# Pan-genome analyses of the species *Salmonella enterica*, and identification of genomic markers predictive for species, subspecies, and serovar.

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# Abstract

Food safety is a global concern, with upwards of 2.2 million deaths due to enteric diarrheal disease every year. Molecular diagnostic networks such as PulseNet have greatly increased the ability to rapidly identify and respond to food-related outbreaks. Until recently, identification of these contaminants relied on traditional microbiology laboratory techniques such as biochemical utilization, molecular characterization through the identification of key virulence markers, or banding patterns from rare-cutting restriction enzymes.

Current whole-genome sequencing platforms allow for routine sequencing for surveillance, and during outbreak situations; however, a critical challenge that remains in food safety is linking the genotype of an organism to its corresponding phenotype, as differences in human illness have been observed within lineages of the same bacterial species, and even within clades of these lineages

We have previously developed the software program Panseq, which identifies the pan-genome for a group of sequences, and the SuperPhy platform, which utilizes this pan-genome information to identify virulence and antimicrobial resistance genes within genome sequences, as well as biomarkers (both the presence / absence of genes, and single-nucleotide polymorphisms within shared genes) that are predictive of groups of bacterial strains.

In this study we use the previously described software to identify and examine the pan-genome of *Salmonella enterica*, an enteric pathogen responsible for the loss of more disability adjusted life years than any other enteric pathogen. We identify regions of the pan-genome that are statistically predictive of the species *Salmonella enterica*, its six subspecies, and serotypes within subspecies enterica. The approach defined in this study is broadly applicable to any species or collection of genomes.

# Introduction

The global burden of bacterial enteric disease, much of it Foodborne, results in an estimated 2.2 million deaths per year, and an annual loss of 112,000 disability adjusted life years in the United States alone (Bergholz *et al.* 2014, Scallan *et al.* (2015)). Nationwide molecular diagnostic networks, such as PulseNet in North America, were designed to enable the rapid identification of outbreaks by fingerprinting the etiological agents of disease, and keeping nationwide databases of previous fingerprints associated with human disease. Since its inception, PulseNet has relied on Pulsed-Field Gel Electrophoresis (PFGE) for fingerprinting of bacterial pathogens. It has been estimated that PulseNet prevents 277,000 illnesses from bacterial pathogens annually in the United States, reducing the costs associated with medical care and lack of productivity due to worker illness (Scharff *et al.* 2016).

Despite the usefulness of PulseNet, the PFGE technique itself is often unable to distinguish between related and unrelated strains, due to its reliance on rare-cutting restriction enzyme sites within the genome (Allard *et al.* 2012). Additionally, the interpretation of the banding patterns among labs requires extensive training and standardization to enable meaningful comparisons. Lastly, the banding patterns provide no information on the actual content of the genomes they represent, so important information regarding human virulence, such as the presence or absence of known toxins, is not available.

Lastly, while the presence of known virulence factors has been correlated with severe human disease in a number of bacterial species, it has also been shown that some lineages or clades within these same species, while possessing the known virulence factors, are rarely associated with human disease (Waryah *et al.* 2016, Lupolova *et al.* (2016)). Thus, other factors within the genome that influence the expression of key virulence factors, or otherwise modulate the virulence of these strains need to be taken into consideration when attempting to predict the strains of a bacterial species that are potential human health threats (Opijnen *et al.* 2012).

Whole-genome sequencing (WGS) has become the de facto standard for the complete characterization for bacterial pathogens for both ongoing surveillance and outbreak investigations (Deng X, den Bakker HC 2016, Franz *et al.* (2016)). It allows clear definition between outbreak-related strains and those from unrelated sources, the ability to identify routes of transmission, and the ability to perform source attribution of bacterial contaminants (Bakker *et al.* 2014). It is currently being utilized worldwide in outbreak and routine surveillance, including the characterization of all *Listeria monocytogenes* isolated in the United States[genometrackr ref], all *Salmonella* isolated by Public Health England as part of routine surveillance (Ashton *et al.* 2016), and a large-scale survey of *Staphylococcus aureus* in continental Europe, which demonstrated the applicability of WGS for the identification of the emergence and spread of clinically relevant *Staphylococcus aureus* (Aanensen *et al.* 2016).

