

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

Zoe A. Dyson<sup>1,2\*</sup>, Duy Pham Thanh<sup>3</sup>, Ladaporn Bodhidatta<sup>4</sup>, Carl Jeffries Mason<sup>4</sup>, Apichai Srijan<sup>4</sup>, Maia A. Rabaa<sup>3,5</sup>, Phat Voong Vinh<sup>3</sup>, Tuyen Ha Thanh<sup>3</sup>, Guy E. Thwaites<sup>3,5</sup>, Stephen Baker<sup>3,5,6†</sup> and Kathryn E. Holt<sup>1,2†\*</sup>

<sup>1</sup> Centre for Systems Genomics, University of Melbourne, Parkville, Victoria 3052, Australia

<sup>2</sup> Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria 3010, Australia

<sup>3</sup> The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam

<sup>4</sup> Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

<sup>5</sup> Centre for Tropical Medicine and Global Health, Oxford University, Oxford, United Kingdom

<sup>6</sup> The London School of Hygiene and Tropical Medicine, London, United Kingdom

\* Corresponding Author

† Joint senior authors

*S. Typhi* in Thailand before and after immunization

# 1     **Abstract**

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

21  
22  
23  
24  
25  
26  
27  
28

## 29 **Author Summary**

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

44

45

46

47

48

49

50

51

52

53

54

55

56

## 57 Introduction

58 *Salmonella enterica* subspecies *enterica* serovar Typhi (*S. Typhi*) is a human restricted  
 59 bacterial pathogen and the etiological agent of typhoid fever. *S. Typhi* is transmitted faeco-  
 60 orally and can establish asymptomatic carriage in a small subset of an exposed population (1).  
 61 Recent estimates (2-4) place the global burden of typhoid fever at 25-30 million cases  
 62 annually, of which 200,000 are associated with deaths. Typhoid fever occurs most commonly  
 63 in industrialising countries, specifically in locations with limited sanitation and related  
 64 infrastructure (5); children and young adults are among the most vulnerable populations in  
 65 these settings (6-8). Immunization and antimicrobial therapy are the major mechanisms by  
 66 which typhoid fever is controlled (9-12). However, neither of these approaches are optimal  
 67 and resistance against antimicrobials has become increasingly common in *S. Typhi* since the  
 68 1970s (13-15). Additionally, while a number of typhoid vaccines are licenced for use (9, 16-  
 69 19), they are not widely used as a public health tools in endemic areas, with the exception of  
 70 controlling severe outbreaks such as those following natural disasters (20-23).

71  
 72 A sustained typhoid fever outbreak occurred in Thailand in the 1970s. A sharp increase in  
 73 cases was observed in 1973-1974, which finally peaked in 1975-1976. In response, the  
 74 government of Thailand established a national typhoid immunization program, which  
 75 represented the first programmatic use of a typhoid vaccine in the country (24). The  
 76 immunization program targeted over 5 million school aged children (7-12 years) in Bangkok  
 77 between 1977 and 1987 (80% of the eligible population). These children received a single  
 78 locally produced heat/phenol-inactivated subcutaneous dose of  $2.5 \times 10^8$  *S. Typhi* organisms  
 79 (9, 24). Data from four teaching hospitals in Bangkok showed a 93% reduction in blood  
 80 culture confirmed infections with *S. Typhi* between 1976 (n=2,000) and 1985 (n=132) (9, 24).  
 81 Notably, no significant decline was observed in isolation rates of *Salmonella* Paratyphi A (*S.*  
 82 Paratyphi A), a *Salmonella* serovar distinct from *S. Typhi* that causes a clinical  
 83 indistinguishable disease to typhoid fever, but for which *S. Typhi* vaccines provide little or no  
 84 cross-protection (9). This observation suggests that the reduction in *S. Typhi* infections was

85 not attributable to improvements in infrastructure and hygiene practices only (5, 9, 21, 24).  
 86 While the inactivated *S. Typhi* vaccine was found to be highly efficacious (23, 24), it is no  
 87 longer used as a consequence of being overly reactogenic (9, 17, 24, 25). A Vi capsular  
 88 polysaccharide vaccine (16) and live-attenuated oral vaccine of strain Ty21a (17) have since  
 89 replaced this vaccine for travellers to endemic locations (5, 22, 25).  
 90  
 91 The typhoid immunization program in Thailand provided a unique opportunity to investigate  
 92 the impact of immunization on *S. Typhi* populations circulating within an endemic area. Here  
 93 we present an analysis of a historical collection of 44 *S. Typhi* isolates obtained from patients  
 94 in Thailand between 1973 and 1992 (before and during the immunization program). As *S.*  
 95 *Typhi* populations demonstrate little genetic diversity, we used whole genome sequencing  
 96 (WGS) to characterise these isolates, and core genome phylogenetic approaches to compare  
 97 the historic isolates from Thailand to a recently published global *S. Typhi* genomic  
 98 framework (4).  
 99

## 100 **Materials and methods**

### 101 ***Ethics statement***

102 *Salmonella Typhi* isolates were collected during febrile disease surveillance studies in  
 103 Thailand. IRB approval was granted for these studies from the Research Ethics Board of  
 104 Health (REBH) and the Walter Reed Army Institute of Research (WRAIR) Institutional  
 105 Review Board, USA. Oral consent was obtained from a parent or guardian at the time of  
 106 enrolment into the study.

### 108 ***Bacterial isolation and antimicrobial susceptibility testing***

109 Forty-four *S. Typhi* isolated from patients with suspected typhoid fever attending hospitals in  
 110 Bangkok, Nonthaburi, Loi, and Srakaew, in Thailand between 1973 and 1992 were available  
 111 for genome sequencing in this study (**Fig 1** and **Table S1**). At the time of original isolation,  
 112 bacterial cultures were transferred on nutrient agar slants to the department of Enteric

113 Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok,  
 114 Thailand for identification and antimicrobial susceptibility testing. At AFRIMS, bacterial  
 115 isolates were subcultured on Hektoen Enteric agar (HE) and identification was performed by  
 116 biochemical testing on Kligler iron agar slants, tryptone broth for indole, lysine decarboxylase  
 117 medium, ornithine decarboxylase medium, urease test, mannitol and motility media (Becker  
 118 Dickenson, Thailand). Serological agglutination was performed using *Salmonella* O antisera  
 119 and *Salmonella* Vi antiserum (Difco, USA). Bacterial strains were stored frozen at -70°C in  
 120 10% skimmed milk or lyophilised in 10% skimmed milk; lyophilized ampoules were stored at  
 121 2-8°C. Prior to DNA extraction for sequencing, lyophilized bacteria was rehydrated with  
 122 trypticase soy broth, inoculated on McConkey agar and incubated at 37°C for 18-24 hours. If  
 123 bacteria was stored frozen in skimmed milk, organisms were inoculated directly onto  
 124 McConkey agar after thawing and then incubated at 37°C for 18-24 hours.

125

126 Antimicrobial susceptibility testing against ampicillin, chloramphenicol, cephalothin,  
 127 gentamicin, kanamycin, neomycin, sulfisoxazole, trimethoprim/sulfamethoxazole, and  
 128 tetracycline was performed by disk diffusion according to Clinical and Laboratory Standards  
 129 Institute (CLSI) (26-29).

130

### 131 ***Genome sequencing and SNP analysis***

132 Genomic DNA from the 44 *S. Typhi* from Thailand was extracted using the Wizard Genomic  
 133 DNA Extraction Kit (Promega, Wisconsin, USA). Two µg of genomic DNA was subjected to  
 134 indexed WGS on an Illumina HiSeq 2000 platform at the Wellcome Trust Sanger Institute, to  
 135 generate 100 bp paired-end reads. For analysis of SNPs, paired end Illumina reads were  
 136 mapped to the reference sequence of *S. Typhi* CT18 (accession no: AL513382) (30) using the  
 137 RedDog (v1.4) mapping pipeline, available at <https://github.com/katholt/reddog>. RedDog  
 138 uses Bowtie (v2.2.3) (31) to map reads to the reference sequence, then high quality SNPs  
 139 called with quality scores above 30 are extracted from the alignments using SAMtools  
 140 (v0.1.19) (32). 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 (33)(code available at <https://github.com/katholt/genotypphi>). SNPs called in phage regions, repetitive sequences (354 kb; ~7.4% of bases in the CT18 reference chromosome, as defined previously (34)), or recombinant regions (~180kb; <4% of the CT18 reference chromosome, identified using Gubbins (v1.4.4) (35)) 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. 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) were subjected to the same SNP analyses, resulting in a final set of 9,700 SNPs for a total of 386 isolates. For each alignment, SNP alleles from Paratyphi A strain 12601 (36) were also included as an outgroup.

# ***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) (37) with a generalized time-reversible model and a gamma distribution to model site specific recombination (GTR+ $\Gamma$  substitution model; GTRGAMMA in RAxML), with Felsenstein correction for ascertainment bias. Support for ML phylogenies was assessed via 100 bootstrap pseudoanalyses of the alignments. For the larger tree containing global isolates, clades containing only isolates from only a single country were collapsed manually in R using the drop.tip() function in the *ape* package (38). Pairwise SNP distances between isolates were

169 calculated from the SNP alignments using the `dist.gene()` function in the *ape* package for R  
170 (38).

