

Is Sharing Caring?

Elucidating the Effects of the Presence of CRISPR-Cas Systems on Rates of Horizontal Gene Transfer Using Network Analysis

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Abstract

Horizontal Gene Transfer (HGT) is a mechanism by which organisms (mainly prokaryotes) can share genetic material outside of inheritance. HGT has proven to have significant effects on bacterial genome evolution, allowing for increased genetic diversity and advanced niche adaptation. CRISPR associated (CRISPR-Cas) is an adaptive immune system in prokaryotes that has garnered a lot of research attention recently, largely due to its applications in gene editing. Due to the nature of how it works, using guide RNA to cut DNA, CRISPR-Cas systems have been thought to affect rates of HGT. Effort has mostly been focused on how CRISPR-Cas systems affect the mechanisms of HGT and thus little is known about its effects on HGT rates. This work uses a network-theoretic approach to better characterize the effects of the presence of CRISPR-Cas systems on HGT rates within bacterial populations. This approach makes use of phylogenetic methods for estimating HGT rates, improving on estimates using methods based on genome composition used previously. Understanding the effects of CRISPR-Cas on HGT may help uncover potential targets for curbing the spread antibiotic resistance genes.

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Contents

1	Background	3
1.1	What is CRISPR-Cas?	3
1.2	Horizontal Gene Transfer	3
1.2.1	Mechanisms	3
1.2.2	Rate Influencing Factors	4
1.3	Phylogenomic Networks	4
1.4	Detection	4
1.5	How Does CRISPR Affect HGT?	5
1.5.1	Interference Mechanisms	5
1.5.2	Complexities And Costs Of CRISPR-Cas Systems	5
1.5.3	Balancing the Cost of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and HGT	5
2	Objectives	6
3	Methods	6
3.1	Summary	6
3.2	Data Collection	8
3.3	Gene Presence/Absence Matrix	8
3.4	Makophylo Rate Estimations	8
3.5	Network Construction	8
3.6	Network Statistics	9
4	Results	10
5	Discussion	16
5.1	Gene Indel Rates are Different for CRISPR and Non-CRISPR Operation Taxonomic Unit (OTU)s	16
5.2	Phylogenomic Networks Have Low Assortativity	16
5.3	HGT Dynamics Vary Across Bacterial Genera	16

1 Background

1.1 What is CRISPR-Cas?

CRISPR-Cas systems are sets of nucleotide motifs (spacers) interspaced with nucleotide repeats (CRISPRs) and CRISPR-associated (Cas) proteins (with sequences usually adjacent to the CRISPR motifs) that have an adaptive immune function in many bacteria and archaea [1]. Each nucleotide motif is the result of a DNA molecule that was previously taken up by the host, serving as a marker for the Cas proteins to degrade any DNA matching the motif [1]. If a bacterium possessing a CRISPR-Cas system is infected with a phage and survives, a motif representative of that phage can be integrated as a spacer. If the bacterium is reinfected with the same phage strain the spacers will guide Cas proteins to the invading phage DNA and degrade it, hence adaptive immunity. Although CRISPR-Cas appears to have evolved to degrade viral DNA system, the majority of CRISPR spacers have been found to match bacterial Mobile Genetic Element (MGE)s with no known viral match [2].

As of 2017, over 45% of bacterial genomes analyzed ($n = 6782$) appear to contain CRISPR motifs [3]. Moreover, CRISPR motifs show significant diversity between individual bacteriums as they represent a chronological history of spacer acquisition (usually via viral infection or MGE “infection”) for that specific bacterium[1]. There still exist many bacterial strains, and even entire genera with no *known* CRISPR-Cas systems, although they may have simply not been discovered yet [4, 5]. Furthermore, the diversification of CRISPR-Cas systems is driven further by HGT acting on CRISPR and Cas components independently, adding another level of complexity to the propagation of CRISPR-Cas systems [1].

1.2 Horizontal Gene Transfer

1.2.1 Mechanisms

HGT can be defined as the exchange of genetic information across lineages [6], as opposed to vertical gene transfer between parents and offspring [7]. It is a source of genetic variation, allowing organisms to adapt quickly by copying a gene with a specific function, rather than evolving it themselves [7, 8]. There are 3 main mechanisms of HGT

Transformation Free floating DNA is taken up by a bacterium and incorporated into the genome [6]. DNA is not always incorporated successfully even if it taken up.

Conjugation The sharing of genetic material through cell-to-cell bridges, the genes for which are usually carried on a plasmid [9].

Transduction Genes or DNA fragments can be transferred through either lytic or lysogenic bacteriophages [10]. Bacterial DNA can be accidentally packaged into the lysogenic phage head during cell lysis and integrate into the next infected host. [10]. Lysogenic phages can take up bacterial DNA flanking the viral sequence and bring it with them to the next host[10].

It should be noted that *successful* HGT requires that a gene be maintained, either by genomic integration or plasmid replication. Frequently, putatively transferred genes are either lost quickly or diverge quickly due to minimal selective pressure maintaining them [11].

1.2.2 Rate Influencing Factors

The rate of HGT in bacteria is constantly in flux[12]. The more exogenous DNA, higher population density or higher phage density means more DNA is available for transfer [6]. Just like mutation rates, HGT rates are also thought to evolve in response to environmental factors or selective pressure [13, 14]. The metabolic cost or the possibility of receiving toxic or incompatible genes can disincentivize a cell to produce the machinery required for HGT [15]. But for bacteria in hospitals, the potential benefit of receiving antibiotic resistance genes can outweigh any potential danger or metabolic cost, inducing increased bacterial competence [16]. It has been suggested that genes acquired via HGT are often quickly lost since they often confer no advantage to a cell's current selective pressures [11]. Ultimately HGT rates are influenced by a variety of factors related to balancing the potential fitness costs and benefits.