It has been shown that *in silico* prediction of antimicrobial resistance (Zhao *et al.* 2016, Tyson *et al.* (2015), McDermott *et al.* (2016)), serotype (Levine *et al.* 2016, Yoshida *et al.* (2016)), and other traditional sub-typing schemes such as multi-locus sequence typing (Sheppard *et al.* 2012) can be accurately reproduced from bacterial genome sequences. However, given the more complex task of identifying bacterial isolates that are most likely to cause disease in humans, methods that can correctly identify markers that predict such strains from the genome sequence alone are needed. In addition, markers that can identify bacteria likely to exhibit particular phenotypes, such as the ability to survive in a particular niche, or the ability to tolerate harsh environments such as those found in food processing plants are also required.

We have previously developed the software platform Panseq, for the analyses of thousands of genomes in a pan-genome context, where both the presence / absence of the accessory genome and SNPs within the shared core-genome are computed (Laing *et al.* 2010). Additionally, we recently released a platform for the predictive genomics of *Escherichia coli*, called SuperPhy, in which markers statistically biased within groups of bacteria, based on any metadata category, can be identified (Whiteside *et al.* 2016).

In this study we use our previously created software to examine the pan-genome of *Salmonella enterica*, a species that contains human-adapted strains responsible for typhoid fever, as well as a large number of non-typhoidal strains responsible for an estimated 93.8 million annual cases of enteric illness worldwide (Gal-Mor *et al.* 2014, Majowicz *et al.* (2010)). We identify *S. enterica* species- and subspecies-specific markers, as well as markers predictive of serotype for subspecies enterica. While this study focused on *S. senterica*, the tools and approach are broadly applicable to any species or collection of genomes.

# Results

## *S. enterica* pan-genome

Initially we determined the size and distribution of the *S. enterica* pan-genome as genome fragments of 1000bp in size. As can be seen in Figure 1, the pan-genome comprised of 4939 *S. enterica* genomes was found to be 25.3 Mbp in size, with 70% of that present in fewer than 100 genomes. Conversely, the core genome was found to be 1.5 Mbp in size, with all but 200 genomes (96%) containing 3.2 Mbp of shared genomic core. Only 17% of the pan-genome was found in greater than 100 genomes, but fewer than 4739 genomes.

## *S. enterica* species-specific regions

To identify regions of *S. enterica* that were likely to be shared among most genomes of the species, we examined all 211 closed genomes of *S. enterica* in GenBank, looking for genomic regions that were present in at least 190 (90%) of these genomes. We identified 3832 regions of 1000 bp that were present in at least 90% of the closed genomes. These regions were subsequently screened against the GenBank nr database, and any present in non-*Salmonella* genomes were removed, leaving 405 putative *S. enterica* species-specifc regions.

Figure 2 shows the carriage of these 405 regions among the 4939 genomes of this study. All but 105 genomes contained at least 330 of these putative *S. enterica* specific regions. A stark difference in carriage of these species specific markers was observed, with 4742 genomes containing at least 350 species-specific markers, while only 2674 genomes contained 360 or more species-specific markers.

## Quality filtering for subsequent analyses

To ensure the quality of the genomes in use for subsequent analyses, we plotted carriage of the 405 species specific regions versus the number of contigs that each sequenced genome was comprised of (Figure 3). As can be seen, the two genomes marked in yellow contained only one, and the same, species-specific region each, despite being comprised of relatively few contigs. Subsequent searches identified these two genomes as *Citrobacter spp.* contamination, mislabeled as *S. enterica* (GCA\_001570325 and GCA\_001570345). The "*Salmonella enterica* species-specific region" found in both of the contaminant *Citrobacter* genomes, did not match any other *Citrobacter spp.* in GenBank above the thresholds used for determining presence / absence in this study. However, due to the presence of this region in what have been identified as *Citrobacter* genomes, the region was removed from subsequent analyses.

The majority of genomes (4913) were from subspecies enterica, with genomes from the five other *S. enterica* subspecies present in drastically fewer numbers (Table 1). All genomes from subspecies enterica contained greater than 250 species-specific regions, which was more than the genomes from any other subspecies, with the exception of enterica genomes that were of poor quality and comprised of many thousands of contigs (Table 2). Genomes from subspecies houtenae and arizone contained fewer than 100 species-specific regions, while genomes from diarizone, indica, and salamae contained between 100 and 200 species-specific regions. All regions were screened against *Salmonella bongori* to ensure specificity to *S. enterica*; one region was found to also be present in genomes from *S. bongori* and was removed from further analyses.