171

## 172 *Accessory genome analysis*

173 Acquired antimicrobial resistance (AMR) genes were detected, and their precise alleles  
174 determined, by mapping to the ARG-Annot database (39) of known AMR genes using SRST2  
175 v0.1.5 (40). Plasmid replicon sequences were identified using SRST2 to screen reads for  
176 replicons in the PlasmidFinder database (41). Raw read data was assembled *de novo* with  
177 SPAdes (v 3.5.0) (42) and circular contigs were identified visually and extracted using the  
178 assembly graph viewer Bandage (v0.7.0) (43). These putative plasmid sequences were  
179 annotated using Prokka (v1.10) (44) followed by manual curation. Where IncHI1 plasmid  
180 replicons were identified using SRST2, and their presence confirmed by visual inspection of  
181 the assembly graphs, IncHI1 plasmid MLST (pMLST) sequence types were determined using  
182 SRST2 (15, 45, 46). Where resistance genes were detected from short read data, Bandage was  
183 used to inspect their location in the corresponding *de novo* assembly graph in order to  
184 determine whether they were encoded in the bacterial chromosome or on a plasmid.  
185 Assembled contigs were concatenated and putative prophage genomes were identified with  
186 the PHAge Search Tool (PHAST) (47), and their novelty determined by BLASTN analysis  
187 against the GenBank database. Pairwise alignments between novel and known prophage  
188 sequences were visualised using the *genoPlotR* package for R (48).

189

## 190 **Nucleotide sequence and sequence read data accession numbers**

191 Raw sequence data have been submitted to the European Nucleotide Archive (ENA) under  
192 project PRJEB5281; individual sample accession numbers are listed in **Table S1**. Assembled  
193 phage and protein sequences were deposited in GenBank, accession numbers are listed in  
194 **Table 1**.

195

196



# 197 **Results**

## 198 ***The population structure of *S. Typhi* in Thailand***

199 All 44 *S. Typhi* isolates collected between 1973 and 1992 were subjected to WGS and SNP  
200 analysis. Genome-wide SNPs were used to construct a ML phylogeny and isolates were  
201 assigned to previously defined genotypes (33) using a subset of SNPs (see **Methods**). These  
202 analyses subdivided the population into ten distinct genotypes, each corresponding to a  
203 specific lineage in the ML phylogeny (**Fig 1**). Genotype 3.2.1 (which includes the reference  
204 genome CT18, isolated from Vietnam in 1993 (30)) was the most common (n=14, 32%),  
205 followed by genotype 2.1.7 (n=10, 23%). Genotypes 2.0 (n=1, 2%) and 4.1 (n=3, 7%) were  
206 observed only in 1973 (pre-vaccine period). Genotypes 2.1.7 (n=10, 23%), 2.3.4 (n=1, 2%),  
207 3.4.0 (n=2, 5%), 3.0.0 (n=3, 7%), 3.1.2 (n=2, 5%), were observed only after 1981 (post-  
208 vaccine period). Each of these post-immunization genotypes was from a single location and  
209 time period (**Fig 1**), consistent with short-term localised transmission. The only exceptions  
210 were the two *S. Typhi* 3.1.2 isolates, that were from Srakaew in 1989 and Bangkok in 1992  
211 and separated by just 4 SNPs. Genotypes 3.2.1 and 2.4.0 were observed amongst both pre-  
212 and post-vaccine isolates.

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

215 Based on the Thai *S. Typhi* genotyping results we hypothesised that the post-immunization  
216 typhoid infections in Thailand resulted from occasional re-introduction of *S. Typhi* from  
217 outside the country, as opposed to long-term persistence of *S. Typhi* lineages within Thailand.  
218 To explore this possibility, and to provide a global context for our analysis, we examined  
219 1,832 *S. Typhi* genomes from a recently published global collection that included isolates  
220 from 63 countries (4). Genome-wide SNP-based ML trees for each of these genotypes,  
221 showing the relationships between Thai and global isolates, are shown in **Fig 2**. In general, all  
222 Thai isolates were closely related to recent isolates sourced from neighbouring countries  
223 including Vietnam, Laos and Cambodia (**Fig 2**), consistent with regional endemic circulation.  
224 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 ( $\geq 60$  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 descent 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: *sulI* (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.

## 253 *Other plasmids and mobile genetic elements*

254 We identified three non-AMR related plasmids amongst the Thai isolates (**Fig 1, Table 1**).

255 Ty004 (genotype 2.2) carried two novel plasmids that assembled into circular sequences,

256 pTy004\_01 and pTy004\_02. The largest, pTy004\_01, was a novel variant of the cryptic

257 plasmid pHCM2 (30, 49) (**Fig 4**). Ty004 was isolated in Bangkok in 1973, making

258 pTy004\_01 the earliest example of a pHCM2-like plasmid reported to date. pTy004\_01 was

259 distant from other pHCM2-like plasmids in the global *S. Typhi* genome collection, sharing

260 92% coverage and 99% nucleotide identity with the reference sequence pHCM2 of *S. Typhi*

261 CT18 (genotype 3.2.1) which was isolated approximately 20 years later in Vietnam (30). The

262 pTy004\_01 sequence (**Fig 4**) appears to be ~2 kbp larger than pHCM2, and encodes an

263 additional tRNA-Lys as well as an insertion of a hypothetical protein (*orf17*) into a putative

264 DNA polymerase gene (HCM2.0015c in pHCM2, divided into *orf16* and *orf18* in

265 pTy004\_01). Plasmid pTy004\_02 was ~38 kbp in size and similar to *E. coli* plasmid pEQ2

266 (65% coverage, 98% nucleotide identity), encoding genes for conjugation, chromosomal

267 partitioning, addiction systems and an abortive infection protein (*orf44*). Three isolates

268 (Ty031, Ty042, and Ty049) all of genotype 3.0.0 and obtained from Srakaew in 1986, carried

269 a ~40 kbp cryptic plasmid that we named pTy031\_01. This plasmid was similar to that carried

270 by *Enterobacter hormaechei* strain CAV1176 (83% coverage, 96% identity) and encoded

271 genes for chromosomal partitioning, addiction systems, and a putative restriction modification

272 system (*orf33-orf34*).

273

274 PHAST analysis revealed the presence of novel intact prophages in three Thai *S. Typhi*

275 isolates (**Fig 1, Table 1**). Two *S. Typhi* 3.1.2, isolated from Srakaew in 1989 and Bangkok in

276 1992, shared a novel phage STYP1 that was similar to fiAA91-ss infective for *Shigella sonnei*

277 (**Fig 5A**). However, the *S. Typhi* phage lacked the cytolethal distending toxin *cdt* genes and

278 the IS21 element found in phage fiAA91-ss (50). This prophage sequence had a mosaic

279 architecture, incorporating a number of putative insertions of phage tail fiber genes that were

280 not present in the fiAA91-ss reference genome (**Fig 5A**). Additionally, a single isolate of

281 genotype 4.1 obtained from Bangkok in 1973 contained a novel SfIV-like phage, here named  
282 STYP2, that lacked the serotype conversion gene *Gtr* cluster and *IS1* element of phage SfIV  
283 (51). Again, the novel Thai phage variant also encoded novel tail fiber genes not in the SfIV  
284 reference genome, as well as a Dam methylase gene (*orf37*) (**Fig 5B**)

## 286 Discussion

287 These data provide a historical insight into the population structure of *S. Typhi* in Thailand in  
288 1973 (pre-immunization program, n=11) and 1981-1992 (post- immunization program,  
289 n=33). It has been reported that the national *S. Typhi* immunization program in Thailand,  
290 which commenced in 1977, was highly effective in reducing the burden of typhoid fever (9).  
291 Our data are consistent with the hypothesis that the vaccine program successfully depleted the  
292 endemic *S. Typhi* population to the extent that most subsequent typhoid cases resulted from  
293 sporadic introduction of non-indigenous *S. Typhi*, rather than long-term persistence of the  
294 pre-vaccine era population. It is apparent that these introductions were sometimes  
295 accompanied by limited local transmissions, resulting in small, localized outbreaks, but we  
296 found no evidence to suggest that these result in the establishment of stable local source  
297 populations. Notably, the post-immunization *S. Typhi* isolates from Loi (in the north of  
298 Thailand near the border with Laos, from which it is separated by the Mekong river) were  
299 most closely related to Laos isolates, whilst those from the capital Bangkok and nearby  
300 Nonthaburi and Srakaew districts were closely related to other isolates from across Southeast  
301 Asia (**Fig 2**), suggesting there may have been multiple routes of import into Thailand.

303 Our study is limited by the sample of isolates available for analysis, which was small and  
304 reflects opportunistic sampling of sporadic local cases in the four sites and historical storage.  
305 However, it is notable that the Thai isolates cluster according to site, consistent with limited  
306 local transmission rather than dissemination of lineages between locations. The only  
307 exception to this was two genotype 3.1.2 isolates, which were collected from Srakaew in  
308 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) (33). Therefore, our genomic snapshot of the Thai *S. Typhi* population is consistent with previous insights and is likely reasonably representative for the study period.

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 (53), they are infrequent compared to the IncHI1 MDR plasmid and the cryptic plasmid pHCM2 (54). 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 (55, 56). 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 (57). 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 (58-61). 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

337 (62-64), thus the variation in these regions may be associated with recent adaptations to the *S.*  
 338 Typhi host. While genomic data from more recent *S. Typhi* collections shows limited  
 339 evidence for genetic exchange with other organisms (4), the detection amongst older Thai  
 340 isolates of both phage and plasmids that have been previously associated with *E. coli/Shigella*  
 341 suggests that genetic exchange may have been more common in the past or in certain  
 342 localized populations.

343

344 Overall, these data provide valuable historical insights into the *S. Typhi* populations  
 345 circulating in Thailand during the 1970s and 1980s, and early examples of the two most  
 346 common *S. Typhi* plasmids, as well as other mobile elements identified within the *S. Typhi*  
 347 population.

