenses, generated from host genetic background and symbiont presence, affects the emergence of pathogen genetic diversity across evolutionary time. We passaged the opportunistic pathogen *Pseudomonas aeruginosa* through populations of the nematode *Caenorhabditis elegans* varying in genetic-based defenses and prevalence of a protective symbiont. After 14 passages, we assessed the amount of genetic variation accumulated in evolved pathogen lineages. We found that diversity begets diversity. An overall greater level of pathogen whole-genome and per-gene genetic diversity was measured in pathogens evolved in mixed host populations compared with those evolved in host populations composed of one type of defense. Our findings directly demonstrate that symbiont-generated heterogeneity in host defense can be a significant contributor to pathogen genetic variation.

Keywords: pathogen evolution, host heterogeneity, genetic diversity, defensive symbionts.

Introduction

Elucidating processes that generate diversity has been a central focus of evolutionary biology (Losos et al. 2013). Such diversity is important for pathogen evolution because it can facilitate pathogen adaptation to new hosts (Ekroth et al. 2021). For example, genetic diversity allows pathogens to evade recognition by the immune system (Cobey 2014), which can impact pathogen fitness, prevalence, and control strategies (He and Pascual 2021). Across host individuals, a diverse pathogen population can increase the likelihood of a pathogen genotype spreading in a homogeneous host population or encountering a susceptible host genotype in a heterogeneous host population (Van Baalen and Beekman 2006; Gibson 2022). Identifying factors that govern pathogen diversity can thus further understanding of the forces shaping pathogen traits, persistence, and evolution.

Pathogen diversity can be favored by host diversity (or diversity in resistance levels) across biological scales. Host heterogeneity at the community level is a positive predictor of parasite species diversity (Hechinger and Lafferty 2005; Janz et al. 2006; Kamiya et al. 2014; Johnson et al. 2016). At the host population level, variation in resistance can also maintain variation in pathogen traits, such as transmission rates (Fleming-Davies et al. 2015). Within individual hosts, the adaptive immune system can select for increased pathogen diversity at both the genetic and the species level (Cobey 2014). Host heterogeneity is thus a critical source of pathogen biodiversity and variation.

Genetic diversity for resistance in host populations confers varying levels of protection against infection (King and Lively 2012; Liston et al. 2016). Many organisms also harbor defensive symbionts that protect them from natural enemies, improving their fitness against these threats and facilitating adaptation across different environments (Jaenike et al. 2010; King 2019). Defensive symbionts are prevalent among animals, from invertebrates to mammals, as well as plants (Khosravi and Mazmanian 2013; Oliver et al. 2013; Selosse et al. 2014; Berasategui et al. 2022). These microbes can protect hosts from biotic stressors through interference and resource competition or by priming host immunity (Gerardo and Parker 2014; Ford and King 2016). Symbionts can alter the phenotypes of their hosts and effectively extend the host genetic repertoire, allowing hosts to occupy new niches (Kitano and Oda 2006; Hurst 2017).

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Variation in symbiont presence in host populations may play a role in increasing pathogen diversity similarly to variation in resistance encoded by the host genome. Within and between host populations, variation in defense could be generated by symbionts similar to the immune system alone (Jaenike et al. 2010; Fenton et al. 2011). Furthermore, the prevalence of symbionts across host populations is influenced by their costs, as well as the selection imposed by the enemies (Gerardo et al. 2020). Few studies have directly examined the role of protective symbionts in favoring enemy diversity. In aphids, microbial symbiont species diversity and parasitoid species diversity are positively correlated (Hafer-Hahmann and Vorburger 2021), but the relationship is not causal in the field (Narayan et al. 2022). Symbiont-mediated protection could act as an additional driver of pathogen evolution beyond host gene-encoded resistance (Ford et al. 2016, 2017). We expect pathogen diversity to emerge in host populations with a mix of different defense strategies.

