Whole genome sequence analysis of *Salmonella* Typhi isolated in Thailand before and after the introduction of a national immunization program

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S. Typhi in Thailand before and after immunization

Abstract

Vaccines against *Salmonella* Typhi, the causative agent of typhoid fever, are commonly used by travellers, however, there are few examples of national immunization programs in endemic areas. There is therefore a paucity of data on the impact of typhoid immunization programs on localised populations of *S.* Typhi. Here we have used whole genome sequencing (WGS) to characterise 44 historical bacterial isolates collected before and after a national typhoid immunization program that was implemented in Thailand in 1977 in response to a large outbreak; the program was highly effective in reducing typhoid case numbers. Thai isolates were highly diverse, including 10 distinct phylogenetic lineages or genotypes. Novel prophage and plasmids were also detected, including examples that were previously only reported in *Shigella sonnei* and *Escherichia coli*. The majority of *S.* Typhi genotypes observed prior to the immunization program were not observed following it. Post-vaccine era isolates were more closely related to *S.* Typhi isolated from neighbouring countries than to earlier Thai isolates, providing no evidence for the local persistence of endemic *S.* Typhi following the national immunization program. Rather, later cases of typhoid appeared to be caused by the occasional importation of common genotypes from neighbouring Vietnam, Laos, and Cambodia. These data show the value of WGS in understanding the impacts of vaccination on pathogen populations and provide support for the proposal that large-scale typhoid immunization programs in endemic areas could result in lasting local disease elimination, although larger prospective studies are needed to test this directly.

Author Summary

Typhoid fever is a systemic infection caused by the bacterium *Salmonella* Typhi. Typhoid fever is associated with inadequate hygiene in low-income settings and a lack of sanitation infrastructure. A sustained outbreak of typhoid fever occurred in Thailand in the 1970s, which peaked in 1975-1976. In response to this typhoid fever outbreak the government of Thailand initiated an immunization program, which resulted in a dramatic reduction in the number of typhoid cases in Thailand. To better understand the population of *S.* Typhi circulating in Thailand at this time, as well as the impact of the immunization program on the pathogen population, we sequenced the genomes of 44 *S.* Typhi obtained from hospitals in Thailand before and after the immunization program. The genome sequences showed that isolates of *S.* Typhi bacteria isolated from post-immunization era typhoid cases were likely imported from neighbouring countries, rather than strains that have persisted in Thailand throughout the immunization period. Our work provides the first historical insights into *S.* Typhi in Thailand during the 1970s, and provides a model for the impact of immunization on *S.* Typhi populations.

Introduction

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi) is a human restricted bacterial pathogen and the etiological agent of typhoid fever. *S.* Typhi is transmitted faeco-orally and can establish asymptomatic carriage in a small subset of an exposed population (1). Recent estimates (2-4) place the global burden of typhoid fever at 25-30 million cases annually, of which 200,000 are associated with deaths. Typhoid fever occurs most commonly in industrialising countries, specifically in locations with limited sanitation and related infrastructure (5); children and young adults are among the most vulnerable populations in these settings (6-8). Antimicrobial therapy together with water sanitation and hygiene (WASH) interventions are the major mechanisms by which typhoid fever is controlled (9, 10). However, none of these approaches are optimal and resistance against antimicrobials has become increasingly common in *S.* Typhi since the 1970s (11-13). A number of typhoid vaccines are licenced for use (14-18), however, they are not widely used as a public health tools in endemic areas, with the exception of controlling severe outbreaks such as those following natural disasters (19-22).

A sustained typhoid fever outbreak occurred in Thailand in the 1970s. A sharp increase in cases was observed in 1973-1974, which finally peaked in 1975-1976. In response, the government of Thailand established a national typhoid immunization program, which represented the first programmatic use of a typhoid vaccine in the country (23). The immunization program targeted over 5 million school aged children (7-12 years) each year in Bangkok between 1977 and 1987 (80% of the eligible population). Thus, Thai school children were eligible to receive a single locally produced heat/phenol-inactivated subcutaneous dose of 2.5×10^8 *S.* Typhi organisms annually (14, 23), before the program was halted in the early 1990s because of high rates of adverse reactions caused by the vaccine (22). To our knowledge this is the only such programmatic use of a vaccine for controlling Typhoid fever in children in Thailand. Data from four teaching hospitals in Bangkok showed a 93% reduction in blood culture confirmed infections with *S.* Typhi between 1976 (n=2,000) and

1985 (n=132) (14, 23). Notably, no significant decline was observed in isolation rates of *Salmonella* Paratyphi A (*S*. Paratyphi A), a *Salmonella* serovar distinct from *S*. Typhi that causes a clinical syndrome indistinguishable from typhoid fever, but for which *S.* Typhi vaccines provide little or no cross-protection (14). This observation suggests that the reduction in *S*. Typhi infections was not attributable to improvements in infrastructure and hygiene practices only (5, 14, 20, 23). While the inactivated *S.* Typhi vaccine was found to be highly efficacious (22, 23), it is no longer used as a consequence of being overly reactogenic (14, 16, 23, 24). A Vi capsular polysaccharide vaccine (15) and live-attenuated oral vaccine of strain Ty21a (16) have since replaced this vaccine for travellers to endemic locations (5, 21, 24).

The typhoid immunization program in Thailand provided a unique opportunity to investigate the impact of immunization on *S*. Typhi populations circulating within an endemic area. Here we present an analysis of a historical collection of 44 *S.* Typhi isolates obtained from patients in Thailand between 1973 and 1992 (before and during the immunization program). As *S*. Typhi populations demonstrate little genetic diversity, we used whole genome sequencing (WGS) to characterise these isolates, and core genome phylogenetic approaches to compare the historic isolates from Thailand to a recently published global *S.* Typhi genomic framework (4).

Materials and methods

Ethics statement

This is a retrospective study of bacterial isolates unlinked to patient information and was not subject to IRB approval.

Bacterial isolation and antimicrobial susceptibility testing

Forty-four *S.* Typhi isolated from patients with suspected typhoid fever attending hospitals in Bangkok, Nonthaburi, Loi, and Srakaew, in Thailand between 1973 and 1992 were available

for genome sequencing in this study (**Fig 1** and **Table S1**). At the time of original isolation, bacterial cultures were transferred on nutrient agar slants to the department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand for identification and antimicrobial susceptibility testing. At AFRIMS, bacterial isolates were subcultured on Hektoen Enteric agar (HE) and identification was performed by biochemical testing on Kligler iron agar slants, tryptone broth for indole, lysine decarboxylase medium, ornithine decarboxylase medium, urease test, mannitol and motility media (Becker Dickenson, Thailand). Serological agglutination was performed using *Salmonella* O antisera and *Salmonella* Vi antiserum (Difco, USA). Bacterial strains were stored frozen at -70°C in 10% skimmed milk or lyophilised in 10% skimmed milk; lyophilized ampoules were stored at 2-8°C. Prior to DNA extraction for sequencing, lyophilized bacteria was rehydrated with trypticase soy broth, inoculated on McConkey agar and incubated at 37°C for 18-24 hours. If bacteria was stored frozen in skimmed milk, organisms were inoculated directly onto McConkey agar after thawing and then incubated at 37°C for 18-24 hours.