348

# 349 **Acknowledgements**

350 This project was funded by the Wellcome Trust of Great Britain (106158/Z/14/Z); SB is a Sir  
 351 Henry Dale Fellow, jointly funded by the Wellcome Trust and the Royal Society  
 352 (100087/Z/12/Z) and ZAD is funded by strategic award #106158. KEH is supported by  
 353 fellowship #1061409 from the NHMRC of Australia. DTP is a leadership fellow funded  
 354 through the Oak Foundation. The funders had no role in study design, data collection and  
 355 analysis, decision to publish, or preparation of the manuscript. The view expressed in this  
 356 article are those of the author(s) and do not reflect the official policy of the Department of the  
 357 Army, Department of Defense, or the US government.

358

# 359 **References**

- 360 1. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. N Engl J Med.  
 361 2002;347(22):1770-82.
- 362 2. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid Fever. Clin Infect  
 363 Dis. 2010;50(2):241-6.

- 364 3. Mogasale V, Maskery B, Ochiai RL, Lee JS, Mogasale VV, Ramani E, et al. Burden  
365 of typhoid fever in low-income and middle-income countries: a systematic, literature-  
366 based update with risk-factor adjustment. *Lancet Glob Health*.2014;2(10):e570-e80.
- 367 4. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, et al.  
368 Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella*  
369 Typhi identifies inter- and intracontinental transmission events. *Nat Genet*.  
370 2015;47(6):632-9.
- 371 5. Connor BA, Schwartz E. Typhoid and paratyphoid fever in travellers. *Lancet Infect*  
372 *Dis*. 2005;5(10):623-8.
- 373 6. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World*  
374 *Health Organ*. 2004;82(5):346-53.
- 375 7. Sur D, von Seidlein L, Manna B, Dutta S, Deb AK, Sarkar BL, et al. The malaria and  
376 typhoid fever burden in the slums of Kolkata, India: data from a prospective community-  
377 based study. *Trans R Soc Trop Med Hyg*. 2006;100(8):725-33.
- 378 8. Karkey A, Arjyal A, Anders KL, Boni MF, Dongol S, Koirala S, et al. The Burden  
379 and Characteristics of Enteric Fever at a Healthcare Facility in a Densely Populated Area  
380 of Kathmandu. *PLoS ONE*. 2010;5(11):e13988.
- 381 9. Bodhidatta L, Taylor DN, Thisyakom U, Echeverria P. Control of Typhoid Fever in  
382 Bangkok, Thailand, by Annual Immunization of Schoolchildren with Parenteral Typhoid  
383 Vaccine. *Rev Infect Dis*. 1987;9(4):841-5.
- 384 10. Fraser A, Paul M, Goldberg E, Acosta CJ, Leibovici L. Typhoid fever vaccines:  
385 systematic review and meta-analysis of randomised controlled trials. *Vaccine*.  
386 2007;25(45):7848-57.

387 11. Khan MI, Ochialy RL, Clemens JD. Population impact of Vi capsular polysaccharide  
388 vaccine. *Expert Rev Vaccines*. 2010;9(5):485-96.

389 12. Bhutta ZA. Current concepts in the diagnosis and treatment of typhoid fever. *BMJ*.  
390 2006;333(7558):78-82.

391 13. Chau TT, Campbell JI, Galindo CM, Van Minh Hoang N, Diep TS, Nga TTT, et al.  
392 Antimicrobial Drug Resistance of *Salmonella enterica* Serovar Typhi in Asia and  
393 Molecular Mechanism of Reduced Susceptibility to the Fluoroquinolones. *Antimicrob*  
394 *Agents Chemother*. 2007;51(12):4315-23.

395 14. Kariuki S, Revathi G, Muyodi J, Mwituria J, Munyalo A, Mirza S, et al.  
396 Characterization of Multidrug-Resistant Typhoid Outbreaks in Kenya. *J Clin Microbiol*.  
397 2004;42(4):1477-82.

398 15. Holt KE, Phan MD, Baker S, Duy PT, Nga TVT, Nair S, et al. Emergence of a  
399 Globally Dominant IncHI1 Plasmid Type Associated with Multiple Drug Resistant  
400 Typhoid. *PLoS Negl Trop Dis*. 2011;5(7):e1245.

401 16. Klugman K, Koornhof H, Schneerson R, Cadoz M, Gilbertson I, Robbins J, et al.  
402 Protective activity of Vi capsular polysaccharide vaccine against typhoid fever. *Lancet*.  
403 1987;330(8569):1165-9.

404 17. Levine M, Black R, Ferreccio C, Germanier R. Large-scale field trial of Ty21A live  
405 oral typhoid vaccine in enteric-coated capsule formulation. *Lancet*. 1987;329(8541):1049-  
406 52.

407 18. Ashcroft MT, Singh B, Nicholson CC, Ritchie JM, Sobryan E, Williams F. A seven-  
408 year field trial of two typhoid vaccines in Guyana. *Lancet*. 1967;290(7525):1056-9.

409 19. Waddington CS, Darton TC, Jones C, Haworth K, Peters A, John T, et al. An  
410 Outpatient, Ambulant-Design, Controlled Human Infection Model Using Escalating



411 Doses of *Salmonella* Typhi Challenge Delivered in Sodium Bicarbonate Solution. Clin  
412 Infect Dis. 2014;58(9):1230-40.

413 20. Scobie HM, Nilles E, Kama M, Kool JL, Mintz E, Wannemuehler KA, et al. Impact  
414 of a targeted typhoid vaccination campaign following cyclone Tomas, Republic of Fiji,  
415 2010. Am J Trop Med Hyg. 2014;90(6):1031-8.

416 21. Thompson CN, Kama M, Acharya S, Bera U, Clemens J, Crump JA, et al. Typhoid  
417 fever in Fiji: a reversible plague? Trop Med Int Health. 2014;19(10):1284-92.

418 22. Whitaker JA, Franco-Paredes C, del Rio C, Edupuganti S. Rethinking typhoid fever  
419 vaccines: implications for travelers and people living in highly endemic areas. J Travel  
420 Med. 2009;16(1):46-52.

421 23. DeRoeck D, Ochiai RL, Yang J, Anh DD, Alag V, Clemens JD. Typhoid vaccination:  
422 the Asian experience. Expert Rev Vaccines. 2008;7(5):547-60.

423 24. Guzman CA, Borsutzky S, Griot-Wenk M, Metcalfe IC, Pearman J, Collioud A, et al.  
424 Vaccines against typhoid fever. Vaccine. 2006;24(18):3804-11.

425 25. Ochiai RL, Acosta CJ, Agtini M, Bhattacharya SK, Bhutta ZA, Do CG, et al. The Use  
426 of Typhoid Vaccines in Asia: The DOMI Experience. Clin Infect Dis.  
427 2007;45(Supplement 1):S34-S8.

428 26. CLSI. Performance standards for antimicrobial disk susceptibility tests. Tentative  
429 standard. CLSI document M02. Wayne, Pa: CLSI; 1979.

430 27. CLSI. Performance standards for antimicrobial disk susceptibility tests. Approved  
431 standard. CLSI document M02. Wayne, Pa: CLSI; 1984.

432 28. CLSI. Performance standards for antimicrobial disk susceptibility tests. Tentative  
433 standard. CLSI document M02-A4. Wayne, Pa: CLSI; 1988.

434 29. CLSI. Performance standards for antimicrobial disk susceptibility tests. Approved  
435 standard. CLSI document M02-A4. Wayne, Pa: CLSI; 1990.

436 30. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, et al. Complete  
437 genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18.  
438 Nature. 2001;413(6858):848-52.

439 31. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Meth.  
440 2012;9(4):357-9.

441 32. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N. The sequence  
442 alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.

443 33. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended  
444 genotyping framework for *Salmonella enterica* serovar Typhi, the cause of human  
445 typhoid. Nat Commun. 2016, *in press*.

446 34. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill F-X, Goodhead I, et al. High-  
447 throughput sequencing provides insights into genome variation and evolution in  
448 *Salmonella* Typhi. Nat Genet. 2008;40(8):987-93.

449 35. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, et al. Rapid  
450 phylogenetic analysis of large samples of recombinant bacterial whole genome sequences  
451 using Gubbins. Nucleic Acids Res. 2014.

452 36. Holt K, Thomson N, Wain J, Langridge G, Hasan R, Bhutta Z, et al. Pseudogene  
453 accumulation in the evolutionary histories of *Salmonella enterica* serovars Paratyphi A  
454 and Typhi. BMC genomics. 2009;10(1):36.

455 37. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses  
456 with thousands of taxa and mixed models. Bioinformatics. 2006;22(21):2688-90.

457 38. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R  
458 language. *Bioinformatics*. 2004;20(2):289-90.

459 39. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et  
460 al. ARG-ANNOT, a New Bioinformatic Tool To Discover Antibiotic Resistance Genes in  
461 Bacterial Genomes. *Antimicrob Agents Chemother*. 2014;58(1):212-20.

462 40. Inouye M, Dashnow H, Raven L-A, Schultz M, Pope B, Tomita T, et al. SRST2:  
463 Rapid genomic surveillance for public health and hospital microbiology labs. *Genome*  
464 *Med*. 2014;6(11):90.

465 41. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of  
466 plasmids by PCR-based replicon typing. *J Microbiol Meth*. 2005;63(3):219-28.

467 42. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al.  
468 SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell  
469 Sequencing. *J Comput Biol*. 2012;19(5):455-77.