We used experimental evolution to directly test the role played by host defense heterogeneity in the generation and maintenance of pathogen genetic diversity. We passaged the opportunistic pathogen *Pseudomonas aeruginosa* through populations of the nematode *Caenorhabditis elegans* varying in gene-based defense and symbiont presence for 14 passages. The *C. elegans* genome encodes immune defenses against pathogen infection (Engelmann and Pujol 2010). The nematode also has a core bacterial microbiome in nature, where each member varies in their impact on nematode fitness (Dirksen et al. 2020). One member of this microbiome, *Pseudomonas berkeleyensis*, was found to improve *C. elegans* survival upon *P. aeruginosa* infection (Montalvo-Katz et al. 2013). *Pseudomonas berkeleyensis* protects nematodes by conferring host immunity prior to *P. aeruginosa* infection, inducing expression of genes regulated by the p38 mitogen-activated protein kinase of *C. elegans* (PMK-1). In our experiment, *P. aeruginosa* evolution occurred in nematode populations that were either homogeneous (same in genetic background and in presence/absence of symbiosis) or heterogeneous (mix of genetic backgrounds and pairing in symbiosis). To quantify the genetic diversity that arose, we sequenced and compared the whole genomes of evolved pathogens. Our main prediction was that heterogeneity in host defenses would favor genetically diverse pathogen populations compared with those evolving within homogeneous host populations.

Methods

Nematode and Bacterial Strains

The wild-type host *Caenorhabditis elegans* strain N2 Bristol and *Escherichia coli* strain OP50 were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). Immunocompromised mutant *pmk-1* (*M03F8.4(op497)*, *pmk-1(km25)*) was provided by Jonathan Hodgkin (University of Oxford). The immunocompromised mutant is a knockout of PMK-1. Without this gene, nematodes no longer differ in expression of immune genes regulated by PMK-1, nor are individuals with the symbiont able to resist *Pseudomonas aeruginosa* (Montalvo-Katz et al. 2013; Hoang et al. 2024). The protective bacterium, *Pseudomonas berkeleyensis* MSPm1 (previously identified as *Pseudomonas mendocina*), was provided by Michael Shapira (University of California, Berkeley). Green fluorescent–labeled *P. aeruginosa* PA14 (PA14-GFP) was provided by Kevin Foster (University of Oxford).

Experimental Evolution

We passaged *P. aeruginosa* PA14-GFP under eight treatments (fig. 1): four homogeneous host treatments (all host individuals share the same genotype and symbiont exposure), three heterogeneous host treatments (each composed of 50:50 mixtures of hosts from the homogeneous populations), and one no-host treatment. To start, one single colony (i.e., all cells being genetically identical to each other) of PA14-GFP was grown overnight in lysogeny broth (LB) and spread onto nematode growth medium (Cold Spring Harbor Protocols 2016), then incubated at 30°C for 1 day. Roughly 1,000 nematodes were transferred from their respective rearing plates (described below) onto the *P. aeruginosa* infection plates, where they were incubated at 20°C. After 1 day, nematodes were washed off each plate. We then transferred 10% of the nematode/M9 mixture to a fresh tube. Following a modified protocol from Vega and Gore (2017), the nematodes were washed three times with M9 buffer, then kept at 4°C for 30 min to stop peristalsis. We then added enough cold bleach, such that the final concentration was 0.3% in the nematode/M9 mixture, to remove surface bacteria. After briefly mixing, the mixture was kept on ice for 10 min, then cold M9 was added to stop the bleaching process. Nematodes were washed once more with cold M9, and the supernatant was plated to check for efficiency of bleaching. The nematode mixture was then crushed using a BeadBug microtube homogenizer (Benchmark Scientific), and homogenates were plated out onto LB plates. After overnight incubation, 100 colonies were picked into broth to start the next passage. Each treatment consisted of five replicate rearing and infection plates across 14 passages.

For the no-host treatment, we followed the same experimental schedule for treatments involving nematodes. We grew the bacteria on nematode growth medium (NGM) and incubated at 30°C for 1 day, then 20°C for 1 day. Since bacterial extraction from nematodes required a light bleaching step, we could not perform this step on the no-host