Antimicrobial susceptibility testing against ampicillin, chloramphenicol, cephalothin, gentamicin, kanamycin, neomycin, sulfisoxazole, trimethoprim/sulfamethoxazole, and tetracycline was performed by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) (25-28).

Genome sequencing and SNP analysis

Genomic DNA from the 44 *S.* Typhi from Thailand was extracted using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA). Two µg of genomic DNA was subjected to indexed WGS on an Illumina Hiseq 2000 platform at the Wellcome Trust Sanger Institute, to generate 100 bp paired-end reads. For analysis of SNPs, paired end Illumina reads were mapped to the reference sequence of *S.* Typhi CT18 (accession no: AL513382) (29) using the RedDog (v1.4) mapping pipeline, available at https://github.com/katholt/reddog. RedDog uses Bowtie (v2.2.3) (30) to map reads to the reference sequence, then high quality SNPs

called with quality scores above 30 are extracted from the alignments using SAMtools (v0.1.19) (31). SNPs were filtered to exclude those with less than 5 reads mapped or with greater than 2.5 times the average read depth (representing putative repeated sequences), or with ambiguous base calls. For each SNP that passed these criteria in any one isolate, consensus base calls for the SNP locus were extracted from all genomes (ambiguous base calls and those with phred quality scores less than 20 were treated as unknowns and represented with a gap character). SNPs with confident homozygous allele calls (i.e. phred score >20) in >95% of the *S.* Typhi genomes (representing a 'soft' core genome of common *S.* Typhi sequences) were concatenated to produce an alignment of alleles at 45,893 variant sites. The resultant allele calls for 68 of these SNPs were used to assign isolates to previously defined lineages according to an extended *S.* Typhi genotyping framework (32) code available at https://github.com/katholt/genotyphi). SNPs called in phage regions, repetitive sequences (354 kb; ~7.4% of bases in the CT18 reference chromosome, as defined previously (33) or recombinant regions (~180kb; <4% of the CT18 reference chromosome, identified using Gubbins (v1.4.4) (34)) were excluded, resulting in a final set of 1,850 SNPs identified in an alignment length of 4,275,037 bp for the 44 isolates. SNP alleles from Paratyphi A strain 12601 (35) were also included as an outgroup to root the tree. For global context, raw read data (4) were also subjected to genotyping analysis and those isolates sharing the genotypes that were observed in the Thai collection (n=340; details in **Table S2**) were subjected to the same SNP analyses, resulting in a final set of 9,700 SNPs for a total of 386 isolates.

Phylogenetic and SNP analysis

Maximum likelihood (ML) phylogenetic trees (**Figs 1-2**) were constructed using the 1,850 and 9,700 bp SNP alignments, respectively, using RAxML (v 8.1.23) (36) with a generalized time-reversible model and a gamma distribution to model site specific recombination (GTR+Γ substitution model; GTRGAMMA in RAxML), with Felsenstein correction for ascertainment bias. Support for ML phylogenies was assessed via 100 bootstrap

169 pseudoanalyses of the alignments. For the larger tree containing global isolates, clades
170 containing only isolates from only a single country were collapsed manually in R using the
171 drop.tip() function in the *ape* package (37). Subtrees were extracted for each subclade, which
172 are therefore each rooted by the other subclades. Pairwise SNP distances between isolates
173 were calculated from the SNP alignments using the dist.gene() function in the *ape* package
174 for R (37).
175
176 ***Accessory genome analysis***
177 Acquired antimicrobial resistance (AMR) genes were detected, and their precise alleles
178 determined, by mapping to the ARG-Annot database (38) of known AMR genes using SRST2
179 v0.1.5 (39). Plasmid replicon sequences were identified using SRST2 to screen reads for
180 replicons in the PlasmidFinder database (40, 41). Raw read data was assembled *de novo* with
181 SPAdes (v 3.5.0) (42) and circular contigs were identified visually and extracted using the
182 assembly graph viewer Bandage (v0.7.0) (43). These putative plasmid sequences were
183 annotated using Prokka (v1.10) (44) followed by manual curation. Where IncHI1 plasmid
184 replicons were identified using SRST2, and their presence confirmed by visual inspection of
185 the assembly graphs, IncHI1 plasmid MLST (pMLST) sequence types were determined using
186 SRST2 (13, 45, 46). Where resistance genes were detected from short read data, Bandage was
187 used to inspect their location in the corresponding *de novo* assembly graph in order to
188 determine whether they were encoded in the bacterial chromosome or on a plasmid.
189 Assembled contigs were concatenated and putative prophage genomes were identified with
190 the PHAge Search Tool (PHAST) (47), and their novelty determined by BLASTN analysis
191 against the GenBank database. Pairwise alignments between novel and known prophage
192 sequences were visualised using the *genoPlotR* package for R (48).
193
194 **Nucleotide sequence and sequence read data accession numbers**
195 Raw sequence data have been submitted to the European Nucleotide Archive (ENA) under
196 project PRJEB5281; individual sample accession numbers are listed in **Tables S1 and S2**.

Assembled phage and protein sequences were deposited in GenBank, accession numbers are listed in **Table 1**.

Results

The population structure of S. Typhi in Thailand

All 44 *S.* Typhi isolates collected between 1973 and 1992 were subjected to WGS and SNP analysis. Genome-wide SNPs were used to construct a ML phylogeny and isolates were assigned to previously defined genotypes (32) using a subset of SNPs (see **Methods**). These analyses subdivided the population into ten distinct genotypes, each corresponding to a specific lineage in the ML phylogeny (**Fig 1**). Genotype 3.2.1 (which includes the reference genome CT18, isolated from Vietnam in 1993 (29)) was the most common (n=14, 32%), followed by genotype 2.1.7 (n=10, 23%). Genotypes 2.0 (n=1, 2%) and 4.1 (n=3, 7%) were observed only in 1973 (pre-vaccine period). Genotypes 2.1.7 (n=10, 23%), 2.3.4 (n=1, 2%), 3.4.0 (n=2, 5%), 3.0.0 (n=3, 7%), 3.1.2 (n=2, 5%), were observed only after 1981 (post-vaccine period). Each of these post-immunization genotypes was from a single location and time period (**Fig 1**), consistent with short-term localised transmission. The only exceptions were the two *S.* Typhi 3.1.2 isolates, that were from Srakaew in 1989 and Bangkok in 1992 and separated by just 4 SNPs. Genotypes 3.2.1 and 2.4.0 were observed amongst both pre- and post-vaccine isolates.