470 43. Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualisation of de  
471 novo genome assemblies. *Bioinformatics*. 2015.

472 44. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*. 2014.

473 45. Phan M-D, Kidgell C, Nair S, Holt KE, Turner AK, Hinds J, et al. Variation in  
474 *Salmonella enterica* Serovar Typhi IncHI1 Plasmids during the Global Spread of  
475 Resistant Typhoid Fever. *Antimicrob Agents Chemother*. 2009;53(2):716-27.

476 46. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at  
477 the population level. *BMC Bioinformatics*. 2010;11(1):1-11.

478 47. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phage search  
479 tool. *Nucleic Acids Res*. 2011;39:W347-52.

480 48. Guy L, Roat Kulima J, Andersson SGE. genoPlotR: comparative gene and genome  
481 visualization in R. *Bioinformatics*. 2010;26(18):2334-5.

482 49. Kidgell C, Pickard D, Wain J, James K, Diem Nga LT, Diep TS, et al.  
483 Characterisation and distribution of a cryptic *Salmonella* Typhi plasmid pHCM2.  
484 *Plasmid*. 2002;47(3):159-71.

485 50. Allue-Guardia A, Imamovic L, Muniesa M. Evolution of a self-inducible cytolethal  
486 distending toxin type V-encoding bacteriophage from *Escherichia coli* O157:H7 to  
487 *Shigella sonnei*. *J Virol*. 2013;87(24):13665-75.

488 51. Jakhetia R, Talukder K, Verma N. Isolation, characterization and comparative  
489 genomics of bacteriophage SfIV: a novel serotype converting phage from *Shigella*  
490 *flexneri*. *BMC Genomics*. 2013;14(1):677.

491 52. Roumagnac P, Weill F-X, Dolecek C, Baker S, Brisse S, Chinh NT, et al.  
492 Evolutionary History of *Salmonella* Typhi. *Science*. 2006;314(5803):1301-4.

493 53. Holt KE, Perkins TT, Dougan G, Kingsley RA. Genomics and pathogenesis of  
494 *Salmonella enterica* serovars Typhi and Paratyphi A. *Salmonella*: From genome to  
495 function. 2011:107-21.

496 54. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill F-X, Goodhead I. High-  
497 throughput sequencing provides insights into genome variation and evolution in  
498 *Salmonella* Typhi. *Nat Genet*. 2008;40.

499 55. Rychlik I, Gregorova D, Hradecka H. Distribution and function of plasmids in  
500 *Salmonella enterica*. *Vet Microbiol*. 2006;112(1):1-10.

501 56. Murray BE, Levine MM, Cordano AM, D'Ottone K, Jayanetra P, Kopecko D, et al.  
502 Survey of plasmids in *Salmonella* Typhi from Chile and Thailand. *J Infect Dis*.  
503 1985;151(3):551-5.

504 57. Jørgensen TS, Xu Z, Hansen MA, Sørensen SrJ, Hansen LH. Hundreds of Circular  
505 Novel Plasmids and DNA Elements Identified in a Rat Cecum Metamobilome. PLoS  
506 ONE. 2014;9(2):e87924.

507 58. Buckling A, Rainey PB. Antagonistic coevolution between a bacterium and a  
508 bacteriophage. P Roy Soc B-Biol Sci. 2002;269(1494):931-6.

509 59. Koskella B, Brockhurst MA. Bacteria-phage coevolution as a driver of ecological and  
510 evolutionary processes in microbial communities. FEMS Microbiol Rev. 2014;38(5):916-  
511 31.

512 60. Lenski R. Coevolution of Bacteria and Phage: Are There Endless Cycles of Bacterial  
513 Defenses and Phage Counterdefenses? J Theor Biol. 1984;108:319-25.

514 61. Schrag SJ, Mittler JE. Host-parasite coexistence: The role of spatial refuges in  
515 stabilizing bacteria-phage interactions. Am Nat. 1996;148(2):348-77.

516 62. Kutter E, Raya R, Carlson K. Molecular mechanisms of phage infection. In: Kutter E,  
517 Sulakvelidze A, editors. Bacteriophages Biology and Applications. Florida: CRC Press;  
518 2005.

519 63. Guttman B, Raya R, Kutter E. Chapter 3 - Basic Phage Biology. In: Kutter E,  
520 Sulakvelidze A, editors. Bacteriophages - Biology and Applications. New York: CRC  
521 Press; 2005. p. 29-66.

522 64. Thomson N, Baker S, Pickard D, Fookes M, Anjum M, Hamlin N, et al. The role of  
523 prophage-like elements in the diversity of *Salmonella enterica* serovars. J Mol Biol.  
524 2004;339(2):279-300.

525

526

527 **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 Modification)
042						
049						
036	3.2.1	pTy036_01	HI1	~215 kbp	N/A.	AMR ( <i>sulI</i> , <i>catA1</i> , <i>tet(B)</i> , <i>aadA1</i> )
046						
051						
052						
054	3.1.2	Prophage STYP1	<i>attL</i> CAAGCTGGTCAG	28,946 bp (39)	KX833211	Cryptic
055			<i>attR</i> CAAGCTGGTCAG			

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

528

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

530 (A) Maximum likelihood phylogenetic tree (outgroup rooted). Strains are labelled with their  
531 three digit name code, year of isolation (pink shading indicates post-vaccine isolates); source  
532 location (shaded by city, as indicated in panel B); and plasmid content (any antibiotic  
533 resistance genes are indicated in italics). Branch lengths are indicative of the number of SNPs.  
534 (B) Locations from which S. Typhi were isolated in Thailand. (C) Total number of positive  
535 blood cultures of S. Typhi (black) and Paratyphi A (grey) between 1970 and 1985;  
536 immunization period is indicated in pink; reproduced using data from reference (9).

537  
538 **Figure 2. Zoomed in phylogenies showing relationships of Thai S. Typhi to global**

539 **isolates.** Midpoint rooted ML trees including S. Typhi isolates from the Thai and global  
540 collections are shown, for each genotype that was observed amongst the Thai isolates.  
541 Colored branches and nodes indicate country of origin, according to the inset legend. Year of  
542 isolation is shown to the left; pink and red, Thai isolates obtained before and after the  
543 introduction of the immunization program; grey and black, non-Thai isolates obtained before  
544 and after the introduction of the immunization program. Thai isolates are also labelled to  
545 indicate their city of origin: L, Loi; B, Bangkok; S, Srakaew; N, Nonthaburi. SNP distances  
546 between isolates as well as AMR plasmids are labelled, with any resistance genes indicated in  
547 italics. Branch lengths are indicative of the number of SNPs.

548  
549 **Figure 3. SNP distances for Thai and global collection isolates.**

550 SNP distance between post-vaccine Thai isolates and their closest pre-vaccine Thai and post-  
551 vaccine global collection relatives, colored points indicate country of origin.

552  
553 **Figure 4. Blast comparison of novel plasmid pTy004\_01 with pHCM2 (AL513383).**

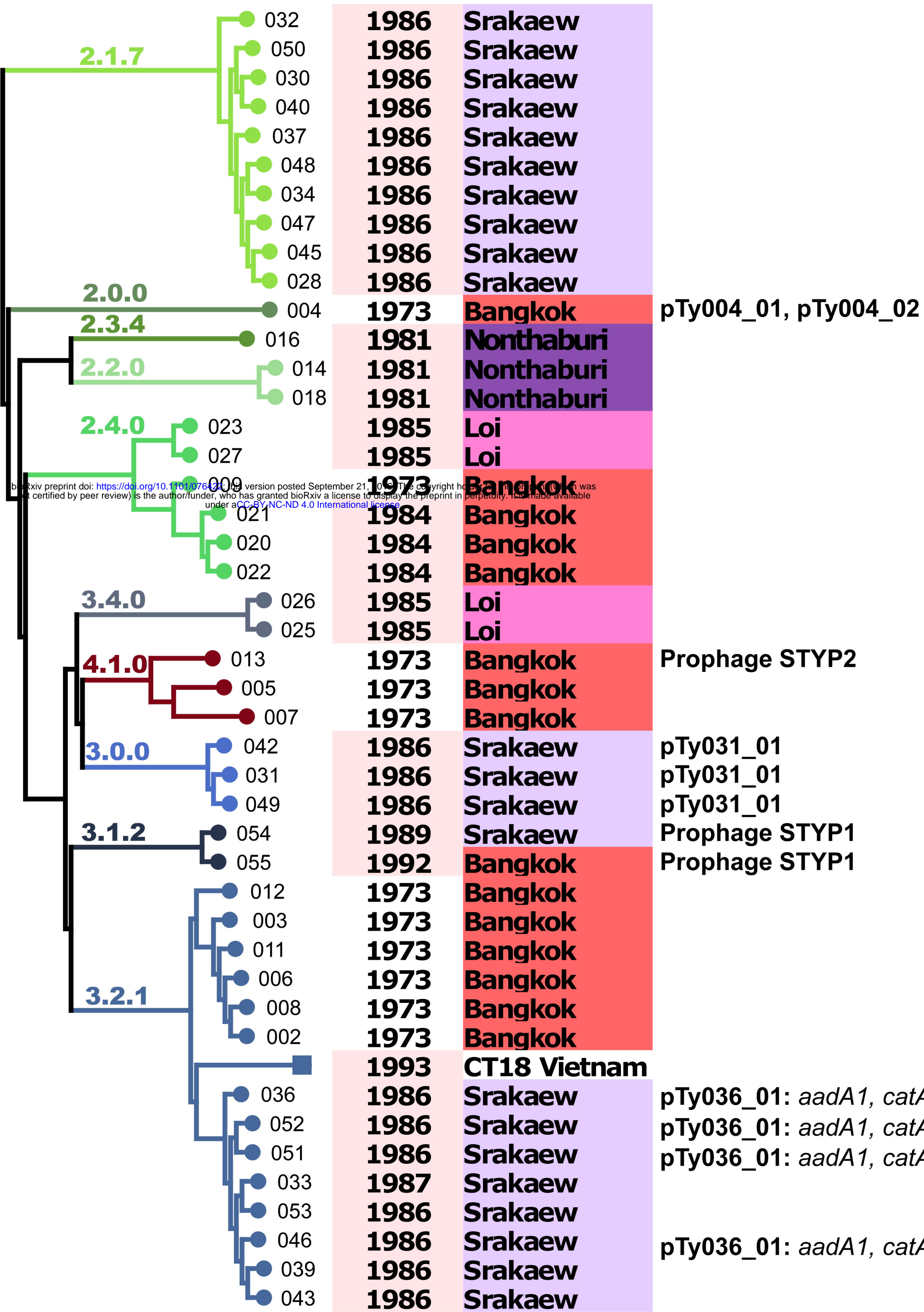
554 Shaded regions indicate areas of sequence homology, intensity of shading indicates relative  
555 nucleotide similarity. Arrows represent protein coding genes, direction indicates coding  
556 strand.