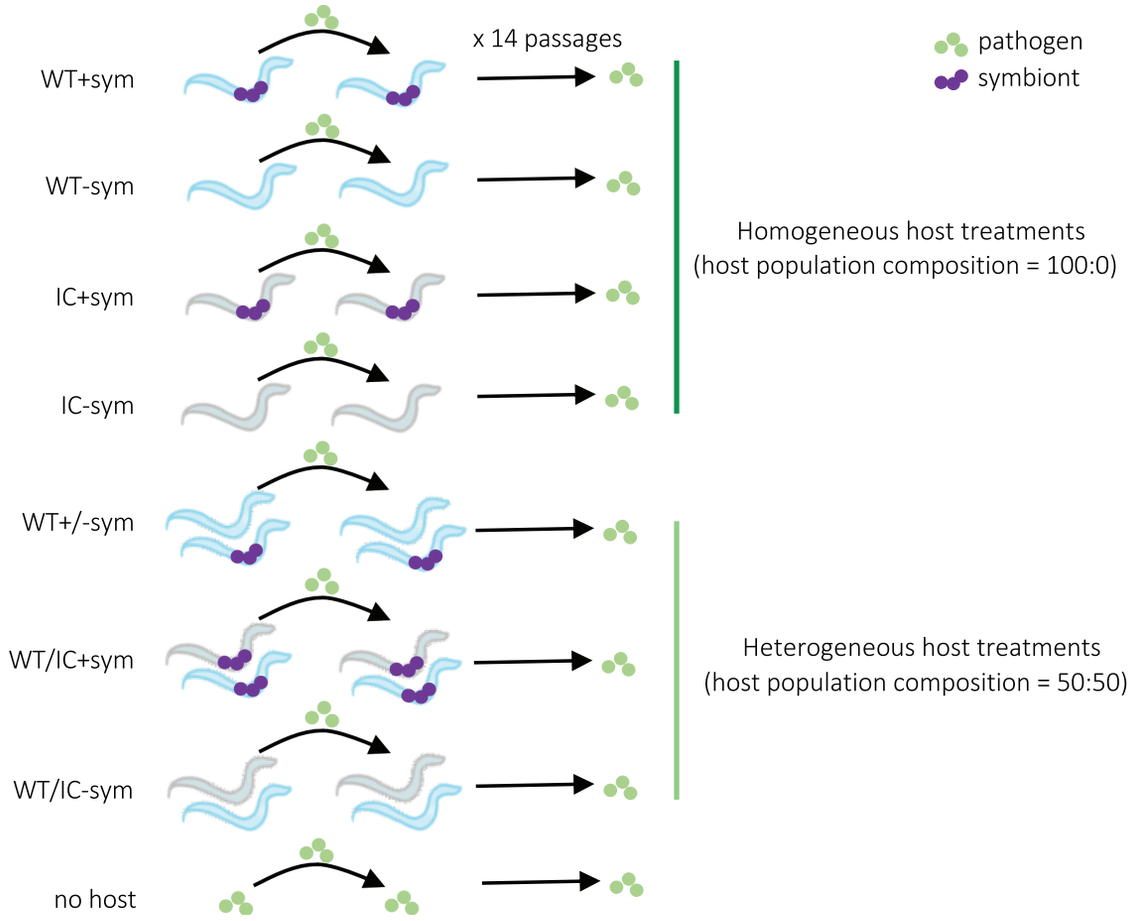

Figure 1: Experimental evolution design. WT = wild-type host; IC = immunocompromised host; sym = symbiont (*Pseudomonas berkeleyensis*).

populations. Instead, we dabbed random areas of the bacteria grown on NGM with a pipette tip to streak onto LB agar plates. We picked 100 colonies into LB broth.

Nematodes were kept evolutionarily static throughout the experiment. Nematode eggs were collected, surface sterilized, and age synchronized following a standard sodium hypochlorite protocol (Stiernagle 2006). After hatching, L1 larvae were spotted onto lawns of either *P. berkeleyensis* or *E. coli* OP50 food grown on NGM plates and incubated at 20°C. After 2 days, L4/young adults were transferred to *P. aeruginosa* plates as described above. For each passage, eggs were collected from stock nematode populations that were regularly resurrected from −80°C to limit accumulation of de novo mutations in host lineages throughout the experiment. We did not include a treatment with 50:50 immunocompromised hosts with or without *P. berkeleyensis* (IC±sym) because we expected these populations to be similar to the homogeneous immunocompromised host treatments (IC+sym, IC−sym) in terms of the level of protection obtained from the symbiont. The ancestral pathogen causes similar levels of mortality in immunocompromised hosts with (IC+sym) or without (IC−sym) the symbiont (Montalvo-Katz et al. 2013; Hoang et al. 2024). Similarity in mortality between the two treatments was expected because immunocompromised hosts lack the pathway being stimulated by the symbiont and cannot be conferred protection. However, it is possible that immunocompromised hosts may allow the symbiont to persist longer in the host and interact with the pathogen, generating some host heterogeneity.