Thai S. Typhi in the context of a global genomic framework

Based on the Thai *S.* Typhi genotyping results we hypothesised that the post-immunization typhoid infections in Thailand resulted from occasional re-introduction of *S.* Typhi from outside the country, as opposed to long-term persistence of *S.* Typhi lineages within Thailand. To explore this possibility, and to provide a global context for our analysis, we examined 1,832 *S.* Typhi genomes from a recently published global collection that included isolates from 63 countries (4). Genome-wide SNP-based ML trees for each of these genotypes,

showing the relationships between Thai and global isolates, are shown in **Fig 2**. In general, post-vaccine Thai isolates were closely related to recent isolates sourced from neighbouring countries including Vietnam, Laos and Cambodia (**Fig 2**), consistent with regional endemic circulation. In contrast, most pre-vaccine isolates had no close neighbours in the global collection, particularly 2.0.0 strains (**Fig 2A**), suggesting they may have been Thailand-specific lineages that have died out following the vaccine program. The *S*. Typhi genomes in the global collection were mainly isolated 2-3 decades after the Thai isolates as we did not have access to contemporaneous isolates from these countries that could identify specific transfer events. However, all but three of the post-vaccine Thai isolates shared shorter SNP distances with isolates from neighbouring countries than they did with pre-vaccination Thai isolates (see **Fig 3**), consistent with these cases being caused by occasional re-introduction of genotypes circulating in the region. Notably, Thai *S*. Typhi 3.2.1 that were isolated in 1986-7 clustered separately from the 1973 pre-vaccine isolates (≥60 SNPs apart), but closely with isolates from Vietnam and Cambodia (differing by as few as 7 SNPs; **Fig 2H**). Post-vaccine Thai *S*. Typhi 2.4 formed two distinct groups that were not consistent with direct descendance from earlier isolates (**Fig 2E**). These data are therefore consistent with transfer of *S*. Typhi into Thailand from neighbouring countries during the post-immunization program era, although the long-term circulation of ancestral populations in Thailand remains an unlikely alternative explanation.

Acquired antimicrobial resistance

We identified acquired AMR genes in the genomes of four *S*. Typhi genotype 3.2.1 that were isolated in Srakaew in 1986 (**Fig 1, Table 1**). These isolates shared the same four AMR genes: *sul1* (sulphonamides), *catA1* (chloramphenicol), *tet(B)* (tetracyclines), and *aadA1* (aminoglycosides) which were carried on near-identical plasmids of IncHI1 plasmid sequence type 2 (PST2). Although the presence of insertion sequences (IS) in these plasmids prevented the complete sequences from being assembled, the regions of these plasmids encoding the AMR genes were identical in all assemblies. This commonality suggests they are a single

plasmid (referred to as pTy036_01 in **Fig 1** and **Table 1**) that was likely acquired in a common ancestor of this clade. The chromosomal and IncHI1 plasmid sequences for these four isolates were very closely related to those of a 1993 Vietnamese isolate (Viety1-60_1993) in the global *S*. Typhi collection (45), consistent with regional transfer.

Other plasmids and mobile genetic elements

We identified three non-AMR related plasmids amongst the Thai isolates (**Fig 1, Table 1**). Ty004 (genotype 2.2) carried two novel plasmids that assembled into circular sequences, pTy004_01 and pTy004_02. The largest, pTy004_01, was a novel variant of the cryptic plasmid pHCM2 (29, 49) (**Fig 4**). Ty004 was isolated in Bangkok in 1973, making pTy004_01 the earliest example of a pHCM2-like plasmid reported to date. pTy004_01 was distant from other pHCM2-like plasmids in the global *S*. Typhi genome collection, sharing 92% coverage and 99% nucleotide identity with the reference sequence pHCM2 of *S*. Typhi CT18 (genotype 3.2.1) which was isolated approximately 20 years later in Vietnam (29). The pTy004_01 sequence (**Fig 4**) appears to be ~2 kbp larger than pHCM2, and encodes an additional tRNA-Lys as well as an insertion of a hypothetical protein (*orf17*) into a putative DNA polymerase gene (HCM2.0015c in pHCM2, divided into *orf16* and *orf18* in pTy004_01). Plasmid pTy004_02 was ~38 kbp in size and similar to *E. coli* plasmid pEQ2 (65% coverage, 98% nucleotide identity), encoding genes for conjugation, chromosomal partitioning, addiction systems and an abortive infection protein (*orf44*). Three isolates (Ty031, Ty042, and Ty049) all of genotype 3.0.0 and obtained from Srakaew in 1986, carried a ~40 kbp cryptic plasmid that we named pTy031_01. This plasmid was similar to that carried by *Enterobacter hormaechei* strain CAV1176 (83% coverage, 96% identity) and encoded genes for chromosomal partitioning, addiction systems, and a putative restriction modification system (*orf33*-*orf34*).

PHAST analysis revealed the presence of novel intact prophages in three Thai *S.* Typhi isolates (**Fig 1**, **Table 1)**. Two *S*. Typhi 3.1.2, isolated from Srakaew in 1989 and Bangkok in

1992, shared a novel phage STYP1 that was similar to fiAA91-ss infective for *Shigella sonnei* (**Fig 5A**). However, the *S*. Typhi phage lacked the cytolethal distending toxin *cdt* genes and the IS*21* element found in phage fiAA91-ss (50). This prophage sequence had a mosaic architecture, incorporating a number of putative insertions of phage tail fiber genes that were not present in the fiAA91-ss reference genome (**Fig 5A**). Additionally, a single isolate of genotype 4.1 obtained from Bangkok in 1973 contained a novel SfIV-like phage, here named STYP2, that lacked the serotype conversion gene Gtr cluster and IS*1* element of phage SfIV (51). Again, the novel Thai phage variant also encoded novel tail fiber genes not in the SfIV reference genome, as well as a Dam methylase gene *(orf37*) (**Fig 5B).**

Discussion

These data provide a historical insight into the population structure of *S*. Typhi in Thailand in 1973 (pre-immunization program, n=11) and 1981-1992 (post- immunization program, n=33). It has been reported that the national *S*. Typhi immunization program in Thailand, which commenced in 1977, was highly effective in reducing the burden of typhoid fever (14). Our data are consistent with the hypothesis that the vaccine program successfully depleted the endemic *S*. Typhi population to the extent that most subsequent typhoid cases resulted from sporadic introduction of non-indigenous *S*. Typhi, rather than long-term persistence of the pre-vaccine era population. It is apparent that these introductions were sometimes accompanied by limited local transmissions, resulting in small, localized outbreaks, but we found no evidence to suggest that these result in the establishment of stable local source populations. Notably, the post-immunization *S*. Typhi isolates from Loi (in the north of Thailand near the border with Laos, from which it is separated by the Mekong river) were most closely related to Laos isolates, whilst those from the capital Bangkok and nearby Nonthaburi and Srakaew districts were closely related to other isolates from across Southeast Asia (**Fig 2**), suggesting there may have been multiple routes of import into Thailand.