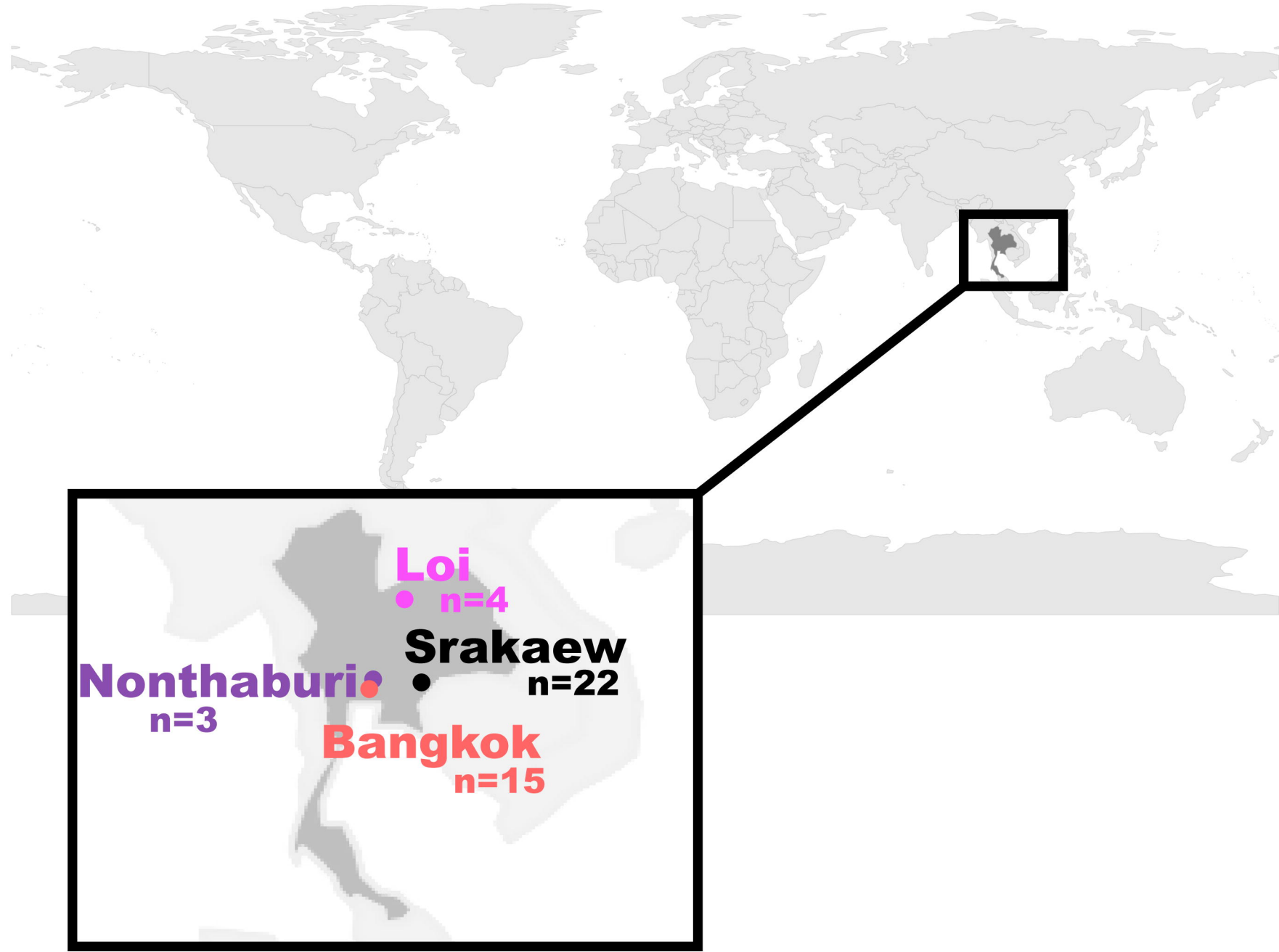
557 **Figure 5. Blast comparison of novel phages observed in Thai S. Typhi isolates to nearest**  
 558 **known phage sequences.**

559 (A) Novel phage STYP1 compared to Shigella sonnei phage fiAA91-ss (NC\_022750). (B)  
 560 Novel phage STYP2 compared to Shigella flexneri phage SfIV (NC\_022749). Shaded regions  
 561 indicate areas of sequence homology, intensity of shading indicates relative nucleotide  
 562 similarity. Arrows represent protein coding genes (direction indicates coding strand), colored  
 563 by encoded protein functions: red, DNA packaging module; orange, virion morphogenesis  
 564 module; yellow, cargo genes; blue, DNA replication and lysogenic cycle maintenance; green,  
 565 lysis module.