DNA Extraction and Sequencing

To evaluate pathogen diversity at the genomic level, we conducted whole-genome resequencing of the evolved populations. For each replicate population, we grew 40 individual clones separately overnight in LB broth, then standardized the OD_{600} (optical density measured at 600 nm) of each clone before pooling them into one tube

to perform DNA extraction. We extracted genomic DNA using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. Sample libraries were prepared using the Illumina DNA Prep kit and sequenced on an Illumina NextSeq 2000. Sequence quality was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and sequences were trimmed using fastP (Chen et al. 2018).

The ancestral PA14-GFP clone was sequenced using Oxford Nanopore Technologies (ONT) in addition to Illumina for hybrid assembly. Quality control and adapter trimming was performed with bcl2fastq (Illumina, n.d.) and porechop (n.d.) using default parameters for Illumina and ONT sequencing, respectively. Hybrid assembly with Illumina and ONT reads was performed with Unicycler (Wick et al. 2017), and the resulting assembly was annotated using the Bakta annotation pipeline (Schwengers et al. 2021). Coverage of mapped reads was calculated using Samtools (Li et al. 2009). Each sample had at least 200× coverage.

Calculating Nucleotide Diversity

To compare the level of genetic diversity across evolved pathogens, we calculated the nucleotide diversity for each population. Nucleotide diversity (also known as π) is the average number of nucleotide differences between all possible pairs of individuals in the population and is a common measure of genetic diversity across a wide range of organisms, including pathogens (Neafsey et al. 2012; Jansen et al. 2015; O'Neill et al. 2015; Mongelli et al. 2022). For example, studies of natural pathogens have used nucleotide diversity to assess drivers of pathogen genetic variation and evolution within hosts (O'Neill et al. 2015; Mongelli et al. 2022). We measured nucleotide diversity across the entire genome and for each gene to quantify the level of genetic diversity in the evolved pathogen populations. To calculate nucleotide diversity across the entire genome of *P. aeruginosa*, we used the software PoPoolation (Kofler et al. 2011) to calculate nucleotide diversity (Tajima's π) using a sliding window analysis. Each window size was 5 kb, with a step size of 2.5 kb. To calculate nucleotide diversity per gene, we also used PoPoolation (Kofler et al. 2011) with gene (coding sequence) coordinates obtained from a gene transfer format (.gtf) file generated from the Bakta annotation of the ancestral clone assembly.

Percent Change Calculation

To calculate the percent change in pathogen genetic diversity between hosts exposed to the symbiont (WT+sym, IC+sym) versus hosts without the symbiont (WT−sym, IC−sym), we calculated the mean of WT+sym and IC+sym populations (mean_{sym}) and WT−sym and IC−sym populations ($\text{mean}_{\text{no\_sym}}$), then used the following formula:

$$\left(\left(\text{mean}_{\text{sym}} - \text{mean}_{\text{no\_sym}}\right)/\text{mean}_{\text{no\_sym}}\right) \times 100.$$

Statistical Analyses

We used a Kruskal-Wallis test to compare the means of genome-wide nucleotide diversity between the two types of host population composition and to compare the means of nucleotide diversity per gene. We used an ANOVA to compare the number of genes with nucleotide diversity greater than zero between the two types of host population composition.

Functional Pathways for Top 1% of Nucleotide Diversity Values

We identified the top 1% of genes with the highest nucleotide diversity values and used the DAVID (Database for Annotation, Visualization and Integrated Discovery; Sherman et al. 2022) tool to map each gene to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto 2000).

Results

We calculated nucleotide diversity across the genome of each evolved pathogen population (fig. 2*A*, 2*B*). Homogeneous host treatments had lower nucleotide diversity across the genome compared with heterogeneous treatments ($\chi^2_1 = 24.67, P < .001$; fig. 2*C*, 2*D*). We determined whether symbiont presence alone can drive pathogen diversity in the absence of host diversity (the first four treatments of fig. 2*C*). Symbiont presence had a marginally insignificant effect on pathogen diversity ($F_{1,16} = 3.59$, $P = .076$), where there was an increase of 8.06% in pathogen nucleotide diversity compared with that in the absence of the symbiont. There was no host or interaction effect (host: $F_{1,16} = 0.69$, $P = 0.42$; interaction: $F_{1,16} = 0.45$, $P = .51$). Including the WT±sym treatment in the comparison increases pathogen nucleotide diversity by 20.53%, suggesting that intermediate symbiont frequencies had a larger influence on pathogen diversity than the presence of symbionts alone.