Our study is limited by the sample of isolates available for analysis, which was small and reflects opportunistic sampling of sporadic local cases in the four sites and historical storage. A larger collection of historical isolates from Thailand and neighboring countries in the 1970s and 1980s would help to further elucidate the epidemiological patterns of *S.* Typhi before and after the vaccination program. However, from our data, it is notable that the Thai isolates cluster according to site, consistent with limited local transmission rather than dissemination of lineages between locations. The only exception to this was two genotype 3.1.2 isolates, which were collected from Srakaew in 1989 and Bangkok in 1992 and differed by only 4 SNPs. This is consistent with either transfer between these cities in Thailand following an initial introduction into the country, or two independent transfers into Thailand from a common source. The phylogenetic structure is most suggestive of the latter, but denser samples from Thailand and/or potential source populations would be required to resolve this with confidence.

While our sample is small, this study is nevertheless the largest to date exploring genetic diversity amongst *S.* Typhi from Thailand. An earlier global haplotyping study that included seven Thai isolates (52) identified five distinct haplotypes in Thailand (H3, 1989; H42, 1990; H50, 2002; Vi- H52, 1990; H79, 2002), three of which are related to genotypes that we identified amongst Thai strains in this study (H79, 2.3.4; H52, 3.4; H42, 3.1.2) (32). Genotype 4.3.1 (H58) was not found amongst our historical Thai isolates. This is consistent with previously published spatiotemporal analyses of the global isolate collection, which showed this rapidly expanding clone only began spreading throughout Asia after 1990 (4). To our knowledge the only evidence to date of the presence of 4.3.1 (H58) in Thailand comes from the global study (4), in which three isolates were identified from 2010-2011, most likely introduced from India. Therefore, our genomic snapshot of the Thai *S.* Typhi population is consistent with previous insights and is likely reasonably representative for the study period. In the years following the vaccination program the prevalence of Typhoid fever in Thailand has continued to decline (53, 54)**.** The vaccination program has been credited with reducing

disease incidence in Thailand and was followed by increased economic development in the region as well as improvements to both water and sanitation systems that have likely improved the control of such outbreaks (53, 54). Consequently, Typhoid fever is longer considered a serious public health threat in Thailand (53).

The presence of novel plasmids and prophages in the Thai isolates is also noteworthy. While small plasmids of unknown function have been observed in *S.* Typhi previously (55), they are infrequent compared to the IncHI1 MDR plasmid and the cryptic plasmid pHCM2 (33) . Presumably, such plasmids are ephemeral; possibly because their maintenance imposes a fitness burden on the host cells so a strong selective advantage is required for retention (56, 57). It is also possible that the lack of previous reports regarding the diversity of small plasmids in *S*. Typhi reflects a technological complexity, however, this is bypassed with high-throughput WGS and we detected negligible small plasmid content in the global collection of 1,832 genomes using the same screening approach (58). Notably, few of the Thai plasmids share nucleotide sequence homology with those previously described in *S.* Typhi, but were closely related to those found in other *Enterobacteriaceae*. The novel pHCM2-like plasmid (pTy004_01) and two additional plasmids (pTy004_02 and pTy031_01) harbored genes associated with phage resistance, which could provide protection against phage predation (59-62). We also observed two novel prophages integrated into Thai genomes, which both showed variation in their phage tail structural regions compared to close neighbors found in *Shigella/E. coli*. These regions are typically responsible for binding of phage to host receptors (63-65), thus the variation in these regions may be associated with recent adaptations to the *S.* Typhi host. While genomic data from more recent *S*. Typhi collections shows limited evidence for genetic exchange with other organisms (4), the detection amongst older Thai isolates of both phage and plasmids that have been previously associated with *E. coli/Shigella* suggests that genetic exchange may have been more common in the past or in certain localized populations.

Overall, these data provide valuable historical insights into the *S*. Typhi populations circulating in Thailand during the 1970s and 1980s, and early examples of the two most common *S*. Typhi plasmids, as well as other mobile elements identified within the *S*. Typhi population. Importantly, while genomic epidemiology has been applied to study typhoid transmission, antimicrobial resistance evolution and antibiotic treatment failure in various settings (66-68), this study provides an important proof-of-principle demonstration that this approach can also provide useful insights into the impact of typhoid vaccines on circulating bacterial populations. This should motivate the adoption of WGS methods to monitor *S*. Typhi populations during future immunization programs and other large-scale interventions, which could potentially identify differential impacts on distinct genotypes.

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564 **Table 1. Summary of mobile genetic elements observed in *S.* Typhi isolates from Thailand**

Isolate	Genotype	Name	Replicons detected and/or attachment sites	Size (no. putative genes)	Accession number	Function
004	2.0.0	pTy004_01	FIB (pHCM2)	108, 998 bp (133)	KX833209	Cryptic, Phage defence (Rha protein)
		pTy004_02	X1	38, 266 bp (49)	KX833212	Phage defence (Abortive Infection)
031	3.0.0	pTy031_01	N/A	40, 835 bp (53)	KX833210	Phage defence (Restriction
042						Modification)
049						
036	3.2.1	pTy036_01	HI1	~215 kbp	N/A.	AMR (*sul1*, *catA1*, *tet(B)*, *aadA1*)
046						
051						
052						
054	3.1.2	Prophage STYP1	*attL* CAAGCTGGTCAG	28,946 bp (39)	KX833211	Cryptic
055			*attR* CAAGCTGGTCAG			

Isolate	Genotype	Name	Replicons detected and/or attachment sites	Size (no. putative genes)	Accession number	Function
013	4.1.0	Prophage STYP2	*attL* ATTCGTAATGCGAAGG TCGTAGGTTCGACTCCT ATTATCGGCACCAT *attR* ATTCGTAATGCGAAGG TCGTAGGTTCGACTCCT ATTATCGGCACCA	34, 780 bp (50)	KX833213	Cryptic

565

566 **Figure 1. Genomic analysis of Thai S. Typhi.**

567 (A) Maximum likelihood phylogenetic tree (outgroup rooted). Strains are labelled with their
568 three digit name code, year of isolation (pink shading indicates post-vaccine isolates); source
569 location (shaded by city, as indicated in panel B); and plasmid content (any antibiotic
570 resistance genes are indicated in italics). Branch lengths are indicative of the number of SNPs.
571 (B) Locations from which S. Typhi were isolated in Thailand. (C) Total number of positive
572 blood cultures of S. Typhi (black) and Paratyphi A (grey) between 1970 and 1985;
573 immunization period is indicated in pink; reproduced using data from reference (14).