Within subspecies enterica, a negative linear relationship was observed among the number of species-specific regions contained within a genome, and the number of contigs the genome was comprised of, with the worst-case genome (GCA\_000495155) being comprised of 6945 contigs, but containing only 13 species-specific regions. Other genomes such as *S. enterica* Bovismorbificans strain GCA\_001114865 contained both few contigs (140) as well as fewer species-specific regions (209) than other enterica genomes. Additional searches discovered sequencing gaps within the genome totalling over 464 Kbp. A final outlier genome harbored nearly 5000 contigs, but also contained 403 of the species-specific regions. It was determined that this sequence (GCA\_000765055) was actually a combination of multiple genomes in a single file.

Given the above information, all genomes from the five subspecies other than enterica were included in subsequent analyses, while the thresholds for inclusion of enterica genomes were set at a maximum of 1000 contigs, and a minimum of 250 species-specific regions. Following this quality filtering, 43 genomes were removed, leaving 4870 *S. enterica* enterica genomes for the following analyses.

## Phylogeny of *S. enterica* using the conserved core genome

Based on the distribution of the pan-genome presented in Figure 1, the "conserved core" of *S. enterica* was set at greater than 4500 genomes, to fully capture the conserved genomic regions within the species. A phylogeny based on the SNPs among these shared regions was created, and is shown along with the distribution of the *S. enterica* species specific regions in Figure 4. As can be seen, the majority of the genomes are subspecies enterica, and the other five subspecies are relatively more distant in the order of diarizonae, salamae, indica, houtenae, and arizonae, which mirrors the declining number of species-specific regions contained within genomes of these subspecies, which is also shown in Figure 3.

The serovar distribution within subspecies enterica was shown to be largely concordant with phylogeny, as demonstrated in Figure 5, where the ten most abundant serovars in the current study are highlighted. However, not all serovars clustered as monophyletic groups, as can be seen with serovar Bareilly; nor were all clades found to be comprised of single serovars, demonstrated by the clade containing genomes of serovars Bareilly and Agona.

The large clades within the phylogenetic tree also demonstrate clade-specific patterns of presence / absence for the 403 species-specific markers. Among the most abundant serovars Typhimiurium, Heidelberg, Newport, and Enteritidis were found to contain the most species-specific markers, and group together near the center of the tree. Likewise, serovars Agona, Welevreden, and Kentucky contained fewer species-specific regions, and group together near the bottom of the tree, closer to the non-enterica sub-species genomes.

Table 4 considers all serovars with at least 10 members in the dataset, and the average number of species-specific markers per serovar. As can be seen, the serovars with the largest average number of species-specific regions were: Enteritidis (401.7), Anatum (401.5), Muenchen (400.5), Hadar (400.3), and Typhimurium (400.1); conversely, the serovars with the fewest average number of species-specific regions were: Derby (360.7), Montevideo (360.1), Typhi (358.1), Bovismorbificans (355.3), and Cerro (342.0).

## Phylogeny of *S. enterica* using the pan-genome

A phylogeny based on the presence / absence of the pan-genome among the 4893 *S. enterica* genomes was created, and is shown along with the distribution of the *S. enterica* species specific regions in 6. As can be seen this phylogeny based on the presence / absence of the entire 25.3 Mbp pan-genome is highly concordant to the phylogeny based on the SNPs found in the conserved core of the same strains (Figure 6). In both trees, the serovars cluster together and in the same relation to each other, for example serovars Typhi and Paratyphi strains form a discrete monophyletic clade. However, the branch lengths in the pan-genome tree are larger than those in the conserved SNP tree, due to the larger variation among the presence / absence of the pan-genome than to sequence variation among shared regions.

## Identification of a minimum set of species-specific genomic markers for the identification of subspecies *S. enterica*.

Within the 404 species-specific markers, there were none that were also specific for any of the subspecies. That is, a marker was always present in genomes from at least two subspecies.

We next determined that the presence of a minimum set of two genomic regions was required to unambiguously identify genomes of *S. enterica*, within the 4893 genomes of the current study. The two genomic regions that met this requirement and were also present in the most genomes are highlighted in Table X. All members of *S. enterica* examined contained at least one of these markers, but many other combinations within the 404 species-specific markers are possible.