Genes (coding sequences) in homogeneous host treatments also have lower nucleotide diversity ($\chi^2_1 = 24.34$, $P < .001$; fig. 3*A*, 3*B*). However, the number of genes with nucleotide diversity greater than zero is lower in heterogeneous treatments (for reference, the number of coding sequences estimated for the annotated ancestral *Pseudomonas aeruginosa* genome was ~5,300; $F_{1,33} = 18.2$, $P < .001$; fig. 3*C*, 3*D*). To better understand the traits

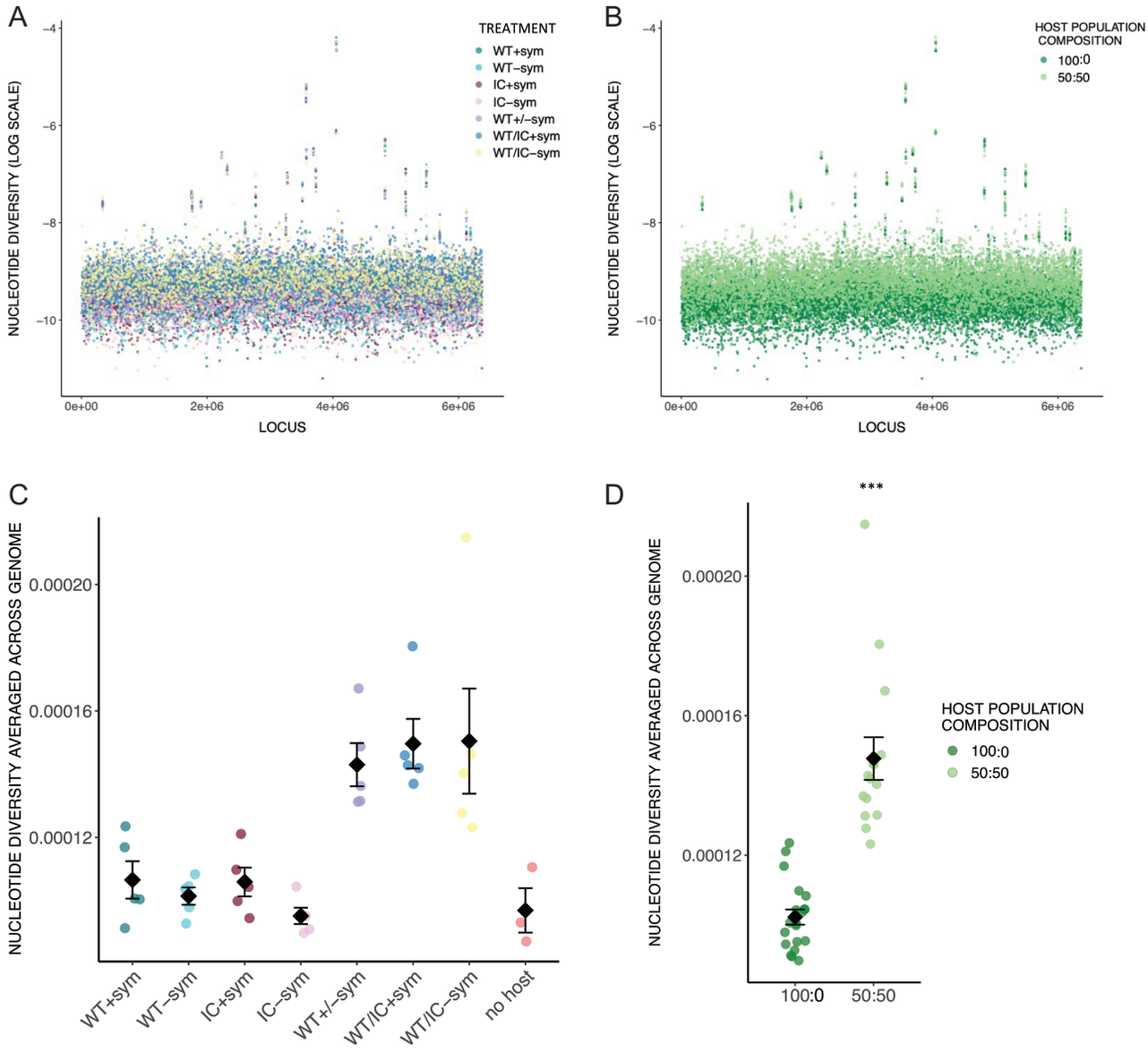

Figure 2: Genome-wide nucleotide diversity of each evolved pathogen population. *A*, Log scale of nucleotide diversity values in 5-kb windows with a step size of 2.5 kb, color coded by treatment. *B*, Same as *A*, color coded by host population composition. *C*, Mean nucleotide diversity values for each treatment. Each data point is the genome-wide mean of a replicate population from the evolution experiment. *D*, Same as *C*, but grouped by host population composition. Error bars indicate standard errors. $^{***}P < .001$.