574

575 **Figure 2. Zoomed in phylogenies showing relationships of Thai S. Typhi to global**
576 **isolates.** Maximum likelihood trees including S. Typhi isolates from the Thai and global
577 collections are shown, for each genotype that was observed amongst the Thai isolates.
578 Colored branches and nodes indicate country of origin, according to the inset legend. Year of
579 isolation is shown to the left; pink and red, Thai isolates obtained before and after the
580 introduction of the immunization program; grey and black, non-Thai isolates obtained before
581 and after the introduction of the immunization program. Thai isolates are also labelled to
582 indicate their city of origin: L, Loi; B, Bangkok; S, Srakaew; N, Nonthaburi. SNP distances
583 between isolates as well as AMR plasmids are labelled, with any resistance genes indicated in
584 italics. Branch lengths are indicative of the number of SNPs.

585

586 **Figure 3. SNP distances for Thai and global collection isolates.**
587 SNP distance between post-vaccine Thai isolates and their closest pre-vaccine Thai and post-
588 vaccine global collection relatives, colored points indicate country of origin.

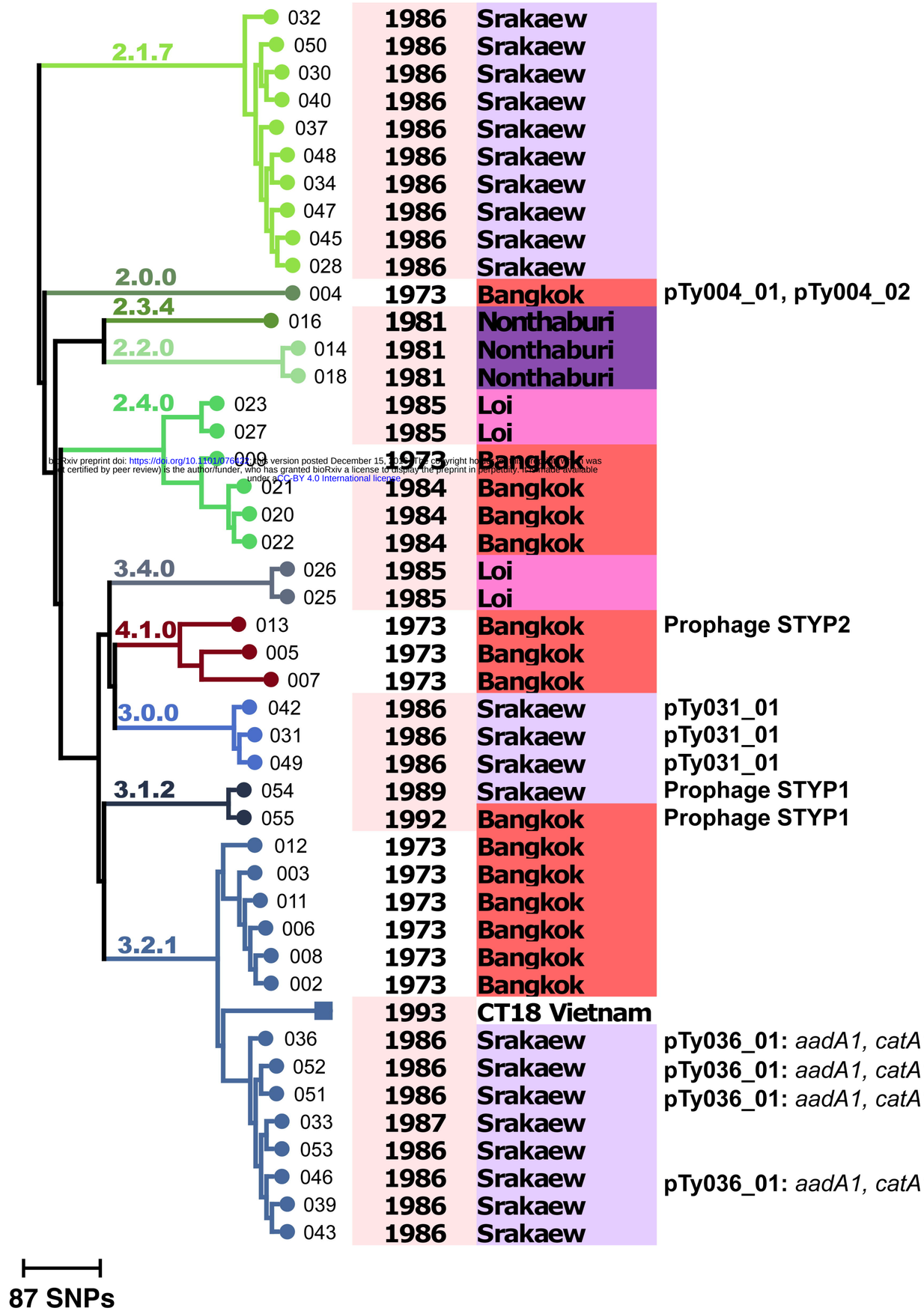
589

590 **Figure 4. Blast comparison of novel plasmid pTy004_01 with pHCM2 (AL513383).**
591 Shaded regions indicate areas of sequence homology, intensity of shading indicates relative
592 nucleotide similarity. Arrows represent protein coding genes, direction indicates coding
593 strand.

Figure 5. Blast comparison of novel phages observed in Thai S. Typhi isolates to nearest known phage sequences.

(A) Novel phage STYP1 compared to Shigella sonnei phage fiAA91-ss (NC_022750). (B) Novel phage STYP2 compared to Shigella flexneri phage SfIV (NC_022749). Shaded regions indicate areas of sequence homology, intensity of shading indicates relative nucleotide similarity. Arrows represent protein coding genes (direction indicates coding strand), colored by encoded protein functions: red, DNA packaging module; orange, virion morphogenesis module; yellow, cargo genes; blue, DNA replication and lysogenic cycle maintenance; green, lysis module.