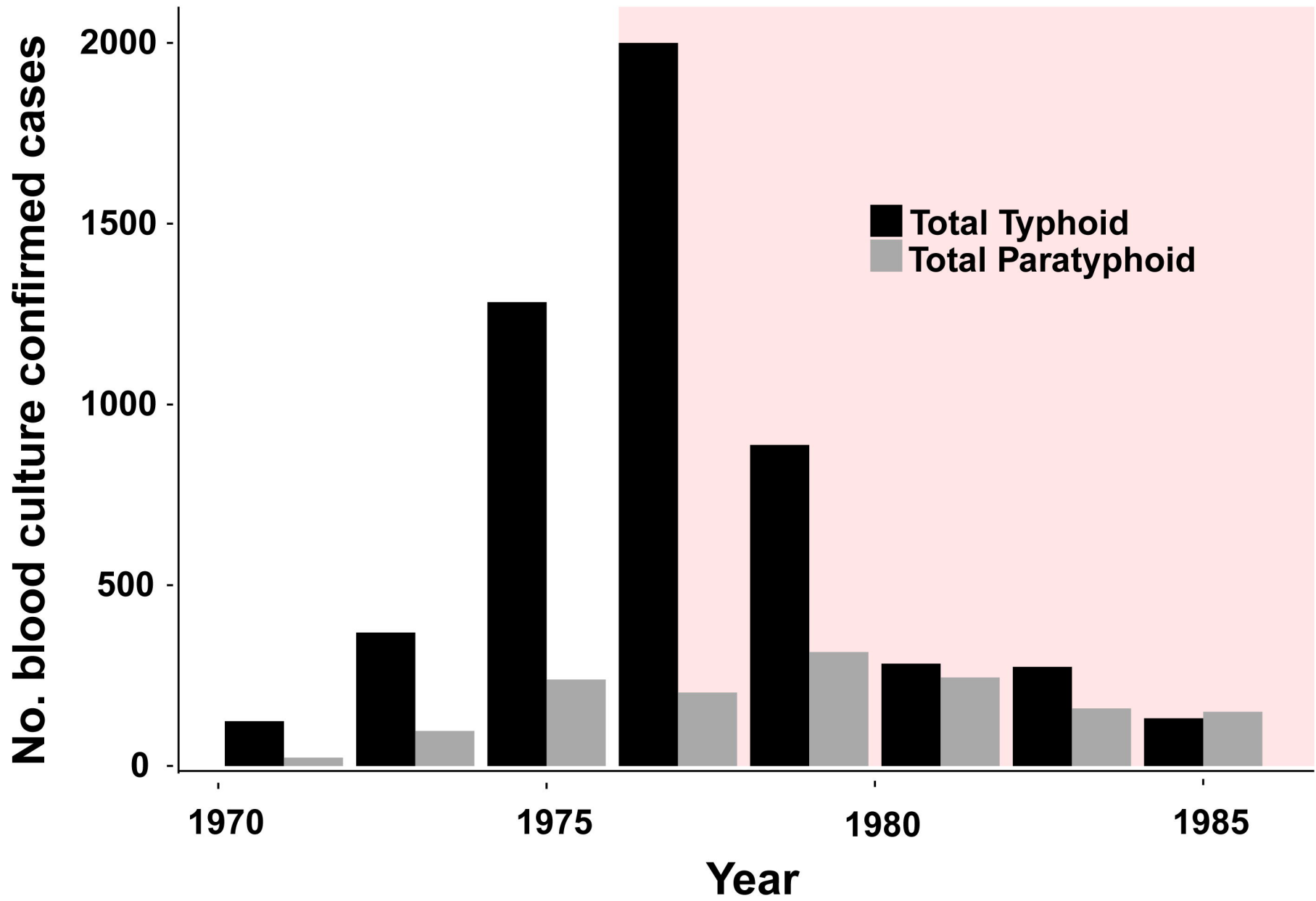
**A**



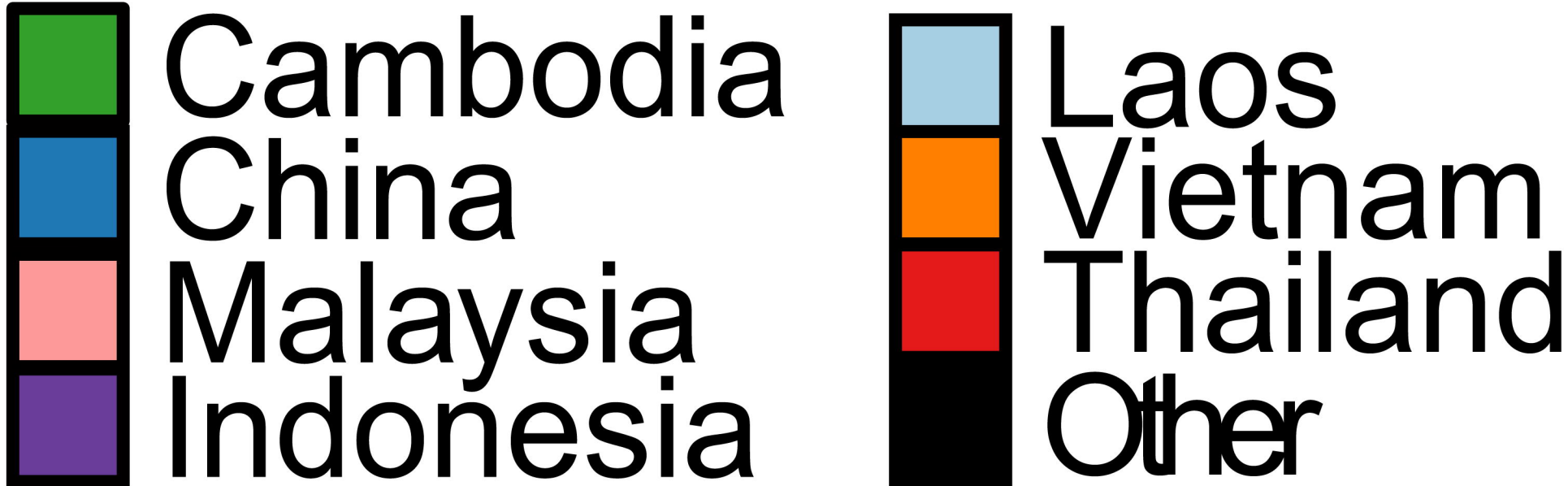
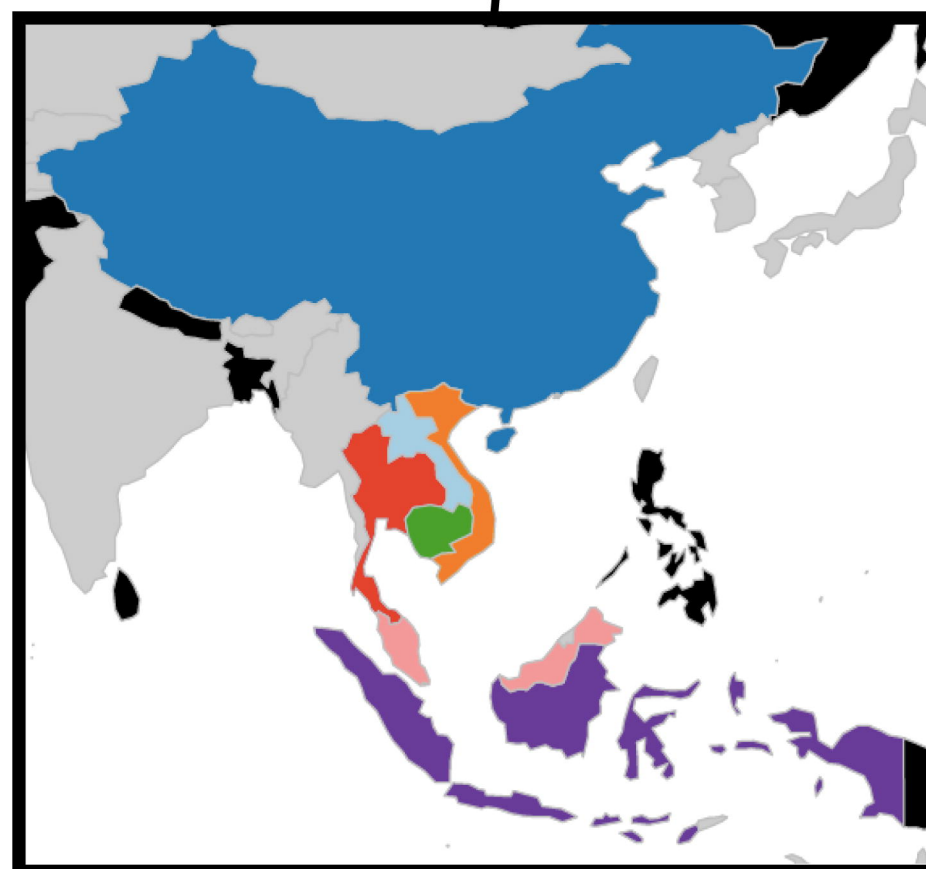