1.3 Phylogenomic Networks

HGT is an important factor in understanding evolution in prokaryotes. In graph theory a tree is defined as a graph where there is only one path between every pair of nodes. In phylogenetics this implies there is only one path for genetic material to transfer between organisms, that path being vertical inheritance. The existence of HGT demonstrates that the tree model is clearly an incomplete representation of genetic relationships between bacterial OTUs. Genetic material can be transferred outside of reproduction, creating multiple paths through which a gene can exist in two different OTUs [6]. The frequency of HGT among prokaryotes has led many to re-evaluate the concept of a "prokaryotic tree of life", which ignores these horizontal interactions [17]. This prompted the idea of a prokaryotic network of life (as opposed to a tree), with edges indicating both vertical and horizontal transfers of genetic material [17]. Edges can now connect closely or distantly related OTUs if HGT has occurred between them.

1.4 Detection

While understanding that HGT is important to bacterial evolution and networks provide a useful theoretic framework to study it, constructing such networks is not trivial. Previously researchers have used methods based on gene composition (GC content, codon usage, di/tri-nucleotide frequency) to detect transferred genes. These methods are often inadequate as there are several reasons why gene composition may differ in one region vs the rest of the genome that are not related to transfer. Further they often cannot discriminate MGEs which may be falsely annotated as transfers. Phylogenetic methods, which rely on recognizing discordance between gene trees and species trees, are currently considered the best for inferring HGT events. If a gene tree is found to have a significantly different topology from a species tree, this difference may be the result of an HGT event [18]. Generally phylogenetic methods are preferred for multiple reasons:

- Can make use of multiple genomes at once [7]
- Require explicit evolutionary models, which come with their own framework for hypothesis testing and model selection [7].

- HGT events identified by parametric methods are often found by phylogenetic methods as well [7].
- In recent years, the requirements of computing power and multiple well sequenced genomes for phylogenetic methods have become easier and easier to meet [7].

While detecting HGT events with high degrees of certainty is still difficult, much progress has been made in recent years, especially using phylogenetic methods [7]. Events that may lead to false diagnosis of HGT are: incomplete lineage sorting, gene duplication followed by loss in one of the descendant lineages or homologous recombination [7, 18].

1.5 How Does CRISPR Affect HGT?

1.5.1 Interference Mechanisms

CRISPR-Cas systems have also been found to interfere with transformation-mediated HGT, by degrading foreign DNA taken up by a cell [19]. They have been shown to interfere with conjugation by targeting genes on conjugative plasmid [20]. They have also been shown to interfere with transduction by creating immunity to phage infection [20]. Thus it been hypothesized that lower rates of HGT will be observed in bacterial strains with CRISPR-Cas systems than without [20].

1.5.2 Complexities And Costs Of CRISPR-Cas Systems

Since antibiotic resistance genes are often transferred on plasmids maintaining a CRISPR-Cas systems can present a large opportunity cost, especially in environments like hospitals or trees [16]. CRISPR-Cas systems incur a metabolic cost, as Cas proteins, guide RNAs and spacer acquisition proteins must all be expressed consistently [1]. Much like the human immune system, CRISPR-Cas systems can have off-target effects, sometimes affecting HGT [21]. While resisting lytic phage infection clearly provides some fitness benefit, CRISPR-Cas has also been shown to help resist prophages which can provide super-infection immunity or reduce competitor populations [21, 22]. It has also been shown that spacers targeting a bacterium's own DNA can be acquired, leading to an auto-immune response [23]. As CRISPR-Cas systems prevent phage infection, mechanisms, denoted as anti-CRISPRs, have evolved in certain phages making them immune to CRISPR [21]. This has a two-fold effect, as it can increase the susceptibility of the host to infection reducing the fitness benefit of CRISPR, but it can also increase transduction-mediated HGT [21].

1.5.3 Balancing the Cost of CRISPR and HGT

Due to the myriad of fitness costs associated with consistently expressing CRISPR-Cas systems, bacteria have appeared to develop strategies to mitigate these costs. It has been posited that CRISPR-Cas systems need only be present in some proportion of a population as they can be horizontally transferred themselves between members [24]. This allows populations to maintain phage immunity while isolating the metabolic cost to only a few organisms [21]. It should also be noted that CRISPR-Cas genes are not necessarily constitutively transcribed, potentially allowing bacteria to tune their CRISPR-Cas to suit their selective pressures [21]. The presence of CRISPR-Cas systems have also

been shown to actually enhance HGT at a population level via transduction by reducing total phage abundance [22].

A bioinformatic analysis has shown increased levels of gene insertion and deletion as in Firmicutes with CRISPR-Cas systems compared to closely related outgroups without [25]. The effects of CRISPR-Cas systems on HGT rates are highly complex, owing in no small part to the broad range of these effects, how CRISPR activity can be modulated and the transfer of CRISPR systems within a population [21]. Taking a systematic approach may help elucidate the dynamics between CRISPR system presence and HGT rate.

2 Objectives

The null hypothesis is that bacterial strains/genera with known CRISPR systems will show no significant differences in network statistics to those strains/genera without known CRISPR systems. Ultimately the goal of this project is to examine the relationship of HGT rates and the presence of CRISPR-Cas systems, using a network theoretic approach.

Within Network Comparisons For genera with strains containing CRISPR and Non-CRISPR species, comparing the network dynamics of those sets of nodes across genera will elucidate if CRISPR-Cas systems affect the HGT rates or the association patterns of individual OTUs.

Gene Indel Rates Vs. Network Statistics Comparing insertion and deletion rates independently can help further specify what mechanisms may be responsible for trends observed in network statistics. If a mixed network is found to be density connected, but also shows a deletion bias, this may imply that most of the genes being transferred may not confer a fitness advantage.