## Putative functional identification of the *S. enterica* species-specific regions

The putative function of the 403 quality-filtered *S. enterica* species-specific regions were determined form the GenBank nr database. The annotation of each of the 403 regions is available as Supplementary File 1. Table 3 summarizes the frequency of functional annotation categories, after annotating each region with the single best match. As can be seen, hypothetical proteins accounted for the majority (64) of the 403 annotations, with secreted effector and membrane proteins being the next most frequent category among the species-specific regions. Other membrane, transport, and secretion proteins were observed. The species-specific regions also included proteins involved in core metabolic functions, protein and DNA synthesis, and response to stress.

## Identification of subspecies specific markers from the pan-genome

Having identified species-specific markers, we employed the same techniques, utilizing the presence / absence of all pan-genome markers, just the 404 species-specific ones, to identify subpecies-specific markers. The number of markers that were completely unique to a subspecies is given in Table 5. Subspecies arizonae contained the most unique markers, at 207, and enterica contained the least, at 9.

## Identification of universal serovar markers within subspecies enterica from the pan-genome

Subspecies enterica genomes were the vast majority of those available, so we attempted to identified serovar-specific markers for the top five serovars, in the same manner that we identified subspecies-specific markers. We found that there were no genomic markers that were unique in presence or absence for any of the serovars; however, there were a number of genomic regions that were universal in there presence or absence among serovars, even though they were present or absent among genomes of other serotype. The number of markers universal for presence or absence among the enterica serovars is shown in Table 6.

# Discussion

## *S. enterica* pan-genome

The species *S. enterica* is divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. Over 99% of human disease caused by *S. enterica* is done so by subspecies *enterica*, with the World Health Organization estimating that *S. enterica* infections from contaminated food alone, constitute a loss of 6.43 million disability adjusted life years worldwide, more than any other enteric pathogen (Kirk *et al.* 2015).

Previous examinations of the *S. enterica* pan-genome were based on relatively small dataset of 45 and 73 genomes (Jacobsen *et al.* 2011,Leekitcharoenphon *et al.* (2012)). While others have analyzed thousands of *S. enterica* genomes, the analyses have not been to examine the population structure. For example, in demonstrating the software program Roary, 1000 *S.* Typhi genomes were used to test the program (Page *et al.* 2015). Likewise, the GenomeTrackR project utilized 32 *S. enterica* genomes to identify a *S. enterica* core, which was subsequently used as the basis for genetic distance estimates for nearly 20,000 genomes (Pettengill *et al.* 2016).

Previous estimates placed the core-genome size of *S. enterica* at ~2800 gene families, and the pan-genome at ~10,000 gene families (Jacobsen *et al.* 2011). The current study identified a strict core of 1.5 Mbp, which given an average gene size of 1000bp is ~1500 genes, and a much larger pan-genome at ~25,300 genes. Previous analyses found *S. enterica* to have a closed pan-genome (Jacobsen *et al.* 2011), and thus the rate of discovery for new genomic regions would decrease for each new genome of the species sequenced (Tettelin *et al.* 2005).

In line with *S. enterica* having a closed pan-genome, when we compared it to *Escherichia coli*, a related bacterial species with an open pan-genome (Tettelin *et al.* 2005), we found that the *E. coli* pan-genome was larger (37.4 Mbp), despite the fact that the *E. coli* study used less than half the number of strains in the current *Salmonella enterica* study. Additionally, more of the pan-genome of *S. enterica* was distributed among more genomes than in *E. coli* (Whiteside *et al.* 2016). Specifically, in *S. enterica* 70% of the pan-genome was found to belong to 100 or fewer of the genomes examined, while in *E. coli* 80% of the pan-genome was found in 100 or fewer genomes.

## *S. enterica* species-specific regions

The host intestinal environment consists of a multitude of bacterial species competing for scare nutritional sources such as carbohydrates, direct antagonistic competition with other bacterial cells, and competition for access to the host intestine, where stable attachment and colonization of the local environment are possible (Sana *et al.* 2016). The normal intestinal microflora offer protection to the host against enteric pathogens such as *S. enterica*, but intestinal disruption from virulence factors and effector proteins secreted by the pathogen itself, or external factors including antibiotics, have been shown to alter the composition of the microbiota, and allow pathogens such as *S. enterica* to capitalize on the fluctuating environment (Ng *et al.* 2013).