A



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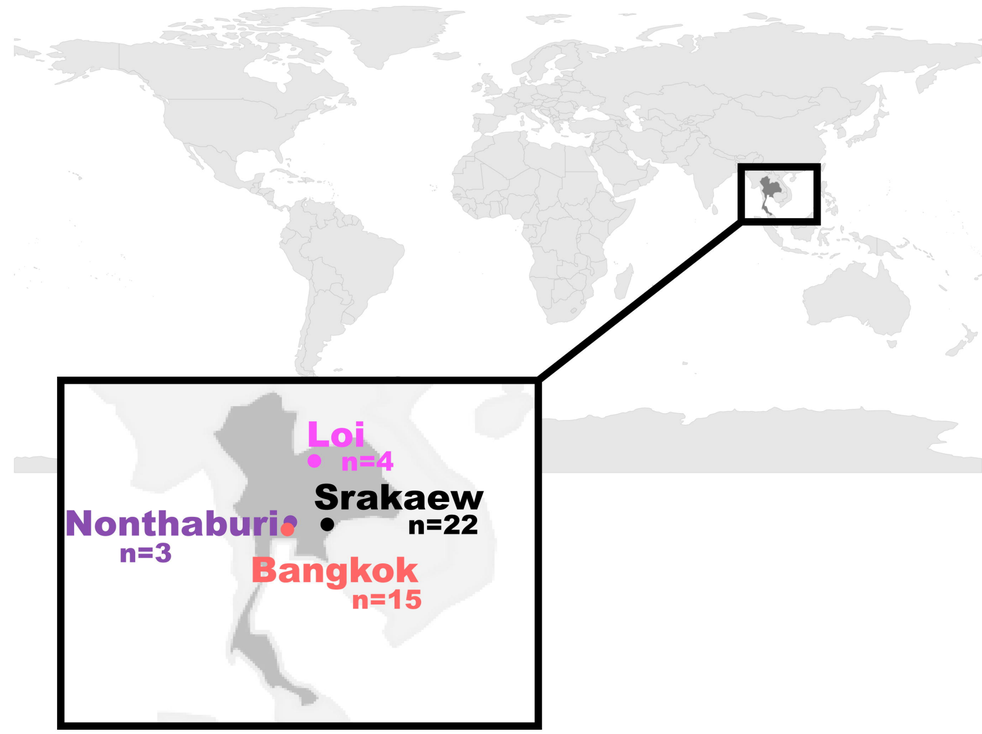

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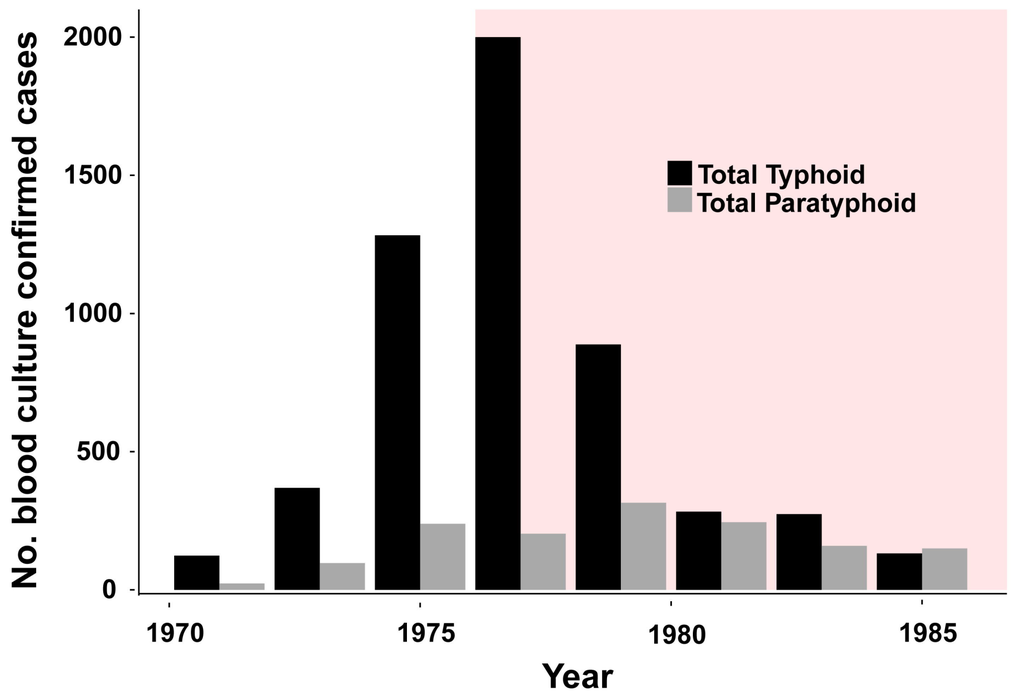

A Genotype 2.0.0

407 SNPs

2003
1996
1973
2003
1998
2000
1999
2010
2000
2003

2011
2003

B Genotype 2.1.7

34-39 SNPs

2008
1998
2010
1994
1998
1988
1939
1994
1986 S
1986 S
1986 S
1986 S
1986 S
1986 S
1986 S
1986 S
1986 S
1986 S

C Genotype 2.2.0

133-145 SNPs

2011
2000
2011
2006
2010
2011
1981 N
1981 N
1994
2003
1980
2011
2001
2004
1999
1999

D Genotype 2.3.4

54 SNPs

1997
2007
1981 N
2009
2011
2009

E Genotype 2.4.0

48-50 SNPs
42 SNPs
78-112 SNPs

2012
2009
2010
1985 L
1985 L
1973
1984 B
1984 B
1984 B

96 SNPs

F Genotype 3.0.0

204-227 SNPs

2008
2011
2011
2003
1999
1986 S
1986 S
1986 S
2001
2012
2011
2012
2011
2010
2006
2011
1994
2012

1984

G Genotype 3.1.2

4 SNPs
71 SNPs

1992 B
1989 S
2008
2010
2002
1983
2003
2012
2009

H Genotype 3.2.1

0-37 SNPs
69-71 SNPs
60-65 SNPs
7-11 SNPs
0-6 SNPs

CT18

2011
1976
1973
1973
1973
1973
1973
1973
1993
1997
1997
2009
2009
2010
2009
1993
2007
2011
1993
1999
2007
1994
1993
1986 S pTy036_01: *aadA1, catA1, sul1, tet(B)*
1986 S pTy036_01: *aadA1, catA1, sul1, tet(B)*
1986 S
1987 S
1986 S pTy036_01: *aadA1, catA1, sul1, tet(B)*
1986 S pTy036_01: *aadA1, catA1, sul1, tet(B)*
1986 S
1986 S