3 Methods

3.1 Summary

The goal of the project is to create a phylogenetic network from a set of protein fasta files and the corresponding nucleotide sequences. In this case all full genomes for a given bacterial genus, for analysis of HGT. The workflow is as follows for a single genus:

1. Download fasta files
2. Filter mobile genetic elements from genomes
3. Cluster all genes into families using Diamond (% identity > 80%)
4. Construct a presence/absence matrix of gene families for organisms
5. Estimate gene family indel rates separately for the CRISPR and non-CRISPR containing genomes using the R package markophylo

6. Construct a species tree using sections from a whole genome alignment of all genomes of the genus
 - (a) Create whole genome alignment with parsnp
 - (b) Filter all aligned regions that are uninformative (identical sequence for every genome)
 - (c) Concatenate all aligned regions into 1 alignment as a nexus file
 - (d) Build the tree using Mr Bayes (10000 generations, 25% burn in rate)
7. Construct the gene trees (≤ 1500)
 - (a) Only consider families with a gene belonging in at least 40% of the genomes analyzed (ex: a family with 6 genes in 6 of 15 genomes)
 - (b) Align each family using mafft with default settings
 - (c) Build a tree for each alignment using Mr Bayes (10000 generations, 25% burn in)
8. Create 1000 subsets of 50 gene trees through bootstrap sampling
9. For each subset, use the program HiDe to infer a phylogenetic network from the species tree and the 50 gene trees.
10. Annotate each network with CRISPR data scraped from the CRISPR-one database.
11. Using the gene indel rates estimated and the annotated networks examine if there are any trends or effects on the dynamics of HGT between organisms with and without CRISPR-Cas systems.

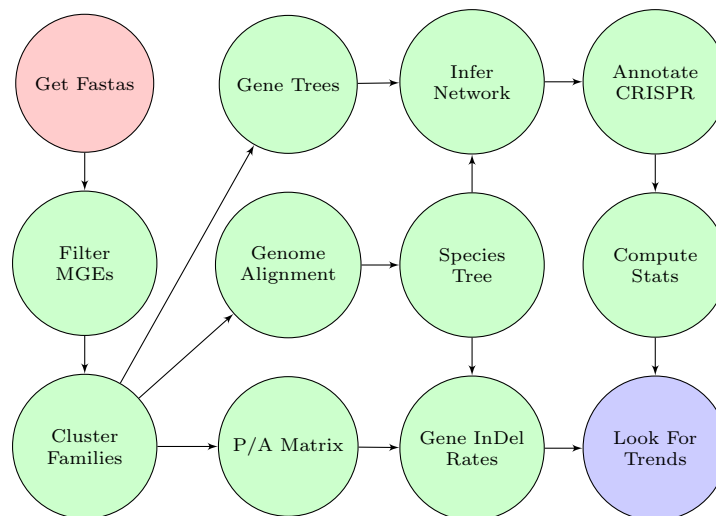


Figure 1: Diagram of the workflow for a single genus

3.2 Data Collection

Protein and nucleotide fastas of all CDS sequences and whole genome fastas for all bacterial strains included in this analysis were downloaded from NCBI RefSeq. Only bacterial data from genomes annotated as complete by RefSeq were used. Data was downloaded using custom scripts available in the github repo `./scripts/scrapingData/{NCBINameScraper.py,get_gbffs.sh}`. CRISPR annotations of Cas and Cfp proteins from the CRISPRone tool from Zhang and Ye were used to determine the presence of CRISPR systems [4]. Data from their online results were parsed and downloaded using a custom python script available in the github repo `./scripts/scrapingData/crispr_one_scraper.py`. An OTU was annotated as having a CRISPR system if it was annotated as having a CRISPR system and at least 1 CRISPR array sequence according to the CRISPRone database [4].

3.3 Gene Presence/Absence Matrix

In order to use the program *markophylo* to estimate indel rates, a Presence/Absence (P/A) matrix of gene families and organisms and a species tree are required. First any genes classified as MGEs (from NCBI annotations) are removed. Next genes are grouped into families by reciprocal BLAST hits and single link clustering. The remaining unclassified genes are compared to the NCBI non-redundant database with BLAST to check if they are genes, and if they are then they are considered their own family with one member. The P/A matrix is constructed as follows, for each OTU a binary vector is created, where each entry represents a gene family and a 1 indicates that that OTU contains at least 1 gene in that family. This is repeated for all OTUs, creating a $G \times O$ binary matrix, where G is the total number of gene families and O is the number OTUs.

There are many ways to construct a species tree, but for this project the tree will be constructed with 16S rRNA genes, using Bayesian methods, as implemented in the program MrBayes.

3.4 Makophylo Rate Estimations

Given a species tree and a gene family P/A matrix for the OTUs of the species tree the R package *markophylo* can provide gene insertion and gene deletion rate estimates [26]. The presence or absence of gene families are considered 2 discrete states, for which a (2×2) transition rate matrix (of a Continuous-time Markov chain with finite state space (CTMC-FFS) model) can be estimated using maximum likelihood techniques. The values in this estimated transition matrix are the insertion rate (transition probability of gene absence \rightarrow presence) and deletion rate (transition probability of gene presence \rightarrow absence) [26].