Nutritional competition exists for free metabolic compounds, such as carbohydrates that are readily available, as well as others that are sequestered in forms such as the intestinal mucus, which is composed of sialic sugar acids (McDonald *et al.* 2016). In the gut, these sugar acids exists as a conjugate in the alpha form, which to be useful for bacteria such as *Salmonella*, need to be converted to the beta form by a mutarotase enzyme [Severi2008]. In this study we identified n-acetylneuraminic acid mutarotases as species-specific genomic regions, along with sialic acid transporters. It is likely the presence of these systems allow *S. enterica* to more efficiently compete with the host microbiota by efficiently utilizing scarce metabolic sources.

It was also previously found that sialic acid on the surface of host colon cells increased colonization by *S.* Typhi, and disialylation of these cells reduced the adherence of the *Salmonella* strains by 41% (Sakarya *et al.* 2010). This was also demonstrated in *S.* Typhimurium, where following antibiotic treatment, the presence of free sialic acid increased, and the ability to utilize it was correlated with levels of bacterial colonization of the host gut (Ng *et al.* 2013).

The ability to utilize sialic acids has previoiusly been shown to be present in 452 bacterial species, including other pathogens such as *Vibrio cholerae*, but the genmoic regions found in the current study were sufficiently diverse at the nucleotide level to be determinative for *S. enterica* (McDonald *et al.* 2016).

In addition to species-specific regions used to gain a metabolic advantage, a number of secretion system and effector proteins were identified as diagnostic of *S. enterica*. These included components of the Type VI secretion system (T6SS), which is a contact-dependent, syringe-like secretion system that allows *S. enterica* to directly kill other competing bacteria that it comes into physical contact with (Brunet *et al.* 2015), and is encoded on the *Salmonella* Pathogenicity Island 6 (Sana *et al.* 2016). It has been demonstrated that silencing the T6SS via H-NS repression (histone-like nucleoid structuring), reduces inter-bacterial killing of *S. enterica* (Brunet *et al.* 2015). It was also previously shown that commensal bacteria are killed by *S. enterica* in a T6SS-dependent manner, that the T6SS was required for *Salmonella* to establish infection in the host gut, and that increased concentrations of bile salts resulted in a concomitant increase in T6SS anti-bacterial activity (Sana *et al.* 2016). The T6SS itself has been shown to be independently acquired from four separate lineages within five of the size *S. enterica* subspecies (Desai *et al.* 2013).

Like the T6SS, the type III sectetion system (T3SS) found within *S. enterica* is a syringe like apparatus that injects effector proteins into host cells (Kubori *et al.* 2000). There are two T3SS found within *S. enterica*: the first is encoded on the Salmonella Pathogenicity Island 1 (SPI1) and is required for invasion into host cells; the second is encoded on Salmonella Pathogenicity Island 2 (SPI2), and is required for survival and proliferation within the host macrophage cells (Hensel *et al.* 1998, Bijlsma2005). The innate host immune system utilizes the inflammatory response to help reduce the proliferation of bacterial pathogens (Sun *et al.* 2016). *S. enterica* has developed a means of regulating host inflammation via the SPI1 T3SS, whereby secreted effector proteins target the NF-kB signalling pathway reduce inflammation and host tissue damage, and allow increased *S. enterica* propogation within the host. *S. enterica* also relies on free long-chain fatty acids within the host to regulate T3SS expression, and help cue the bacteria for host intestinal colonization (Golubeva *et al.* 2016).

The current study identified many secretion system and effector proteins as being species-specific, as well as proteins for attachment to the host, such as fimbriae. These proteins allow *S. enterica* to be competitive within the intestinal environment, and take up residence within the host, where it can proliferate.

Effector proteins and other virulence factors aid in the colonization of the host, and are frequently horizontally acquired and present on mobile elements such as integrated bacteriophages (Moreno Switt *et al.* 2013). Previous work identified clusters of phages that carried virulence factors such as adhesins and antimicrobial resistance determinants within *S. enterica* (Moreno Switt *et al.* 2013). Additionally, many of the genes associated with bacteriophage in *S. enterica* have been found to be of the putative and hypothetical class (Penadés *et al.* 2015).