affected by these genes, we mapped them to *P. aeruginosa* biological pathways in the KEGG database (fig. 4). Several pathogen genes had high nucleotide diversity in homogeneous host treatments but not heterogeneous treatments. Many of these are involved in metabolic functions in *P. aeruginosa* (figs. 4, S1; fig. S1 is available online), such as metabolism of propanoate and β-alanine and degradation of valine, leucine, and isoleucine. Some genes belong to the LysR transcriptional regulator family of proteins (fig. S1), which are involved in bacterial quorum sensing and motility (Maddocks and Oyston 2008).

Discussion

We determined whether heterogeneity in host defenses—either generated by gene-based traits or microbial symbionts—across a population can drive the emergence of pathogen genetic diversity. We found higher genome-wide

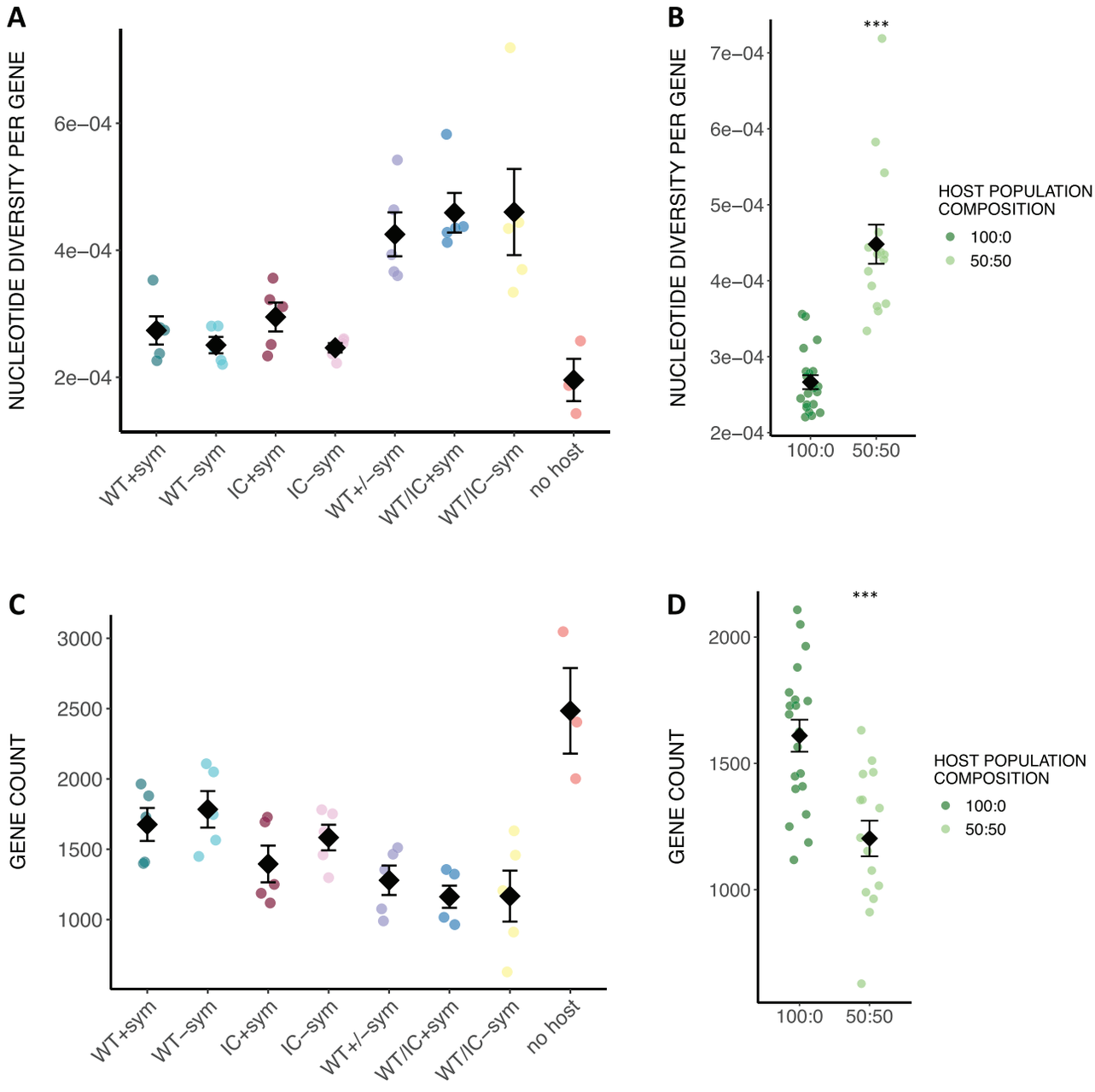

Figure 3: Nucleotide diversity per gene for each evolved population. *A*, Mean nucleotide diversity per gene for each treatment. *B*, Same as *A*, but grouped by host population composition. Each data point is the mean of a replicate population from the evolution experiment. *C*, Number of genes where nucleotide diversity was greater than zero for each treatment. *D*, Same as *C*, but grouped by host population composition. Error bars indicate standard errors. $^{***}P < .001$.

nucleotide diversity of evolved pathogens in all heterogeneous host populations compared with host populations homogeneous in defense type. Specifically, heterogeneity in host genotypes and symbiont presence generated similar levels of nucleotide diversity. These results suggest that symbionts may be a cryptic form of selection shaping the genetic variation in pathogens. Such high levels of genetic diversity could impact the potential for pathogens to adapt and perform new functions (Neafsey et al. 2012). New mutations may promote pathogen transmission and emergence in host populations. Host diversity may then be favored to limit these dynamics and strengthen resistance

Figure 4: Top 1% of genes with highest nucleotide diversity values in each evolved population mapped to Kyoto Encyclopedia of Genes and Genomes pathways. Darker bars at the bottom indicate homogeneous host treatments; lighter bars indicate heterogeneous treatments.

against pathogens (King and Lively 2012; Van Houte et al. 2016; Chabas et al. 2018; Broniewski et al. 2020).

Greater nucleotide diversity per gene also emerged in heterogeneous hosts, but fewer genes were polymorphic. Some of these genes are involved in bacterial metabolism and motility. These pathways can be necessary for pathogen population growth and virulence (Feinbaum et al. 2012). Higher variation in fewer genes may underlie differences between the selection imposed by the homogeneous and heterogeneous host populations. Previous studies have similarly