3.5 Network Construction

Quartet decomposition is method by which HGT events can be identified using a set of gene trees and a species tree. Given a tree T a quartet is a subtree contain 4 of the leaf nodes in T , meaning that for a tree with N leaf nodes (or OTUs) there are $\binom{N}{4}$ unique quartets in that tree. A quartet Q is considered consistent with a tree if $Q = T|Le(Q)$ where $T|Le(Q)$ is the tree obtained by suppressing all degree-two nodes in $T[X]$ and $T[X]$ is the minimal subtree of T with all nodes in X , which is a

leaf set of T [27]. To calculate the weight of an edge for the network, given a species tree S and a set of gene trees G [27]:

1. Pick a horizontal edge $H = ((u, v), (v, u))$ from S
2. Pick a gene tree G_i in G
3. Decompose G_i into it's set of quartets ϕ_i
4. Remove all quartets from ϕ_i either consistent with S or discordant with S but accounted for previously by a quartet set from another tree $G_j \in G$
5. Set $RS((u, v), \phi_i)$ to be the number of quartets in ϕ_i that support the existence of edge (u, v)
6. Set $NS((u, v), \phi_i) = \frac{RS((u, v), \phi_i)}{\lambda}$, where λ is the total number of quartets in S that are consistent with the existence of edge (u, v) .
7. The score for the edge H for tree G_i is $\max\{NS((u, v), \phi_i), NS((v, u), \phi_i)\} \in [0, 1]$
8. The total score for the edge H is the sum of scores for each tree $G_i \in G$

This total score calculation is repeated for each horizontal edge H_i in S , resulting in a list of edges, which is a complete description of the network. This is further explained in the original work, [27].

3.6 Network Statistics

All networks will be comprised of nodes representing OTUs and weighted edges represent the estimated amount of HGT events between the two incident nodes. Bootstrap replicates of a network for each genus can be computed by creating a set of networks from a set of random samples of gene trees and the same species tree. For each genus 1000 bootstrap replicate networks were produced, each with 50 gene trees sampled randomly from all gene trees produced for that genus. The population of gene trees for a genus is all gene families that contained genes such that at least 40% of all OTUs in the genus had 1 gene member of the family. If the genus *Proteus* contains 20 OTUs (genomes) then all gene trees (up to a max of 3000) of *Proteus* genes with at least $0.4 * 20 = 8$ different OTUs are sampled from. In addition extra samples are computed beyond the 1000 replicates if there are gene trees that were not sampled at least once in any sample i.e sampling is continued until the union of all samples is equal to the set of all gene trees produced, although there were no cases of additional sampling required. Given a network, with a set of nodes $V = \{V_0 \dots V_i\}$ of cardinality N and a set of weighted edges (an unordered 2-tuple and weight) $T = \{((V_1, V_2), W_{1,2}) \dots ((V_i, V_j), W_{i,j})\}$ with cardinality E descriptive statistics can be computed as follows [28]:

- **Average Node Degree:** $\frac{1}{|N_u|} \sum_{uv}^{N_u} w_{uv}$ where N_u is the set of nodes incident to u
- **Average Edge Weight:** $\frac{1}{N_c} \sum_i w_i$, The average edge weight for all nodes with CRISPR or without CRISPR
- **Node Clustering Coefficient:** $\frac{1}{k_u(k_u-1)} \sum_{vw}^{T(u)} (\hat{w}_{uw}\hat{w}_{vw}\hat{w}_{uv})^{\frac{1}{3}}$ where $T(u)$ is the set of triangles containing u [29]

- **Node Assortativity:** $A = \frac{Tr(M) - ||M^2||}{1 - ||M^2||}$ Where M is the mixing matrix of a given attribute and $||M||$ is the sum of all elements of M . $A \in [-1, 1]$. [30]
- **Network Modularity:** $Q = \frac{1}{2m} \sum_{uv} [W_{uv} - \frac{k_u k_v}{2m}] \delta(u, v)$ where m is the total weight of all edges, k_u is the degree of u and $\delta(u, v)$ is 1 if u and v both have or do not have CRISPR systems and 0 otherwise. $Q \in [-1, 1]$ [31]

For the statistics estimated separately for CRISPR and Non-CRISPR nodes, the average and standard error was calculated across all replicates. All network statistics were calculated using the networkx python package, except for modularity which was calculated using my own implementation as no modularity function was available. All subsequent statistical analysis was done using base R and all figures were produced using ggplot2.

4 Results

Note: The term indel refers to gene insertion/deletion events. It is impossible to tell between two OTUs if a gene was deleted from one or inserted in the other, leading to an ambiguity. Also a CRISPR OTU is an OTU that I annotated as having a CRISPR system as described in the methods section.