The current study identified a large accessory gene pool that contained many hypothetical and putative genes, which were also the most abundant category of species-specific genomic regions. The proteins of putative and unknown function may aid in colonizing warm-blooded animals, or specific animal or environmental niches. Previous studies identified genotype / phenotype correlations of *S.* Typhimurium\_ that had particular gene complements associated with specific food sources (Hayden *et al.* 2016). The same study also postulated that specific phage repertoires may give phylogenetically distant strains a similar accessory gene content, and therefor similar niche specificity. Previously, 285 gene families were identified as being recruited into *S. enterica*, where most of these genes had unknown function, but were postulated to be important for its survival and infection of its host (Desai *et al.* 2013). It is therefore not surprising to find that the most abundant species-specific category of genomic regions are those of unknown or putative function; they likely represent genes enhancing the ability of *S. enterica* to propogate within warm-blooded animals, but have not yet been fully characterized. The other genomic regions diagnostic of *S. enterica* include means for disseminating these fitness genes within the population, competing for resources in the host, and attaching and proliferating. The *S. enterica* species-specific regions give a good overview of what make it such an effective pathogen and intestinal inhabitant.

### Quality of public data / implications

## Core and Pan-genome comparison

## Specific regions for species, subspecies and serovar

# Tables

Table 1. The frequency of the subspecies observed within the study set of 4937 *Salmonella enterica* genomes, prior to any quality filtering.

|  |  |
| --- | --- |
| Subspecies | No. |
| enterica | 4913 |
| arizonae | 7 |
| diarizonae | 7 |
| houtenae | 4 |
| salamae | 4 |
| indica | 1 |

Table 2. The serovars with more than 20 representatives in the current study set of 4937 *Salmonella enterica* genomes, and their frequency, prior to any quality filtering. The list of all serovars and their frequency within the current study is available as Supplementary File 2.

|  |  |
| --- | --- |
| Serovar | No. |
| Typhi | 1977 |
| Typhimurium | 758 |
| Enteritidis | 413 |
| Heidelberg | 201 |
| Paratyphi | 158 |
| Kentucky | 155 |
| Agona | 136 |
| Weltevreden | 120 |
| Bareilly | 106 |
| Newport | 82 |
| Tennessee | 77 |
| Montevideo | 69 |
| Saintpaul | 48 |
| Infantis | 39 |
| Senftenberg | 35 |
| Bovismorbificans | 34 |
| Hadar | 33 |
| Muenchen | 30 |
| Anatum | 27 |
| Schwarzengrund | 27 |
| Dublin | 24 |
| Cerro | 21 |

Table 3. The putative function of the *S. enterica* species-specific regions for functions that were identified more than once, utilizing the best hit for each region. The complete list of all putative functions is available as Supplemental File 1.

|  |  |
| --- | --- |
| Putative protein function | Frequency |
| hypothetical | 64 |
| secreted effector | 10 |
| membrane | 7 |
| secretion system apparatus | 5 |
| uncharacterised | 5 |
| fimbrial | 5 |
| pathogenicity island 2 effector | 4 |
| fimbrial assembly | 4 |
| outer membrane usher | 4 |
| mfs transporter | 3 |
| oxidoreductase | 3 |
| histidine kinase | 3 |
| putative inner membrane | 3 |
| putative cytoplasmic | 3 |
| lysr family transcriptional regulator | 3 |
| transcriptional regulator | 2 |
| permease | 2 |
| outer membrane | 2 |
| type iii secretion | 2 |
| phosphoglycerate transport | 2 |
| arac family transcriptional regulator | 2 |
| conserved hypothetical | 2 |
| methyl-accepting chemotaxis | 2 |
| hybrid sensor histidine kinase/response regulator | 2 |
| glycosyl transferase, partial | 2 |
| phenylacetaldehyde dehydrogenase | 2 |
| pathogenicity island 1 effector | 2 |
| n-acetylneuraminic acid mutarotase, partial | 2 |
| type iii secretion system | 2 |
| transcriptional regulator, partial | 2 |
| cytoplasmic | 2 |
| fimbrial chaperone | 2 |
| putative sialic acid transporter | 2 |

Table 4. The average number of species-specific genomic regions found among serovars of subspecies enterica, that contained at least 10 representative genomes, within the 4870 quality filtered subpecies enterica genomes of this study.