shown that immunocompromised *Caenorhabditis elegans* populations can favor genetic diversity (Jansen et al. 2015; Hoang et al. 2024). Activities performed by individual cells can affect the traits expressed by the entire bacterial population (Bassler 2002; O'Neill et al. 2015; Davis and Isberg 2016), and allelic composition can determine the function of the community as a whole (Azimi et al. 2020). Synergy between pathogen genotypes can favor genetic diversity within the host (Van Houte et al. 2016). For example, cooperation between nonvirulent pathogen strains can generate virulence collectively, suggesting that community interactions are an important aspect of pathogen function and disease dynamics (Ruiz-Bedoya et al. 2023). Similar to abiotic environments, hosts can shape the genetic composition and interactions between bacteria over time (Sun et al. 2024).

We found that symbiont presence at intermediate frequency had a larger contribution to pathogen diversity than when the symbiont was fixed or absent in the host population. Similarly, symbiont presence in host populations composed of both wild-type and immunocompromised hosts favored greater pathogen diversity compared with host populations composed of either host genotype alone. Similar variation may impact pathogen diversity in other symbiotic systems. Protective symbionts and host gene-encoded defenses can vary in frequency and efficiency across host populations (Jaenike et al. 2010; Vorburger et al. 2013; Liston et al. 2016). A potential reason for such variation may be related to the metabolic and fitness costs associated with host gene-encoded defenses and symbiont-encoded defenses (Gerardo et al. 2020). In the absence of parasitism, symbiont frequency can decline across host generations (Oliver et al. 2008). Symbiont protection can also fail at higher temperatures because of their sensitivity to heat, while gene-coded defenses remain robust (Corbin et al. 2017; Doremus et al. 2018). Conversely, pathogen resistance alleles can decrease over time when protective symbionts are pervasive in host populations (Martinez et al. 2016). Furthermore, greater symbiont protection increases the likelihood of reduced immune vigilance in ecological and evolutionary time (Rafaluk-Mohr et al. 2018, 2022; Metcalf and Koskella 2019; Ford and King 2021). Alternatively, it is possible to maintain both host-encoded defenses and symbiont defenses. Greater protection conferred by the symbiont does not necessarily induce greater costs to hosts (Cayetano et al. 2015), and high pathogen pressure can select for both protective symbionts and robust innate defenses (Hrček et al. 2018). Experiments with additional host genotypes and varying pathogen virulence will help to disentangle the contributions of host genetic variation, symbiont presence, and their interaction in generating diverse pathogens.