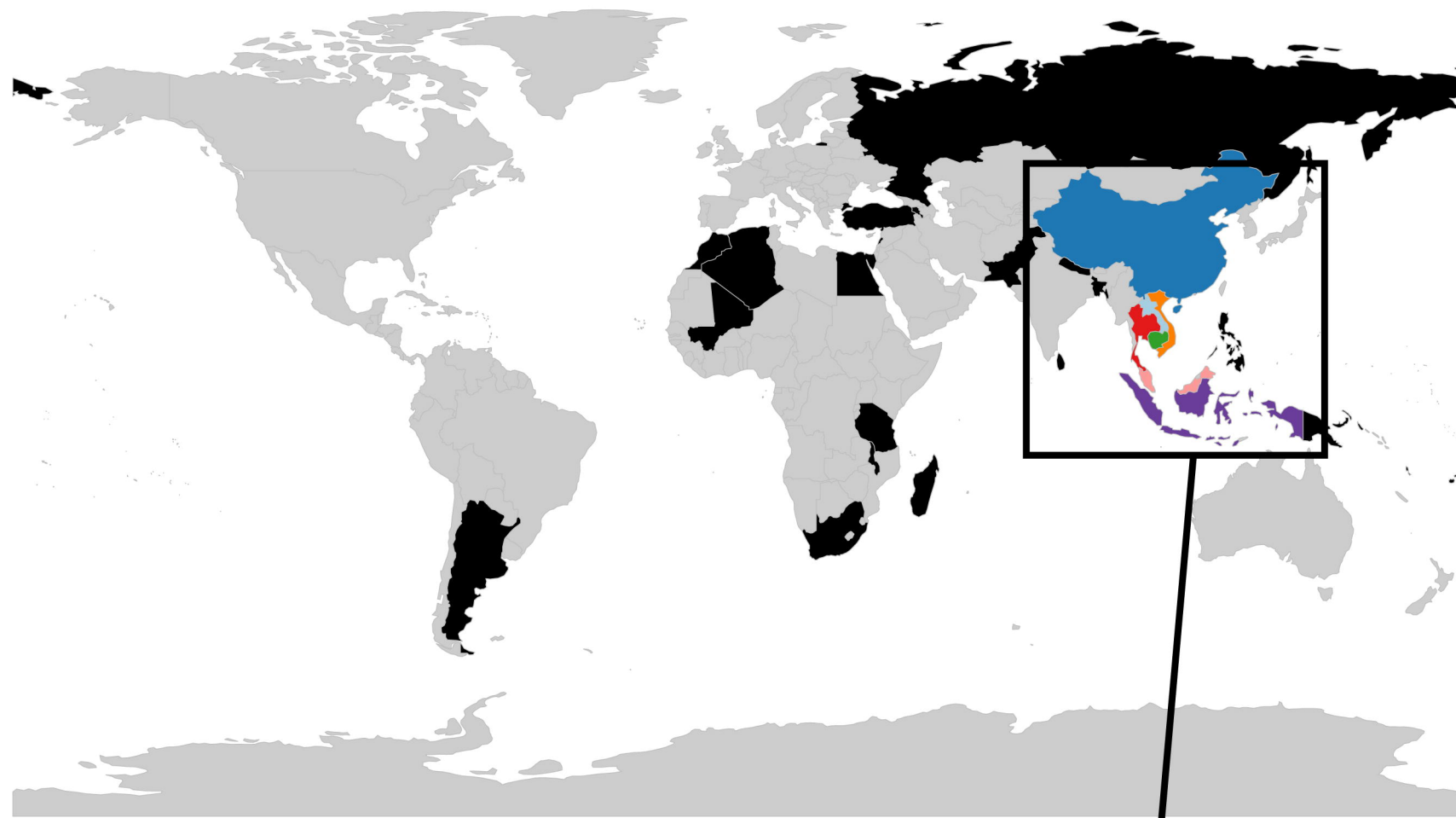
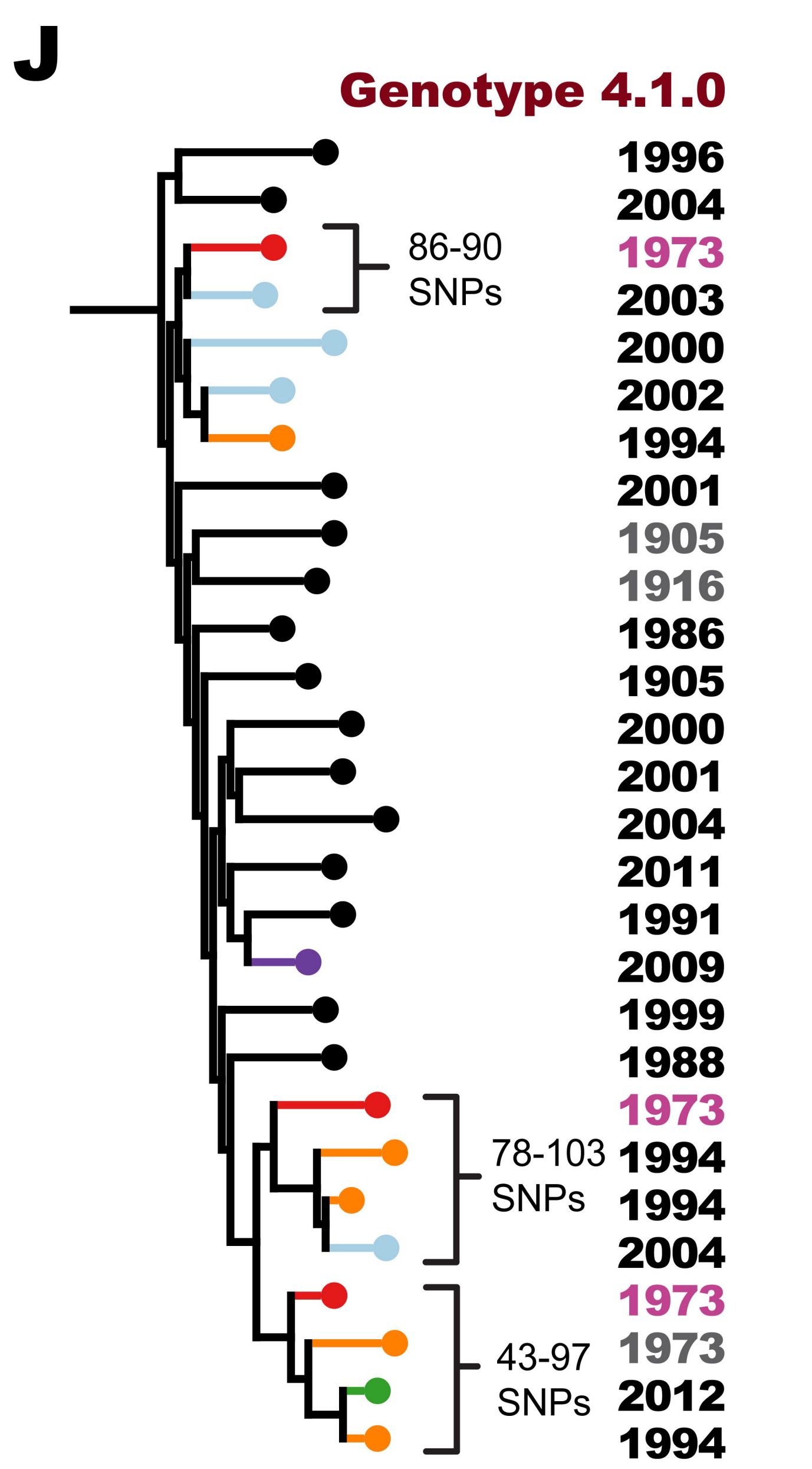
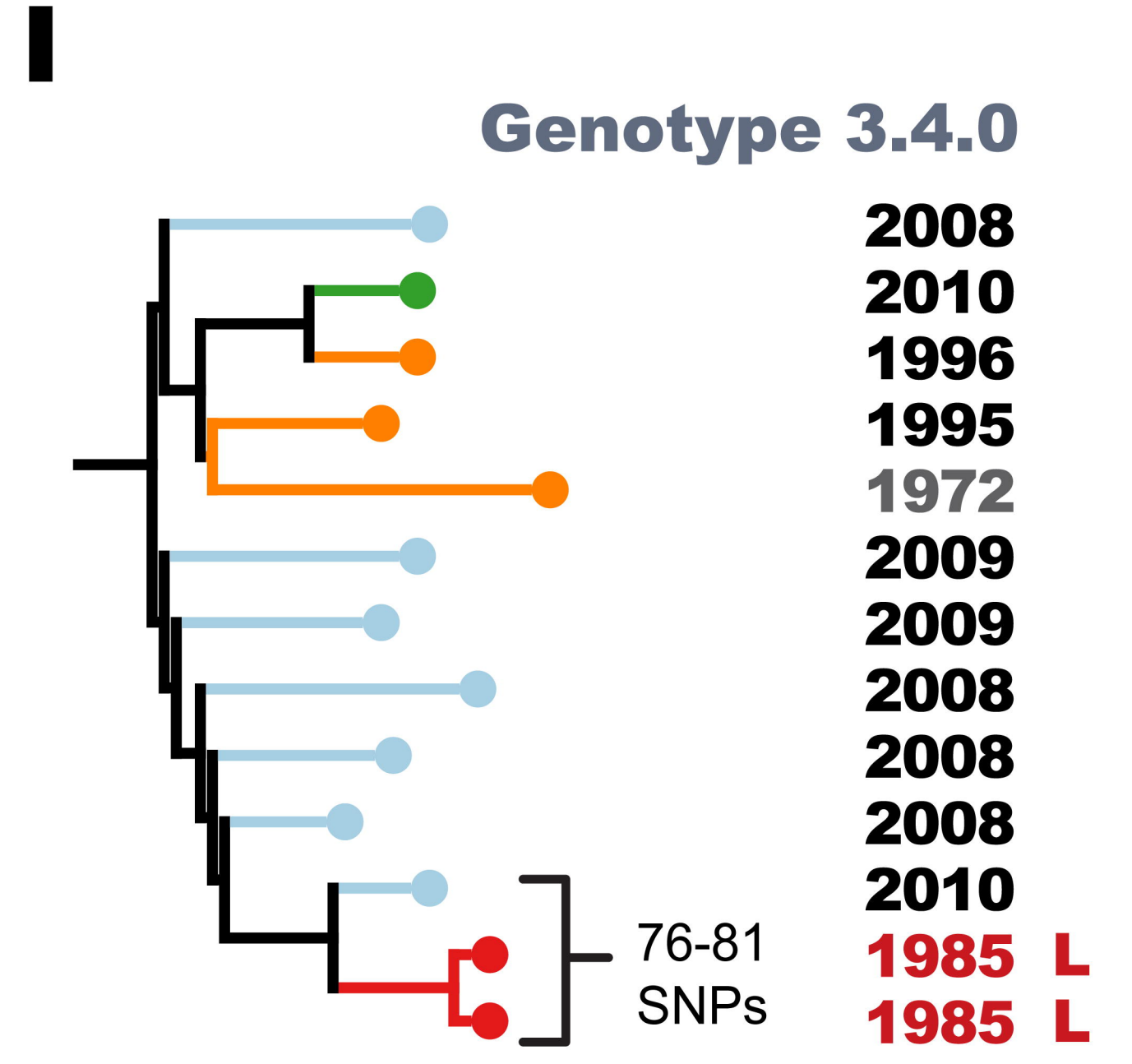