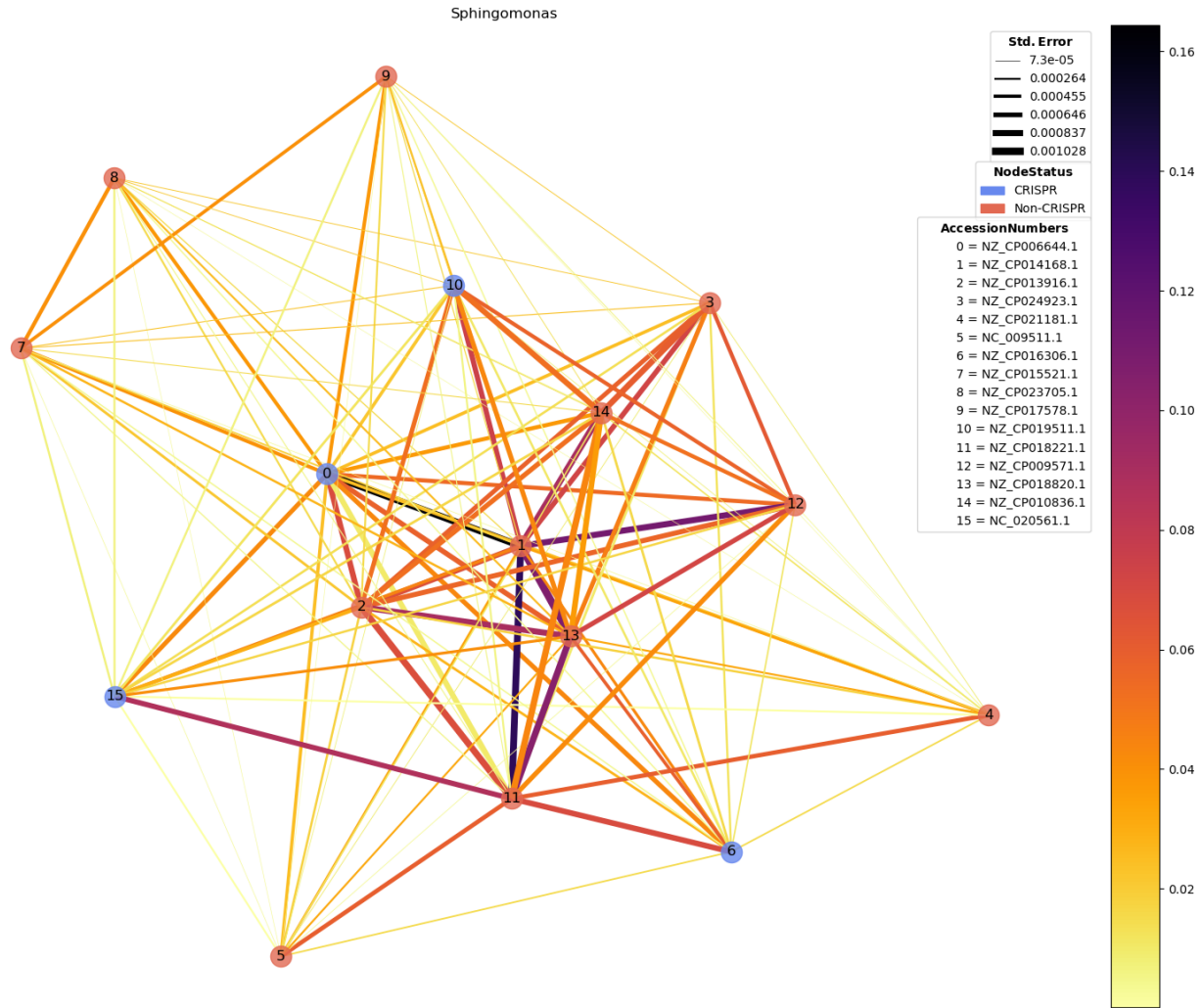


Figure 2: Example of a HGT network produced by HiDe. This network is a “consensus” over 1000 bootstrap replicates, only including edges with a mean value > 0 across all bootstraps. Each bootstrap replicate was produced from 50 randomly sampled gene trees from a total of 376 individual gene trees. Blue nodes were classified as having a CRISPR system, red nodes were not. Color represents the fraction of genes examined that were transferred along that edge. Width represents the standard error of the edge value over the 1000 bootstraps. The maximum value for an edge is 1.00, meaning all examined genes were transferred along that edge)

In Figure 2 it appears that several nodes have weak connections with most other nodes but strong connections with a few nodes. Further both CRISPR and non-CRISPR nodes both show distributions of strong and weak connections with other CRISPR and non-CRISPR nodes both. Also the standard error of each edge appears proportional to it's weight. This is likely due to the sampling, as if more genes were transferred along an edge, the more likely some of those genes were left out of any individual bootstrap sample, as the size of each bootstrap sample was $\frac{50}{376}$ of the total number of gene trees.