Studies directly comparing the efficacy of defensive symbionts versus that of the immune system are available for only a few model systems (e.g., members of the aphid family; Hrček et al. 2018; Weldon et al. 2019). Research on other symbiosis models would provide broader context into how variation in symbiont frequency can favor pathogen diversity. Future directions may include systems where symbiont-mediated immune priming has been shown to be beneficial. For example, exposure to native microbiome or probiotics can stimulate the immune system of bees and increase resistance against parasite infection (Evans and Lopez 2004; Näpflin and Schmid-Hempel 2016; Emery et al. 2017). Monitoring parasite genetic diversity may be important for bee health in the future, particularly given variation in the prevalence of protective microbes among colonies. A related question is whether more complex microbiomes (i.e., more than one symbiont) would have similar effects on pathogen genetic diversity across evolutionary time. Potential systems to address these questions include *C. elegans*, *Drosophila*, zebrafish, and mice, each with well-characterized host gene-encoded defenses and tractable microbial communities (Douglas 2019; Proctor et al. 2023).

Our findings have demonstrated that defensive symbionts can be a source of selection favoring pathogen genetic diversity. Given that symbionts can generate heterogeneity in host defense in addition to that encoded by the host genome, genes and symbionts may similarly shape pathogen evolution, with consequences for the long-term efficacy of disease control using protective symbionts (Ford and King 2016). Furthermore, pathogen diversity likely has played an important role in the evolution of diverse defense mechanisms. Vertebrate hosts activate the innate immune system to respond to immediate threats and deploy the adaptive immune system to differentially respond to pathogens (Robertson 1998; Sela et al. 2018). Similar to the immune system, hosts can be coinfected by different symbionts conferring protection against different enemies (Kaltenpoth and Engl 2014), and pathogen diversity can contribute to the maintenance of symbiont diversity among hosts (Hafer and Vorburger 2019; Hafer-Hahmann and Vorburger 2020). Overall, our study suggests that the pathogen dynamics occurring within individual hosts can mediate long-term ecological and evolutionary patterns of interacting entities.

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Statement of Authorship

K.L.H. and K.C.K. conceived and designed the study. K.L.H. collected the data and conducted the data analysis, with guidance from T.D.R. and K.C.K. K.L.H. drafted the manuscript, with critical revisions provided by all authors.

Data and Code Availability

Raw sequences have been deposited in the National Center for Biotechnology Information Sequence Read Archive under BioProject accession number PRJNA998467, and processed data are available from the Mendeley Data repository (https://doi.org/10.17632/w8zmyf7hc5.1; Hoang 2024).

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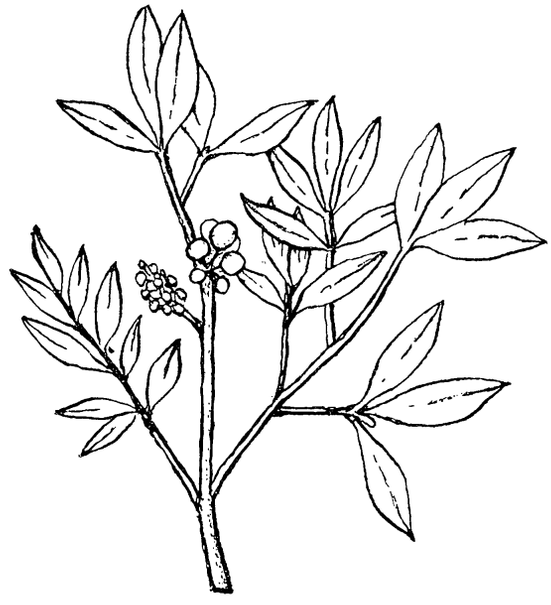

"These mistletoes differ from the members of all the other parasitic groups in being perennial woody parasites upon woody hosts, and also in their method of parasitism." Figured: *Phoradendron flavescens*. From "The Phenogamous Parasites" by Charles A. White (*The American Naturalist*, 1908, 42:12–33).