I Genotype 3.4.0

76-81 SNPs

2008
2010
1996
1995
1972
2009
2009
2008
2008
2008
2010
1985 L
1985 L

J Genotype 4.1.0

86-90 SNPs
78-103 SNPs
43-97 SNPs

1996
2004
1973
2003
2000
2002
1994
2001
1905
1916
1986
1905
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2011
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1988
1973
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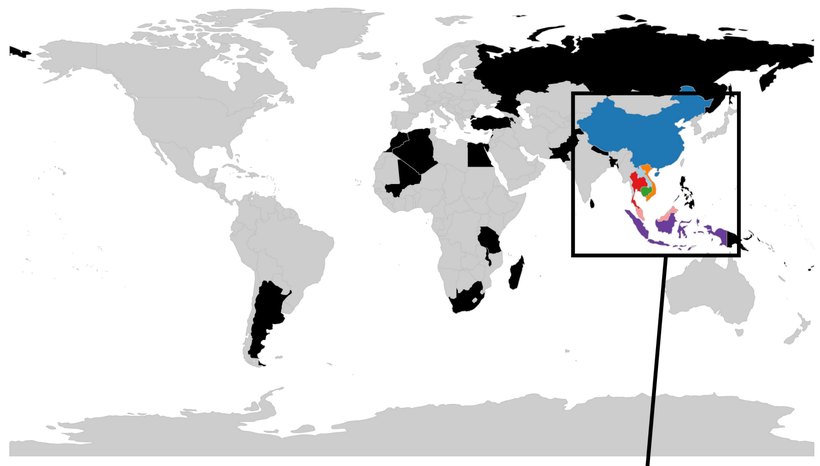
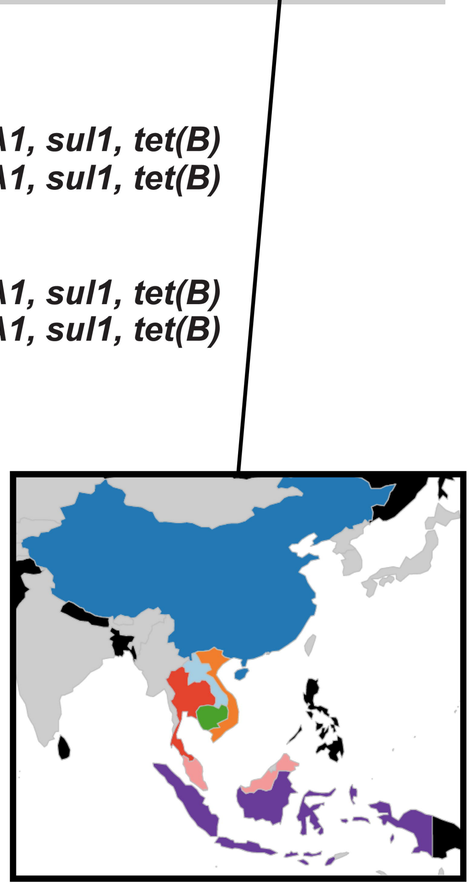
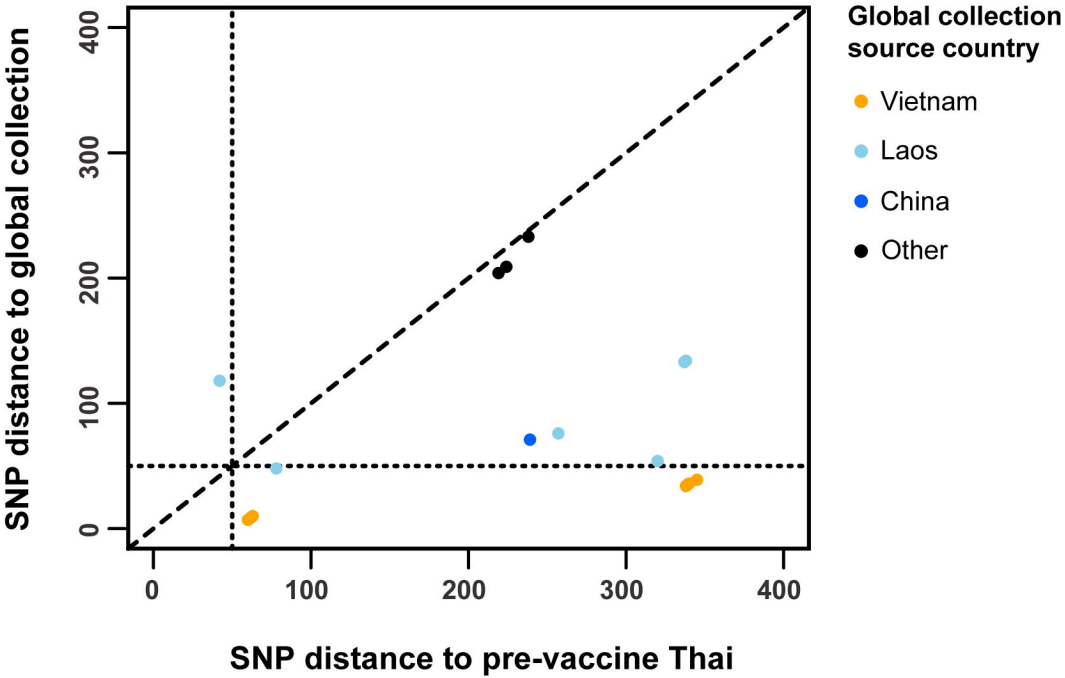
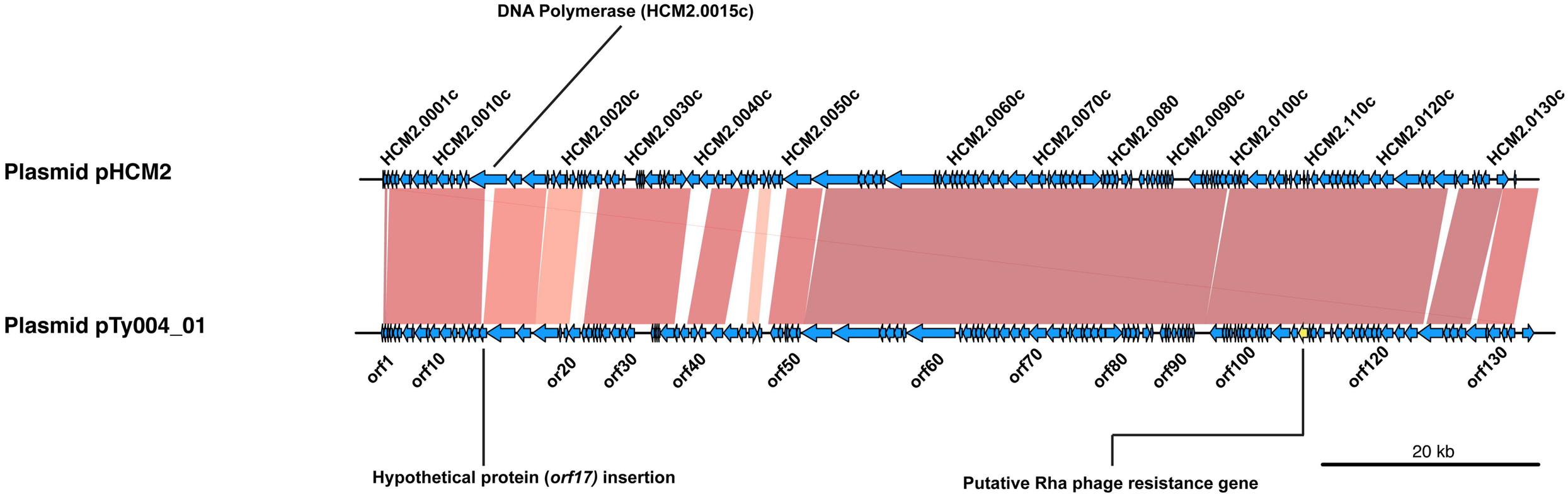
1973
1973
2012
1994