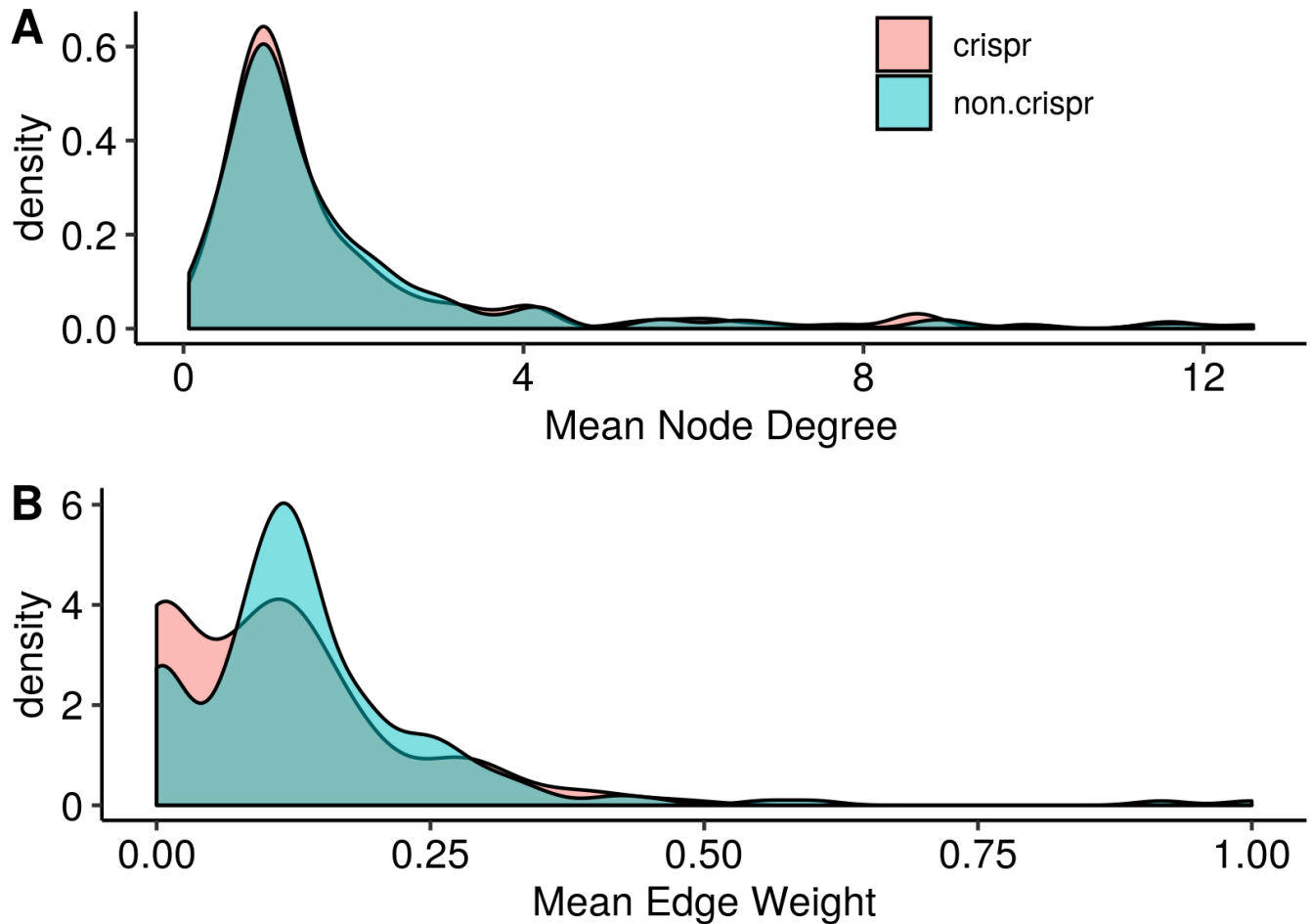


Figure 3: **A)** Distribution of the degree of all nodes with/without a CRISPR system. This includes the mean across all nodes in all 1000 replicates for each genus. **B)** Distribution of the edge weight of all edges connected to a node with/without a CRISPR system. This includes the mean across all edges in all 1000 replicates for each genus.

What is immediately clear from Figure 3 is that the node degrees are much more similar than the mean edge weights for the CRISPR and non-CRISPR OTUs. This is also supported by performing a Wilcoxon Rank Sign test comparing the CRISPR and non-CRISPR means. For the mean node degree the Wilcoxon p value is 0.07412 (n=197 genera). For the mean edge weight the Wilcoxon p value is 0.001981 (n=197 genera). So there appears to be a significant difference between the mean edge weight for all edges but not for the degree.

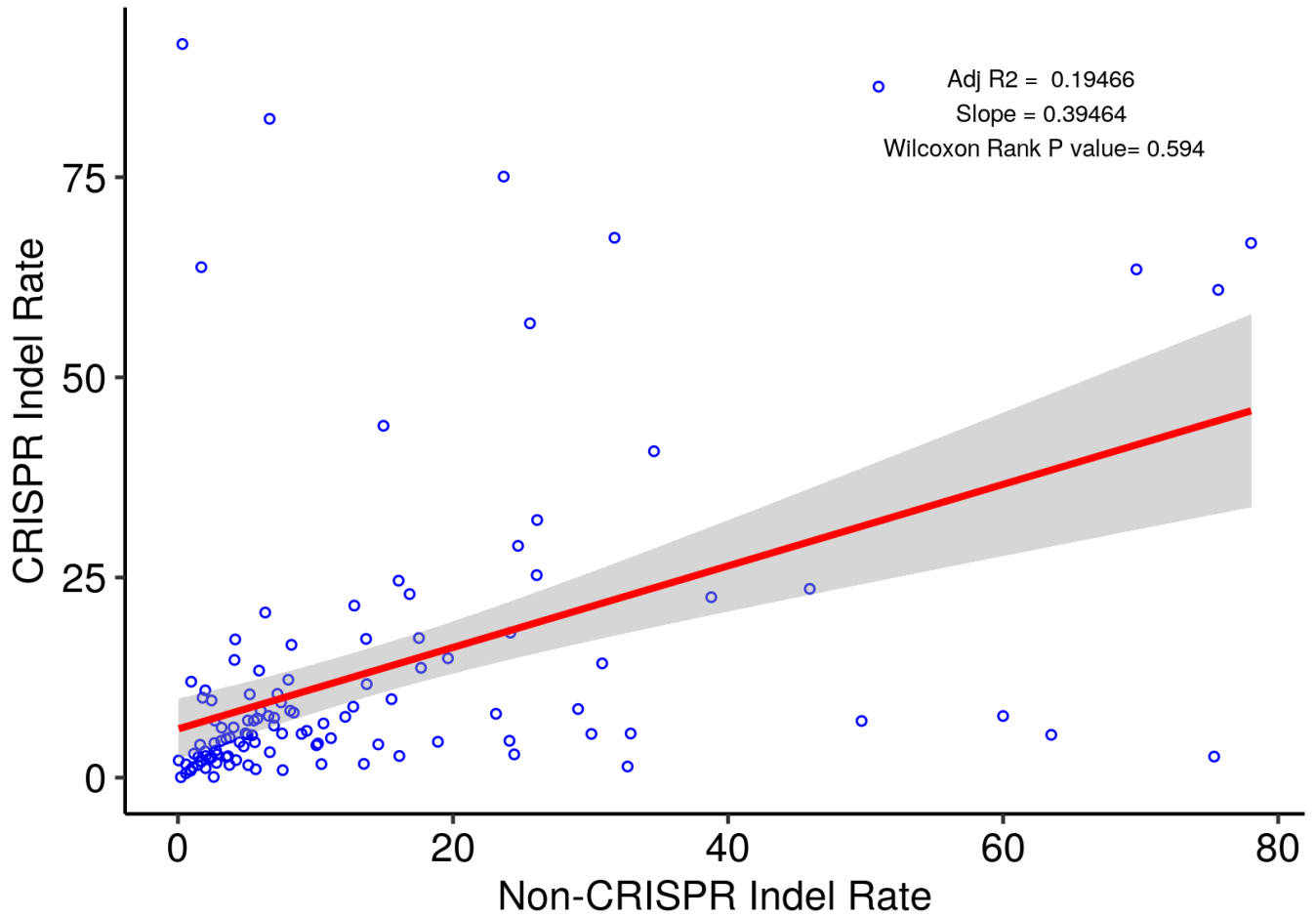


Figure 4: Markopholo estimate of gene insertion/deletion rates for the partitions of CRISPR and Non-CRISPR OTUs for each genus. The Rate is the number of indel events per base pair substitution. Each point represents a genus. There are 128 genera shown.