|  |  |
| --- | --- |
| Serovar | Average no. species-specific regions |
| Enteritidis | 401.7 |
| Anatum | 401.5 |
| Muenchen | 400.5 |
| Hadar | 400.3 |
| Typhimurium | 400.1 |
| Newport | 399.8 |
| Thompson | 399.7 |
| Saintpaul | 399.6 |
| I | 399.0 |
| Heidelberg | 397.4 |
| Dublin | 395.2 |
| Infantis | 394.9 |
| Braenderup | 392.8 |
| Weltevreden | 390.0 |
| Bareilly | 388.5 |
| Kentucky | 380.3 |
| Plymouth/Zega | 377.9 |
| Senftenberg | 376.5 |
| Mbandaka | 374.5 |
| C1:g | 374.1 |
| Reading | 370.4 |
| Agona | 369.5 |
| Tennessee | 368.3 |
| Schwarzengrund | 362.3 |
| Paratyphi | 361.5 |
| Derby | 360.7 |
| Montevideo | 360.1 |
| Typhi | 358.1 |
| Bovismorbificans | 355.3 |
| Cerro | 342.0 |

Table 5. The number of subspecies-specific pan-genome markers that were universally present or absent among members of the subspecies, and not absent or present among genomes from any other subspecies.

|  |  |
| --- | --- |
| Subspecies | No. Markers |
| arizonae | 207 |
| diarizonae | 93 |
| enterica | 9 |
| houtenae | 134 |
| indica | 192 |
| salamae | 135 |

Table 6. The number of pan-genome regions that were universally present and absent, as well as statistically over- or under-represented in comparison to all other genomes, within the 4870 subspecies enterica genomes of this study.

|  |  |  |
| --- | --- | --- |
| Serovar | No. universally present | No. universally absent |
| Typhi | 288 | 2720 |
| Typhimurium | 41 | 698 |
| Enteritidis | 18 | 440 |
| Heidelberg | 121 | 840 |
| Paratyphi | 65 | 202 |
| Kentucky | 177 | 331 |
| Agona | 161 | 638 |
| Weltevreden | 426 | 608 |
| Bareilly | 87 | 436 |
| Newport | 226 | 360 |

# Figures

1. Figure 1 The distribution of the *Salmonella enterica* pan-genome, as 1000bp fragments, among 4939 whole-genome sequences.
2. Figure 2 The carriage of the 405 *S. enterica* species-specific regions among each of the 4939 genomes of this study. Each dot represents a single *S. enterica* genome, which are arranged in order from those that contain the fewest species-specific regions to those that contain the most.
3. Figure 3 The carriage of the 405 *S. enterica* species-specific regions, and the number of contigs that each of the 4939 genomes of this study were distributed amongst. Colours indicate the subspecies within *S. enterica*, or contamination from ther species as follows: red: arizonae, yellow: *Citrobacter* contamination, lime: diarizonae, teal: enterica, blue: houtenae, lavender: indica, magenta: salamae.
4. Figure 4 The phylogeny of the 4893 *S. enterica* genomes post quality-filtering. The six subspecies are highlighted as follows: red: arizonae, yellow: diarizonae, lime: enterica, teal: houtenae, blue: indica, magenta: salamae. The matrix to the right of the phylogeny represents the 404 species-specific regions, with red being the absence of the region, and black being the presence of the region, for each of the genomes of the study.
5. Figure 5 The phylogeny of the 4893 *S. enterica* genomes post quality-filtering based on SNPs found within the conserved core genome. The ten most abundant serotypes of subspecies enterica in the current study are highlighted as follows: dark green: Agona, dark orange: Bareilly, purple: Enteritidis, magenta: Heidelberg, light green: Kentucky, yellow: Newport, brown: Paratyphi, grey: Typhi, pink: Typhimurium, light blue: Weltevreden. The matrix to the right of the phylogeny represents the 404 species-specific regions, with red being the absence of the region, and black being the presence of the region, for each of the genomes of the study.
6. Figure 6 The phylogeny of the 4893 *S. enterica* genomes post quality-filtering based on the presence / absence of the entire pan-genome as 1000bp fragments. The ten most abundant serotypes of subspecies enterica in the current study are highlighted as follows: dark green: Agona, dark orange: Bareilly, purple: Enteritidis, magenta: Heidelberg, light green: Kentucky, yellow: Newport, brown: Paratyphi, grey: Typhi, pink: Typhimurium, light blue: Weltevreden. The matrix to the right of the phylogeny represents the 404 species-specific regions, with red being the absence of the region, and black being the presence of the region, for each of the genomes of the study.

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