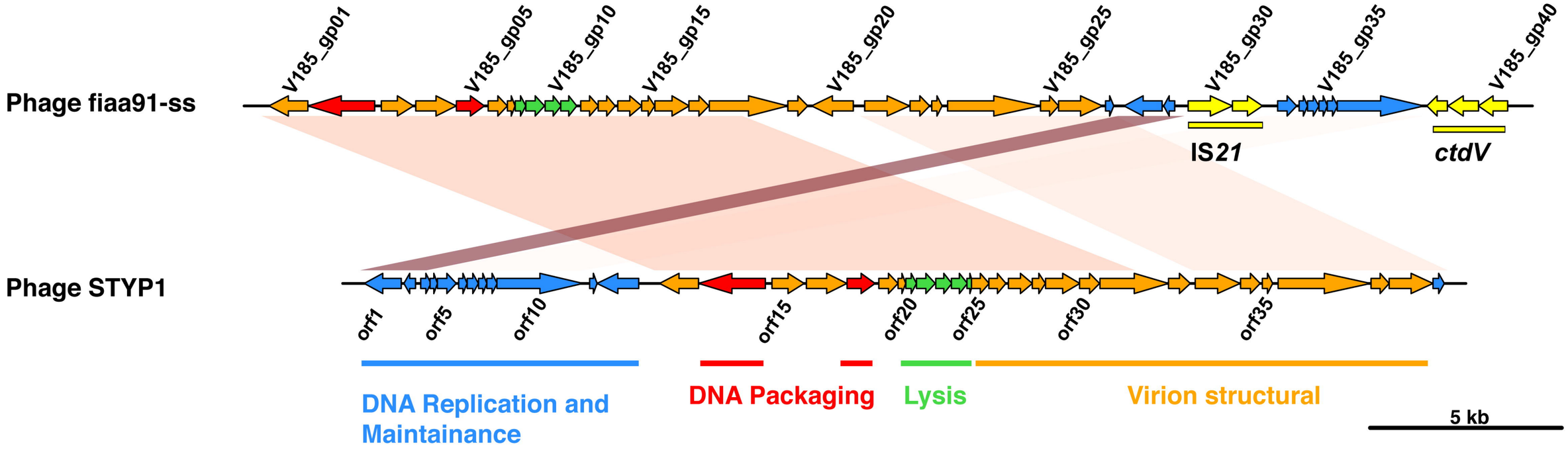


Cambodia
China
Malaysia
Indonesia
Laos
Vietnam
Thailand
Other

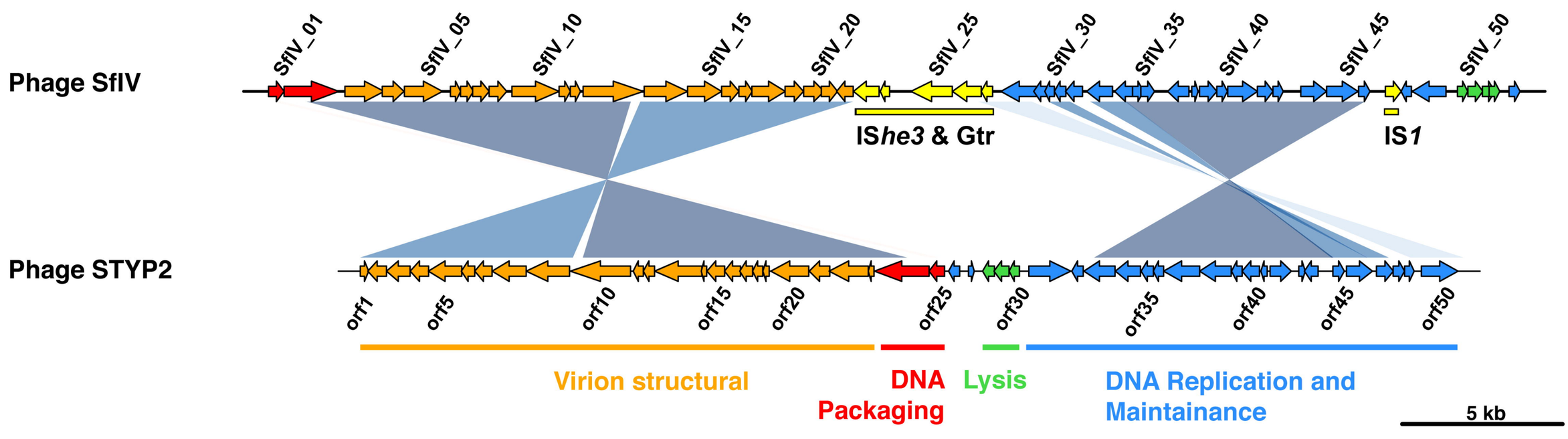

SNP distance to global collection
0
100
200
300
400
SNP distance to pre-vaccine Thai
0
100
200
300
400
Global collection source country
Vietnam
Laos
China
Other


DNA Polymerase (HCM2.0015c)
Plasmid pHCM2
Plasmid pTy004_01
HCM2.0001c
HCM2.0010c
HCM2.0020c
HCM2.0030c
HCM2.0040c
HCM2.0050c
HCM2.0060c
HCM2.0070c
HCM2.0080
HCM2.0090c
HCM2.0100c
HCM2.110c
HCM2.0120c
HCM2.0130c
orf1
orf10
or20
orf30
orf40
orf50
orf60
orf70
orf80
orf90
orf100
orf120
orf130
Hypothetical protein (orf17) insertion
Putative Rha phage resistance gene
20 kb

A


Phage fiaa91-ss
V185_gp01
V185_gp05
V185_gp10
V185_gp15
V185_gp20
V185_gp25
V185_gp30
V185_gp35
V185_gp40
IS21
ctdV
Phage STYP1
orf1
orf5
orf10
orf15
orf20
orf25
orf30
orf35
DNA Replication and Maintainance
DNA Packaging
Lysis
Virion structural
5 kb

B


Phage SfIV
SfIV_01
SfIV_05
SfIV_10
SfIV_15
SfIV_20
SfIV_25
SfIV_30
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SfIV_40
SfIV_45
SfIV_50
ISShe3 & Gtr
IS1
Phage STYP2
orf1
orf5
orf10
orf15
orf20
orf25
orf30
orf35
orf40
orf45
orf50
Virion structural
DNA Packaging
Lysis
DNA Replication and Maintainance
5 kb