Figure 4 shows the estimated gene indel rates for the CRISPR and non-CRISPR branch partitions for 128 genera. The Wilcoxon signed rank test gives a p-value of 0.594, pointing to a lack of a significant difference in indel rates for CRISPR and non-CRISPR OTUs. This is despite the fact that the slope of this fitted trend line is only 0.39464, but this may be because the trend line is influenced by specific outliers.

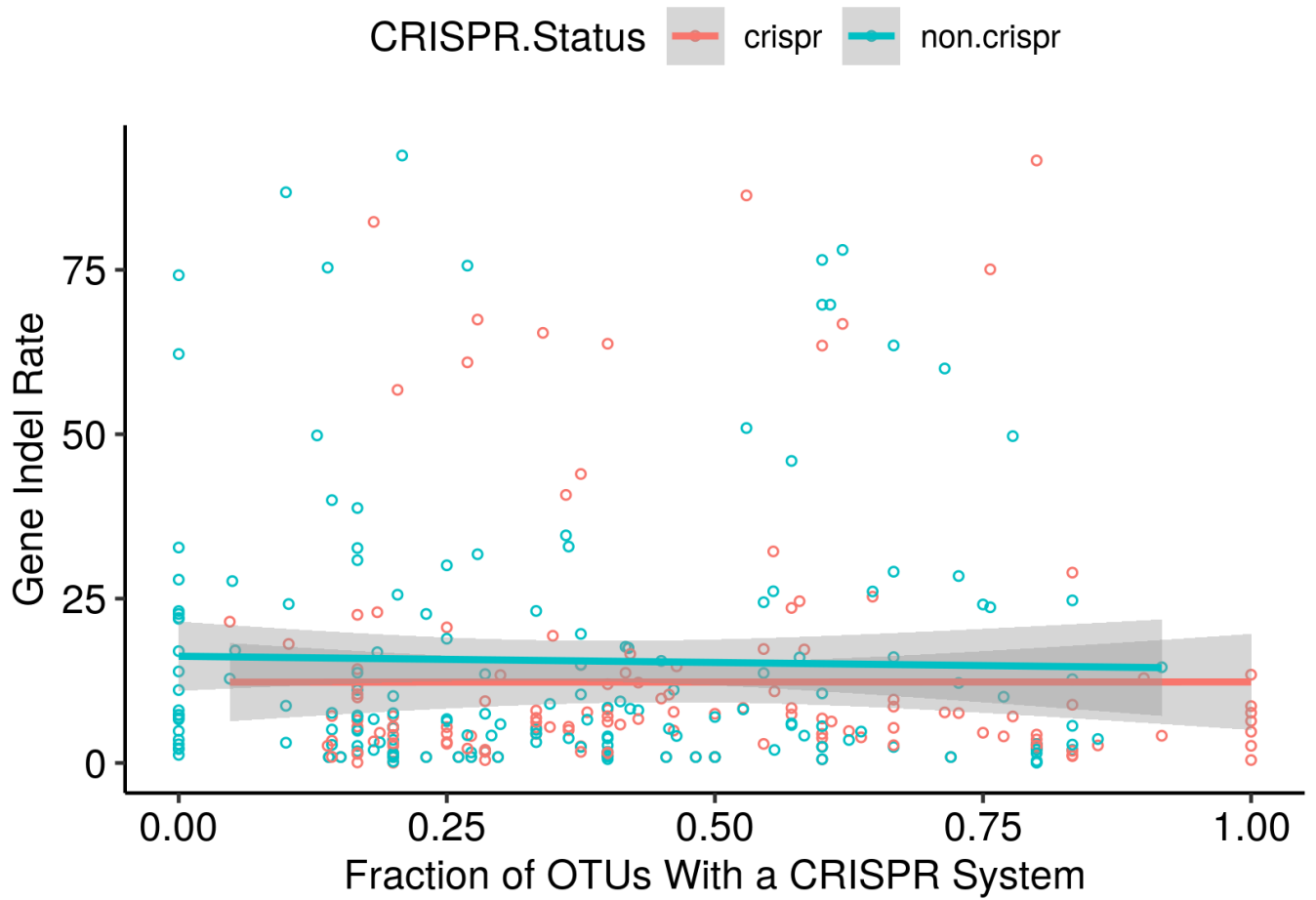


Figure 5: Markopholo estimate of gene indel rates for the partitions of CRISPR and non-CRISPR OTUs for each genus against the fraction of all OTUs in that genus that are annotated as having a CRISPR system. (n=311)

Figure 5 show that the fraction of all OTUs in a genus with a CRISPR system appears to be independent of the gene indel rate. Neither the CRISPR or non-CRISPR show a relationship with CRISPR fraction, having regression line slope and p values of 6.099065e-06, 0.9956342 and -0.0003156696, 0.7553688 respectively.

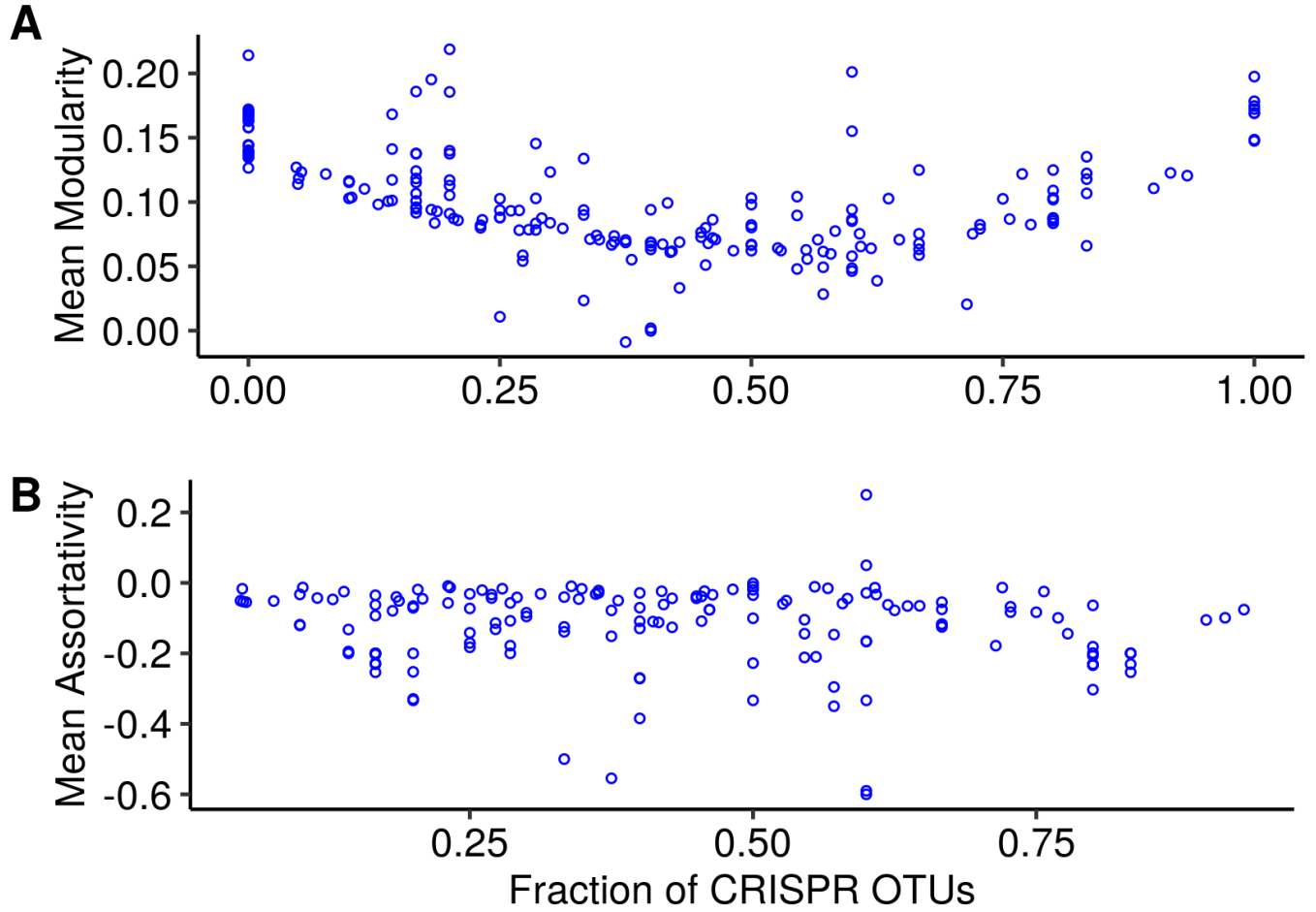


Figure 6: **A)** Mean network modularity over 1000 bootstrap replicates for each genus against the fraction of all OTUs in that genera with a CRISPR system (n=197). **B)** Distribution of network assortativity by CRISPR status (either CRISPR or non-CRISPR) over 1000 bootstrap replicates for each genus against the fraction of all OTUs in that genera with a CRISPR system (n=157).

Figure 6 **A)** shows that the network modularity is centered near 0 for most networks, implying that CRISPR and non-CRISPR OTUs do not form distinct communities. There does appear to be some relationship between CRISPR fraction and modularity, where the closer the CRISPR fraction is to 0.5 the lower the modularity is. This makes intuitive sense, as if more members of the network share an attribute, the more terms are included in the sum in the definition of modularity. Figure 6 **B)** shows distribution of network assortativity is centered near 0.10 for most networks, implying non-assortative mixing between CRISPR and non-CRISPR OTUs. Assortativity is usually defined in terms of a node's degree but here node similarity is defined as whether 2 nodes are both CRISPR or both non-CRISPR. This would suggest that there is no clear separation in how CRISPR and non-CRISPR OTUs edges are organized in a network, with some specific exceptions.

5 Discussion

5.1 Gene Indel Rates are Different for CRISPR and Non-CRISPR OTUs

For most genera, the gene indel rate for non-CRISPR genera is larger than for CRISPR genera. This is in-line with literature surrounding the mechanisms of HGT as CRISPR-Cas systems are meant to stop the integration of foreign DNA into the bacterial genome. Despite this, the mean node degree of CRISPR and non-CRISPR OTUs is relatively similar across genera. One reason for this discrepancy may be that there are often more non-CRISPR OTUs than CRISPR OTUs, thus more genes are exchanged between all non-CRISPR OTUs, but each non-CRISPR OTU transfers genes at a similar rate to CRISPR OTUs. One possible explanation for certain genera having very high gene indel rates for CRISPR OTUs may be that it is an efficient way to acquire new spacers. CRISPR-Cas systems may enhance HGT to preemptively acquire new spacers from the environment in response to environmental phage density.

5.2 Phylogenomic Networks Have Low Assortativity

There seems to be significant HGT between CRISPR and non-CRISPR OTUs, with no clear clustering, assortativity or modularity among most of the networks examined. CRISPR-Cas systems do not appear to have a segregating effect on the network, but do appear to have a population level effect of decreasing the rate of HGT. As suggested by [22] CRISPR-Cas systems may have a population level effect on HGT rate, but it appears to be suppressive in this case, as opposed to theirs.

5.3 HGT Dynamics Vary Across Bacterial Genera

Despite some trends being observable, the one constant is that there is significant variability between genera with regards to HGT. HGT rates can be similar or significantly different between CRISPR and non-CRISPR OTUs, either can be larger, both can be similar and either large or small. While the means are similar, the shapes of the distributions of network assortativity and modularity are not homogeneous.

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