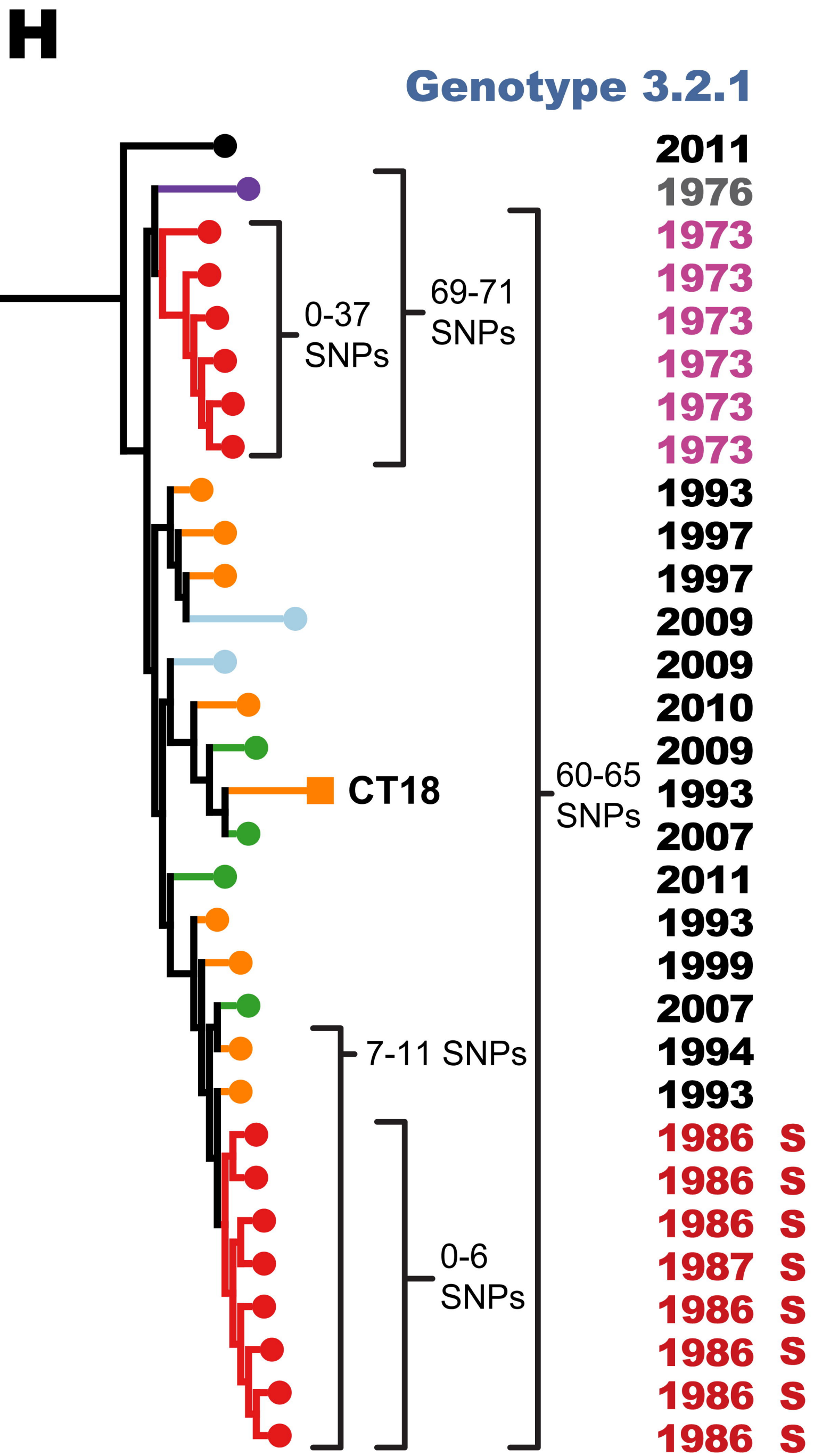
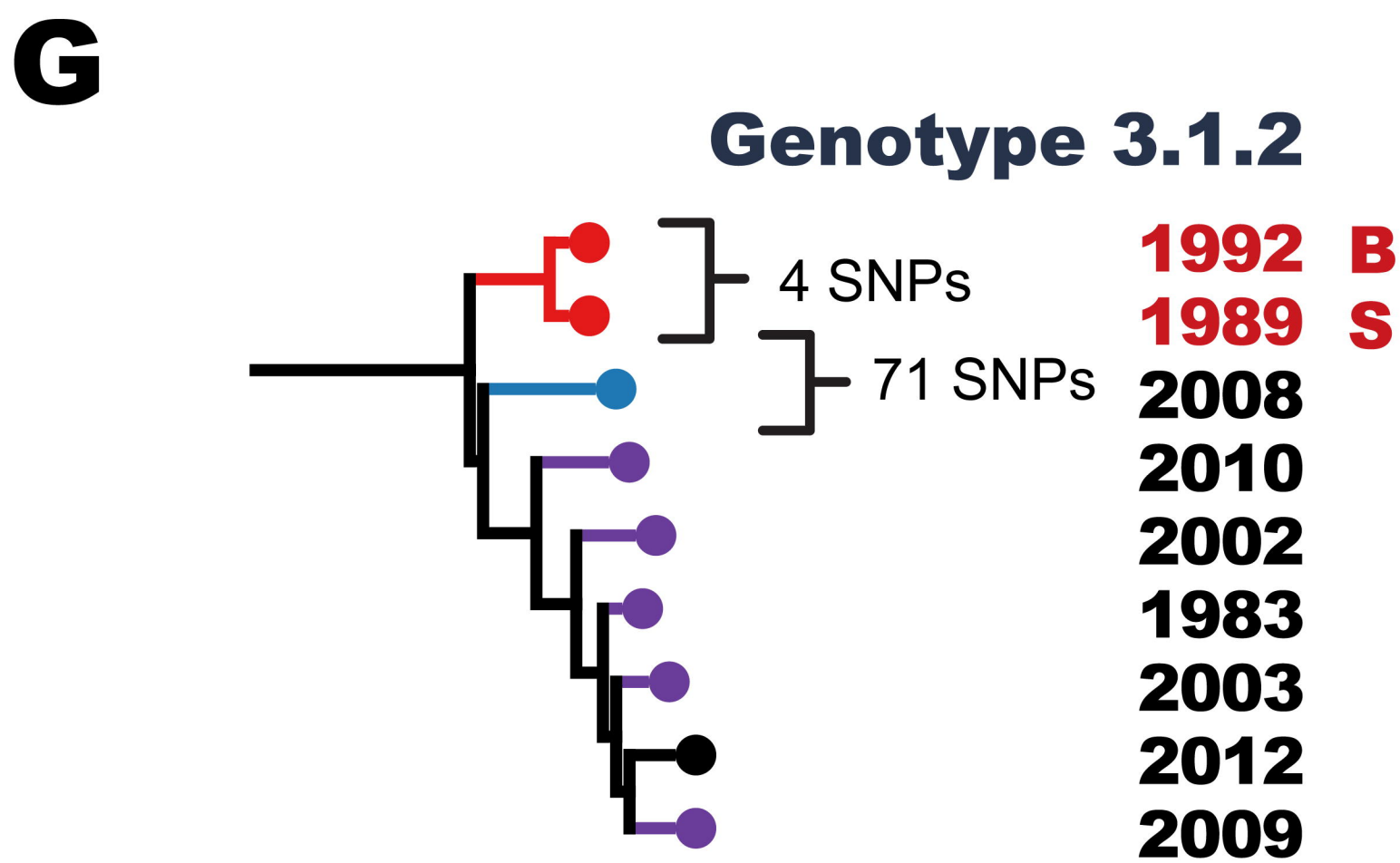
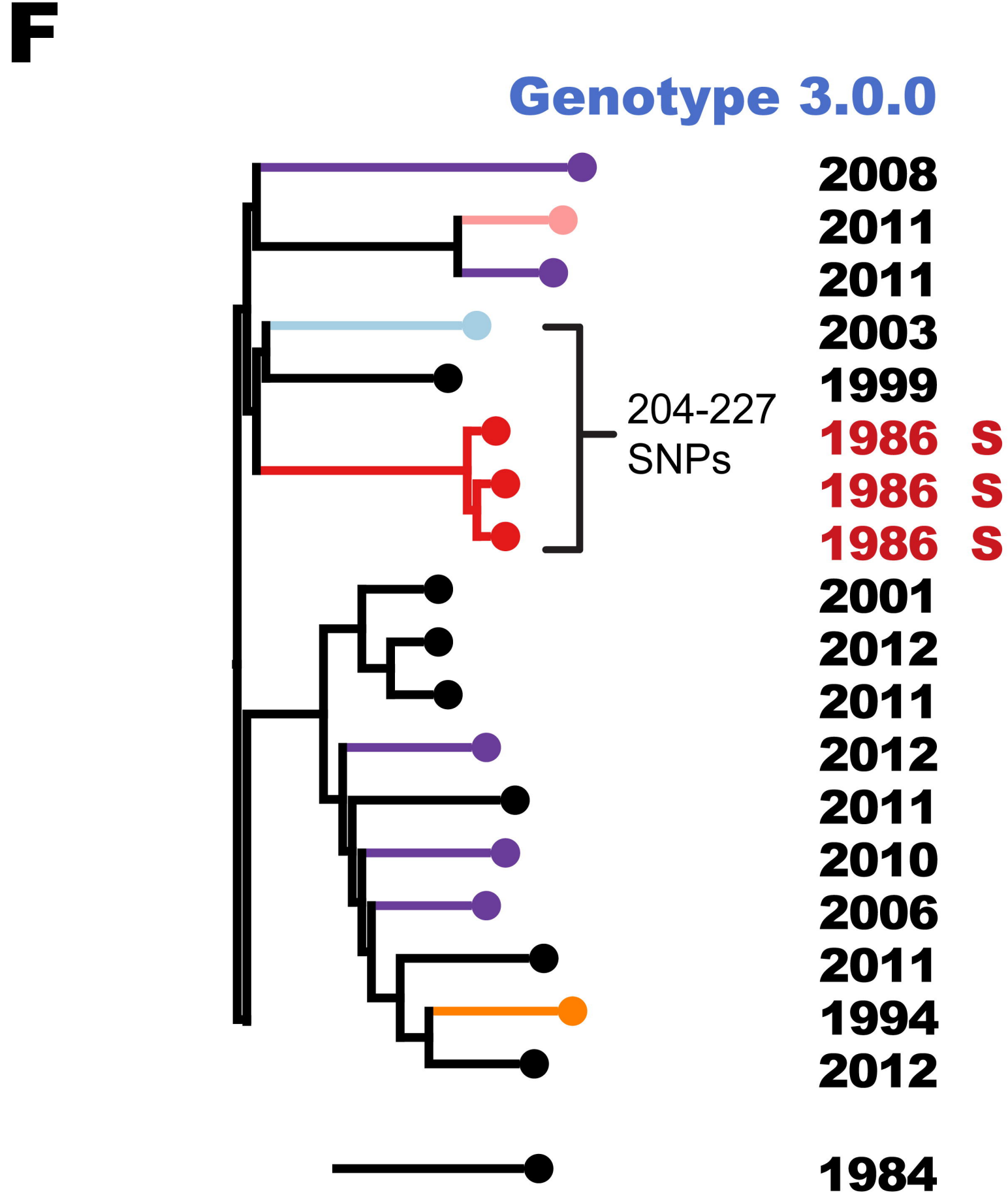
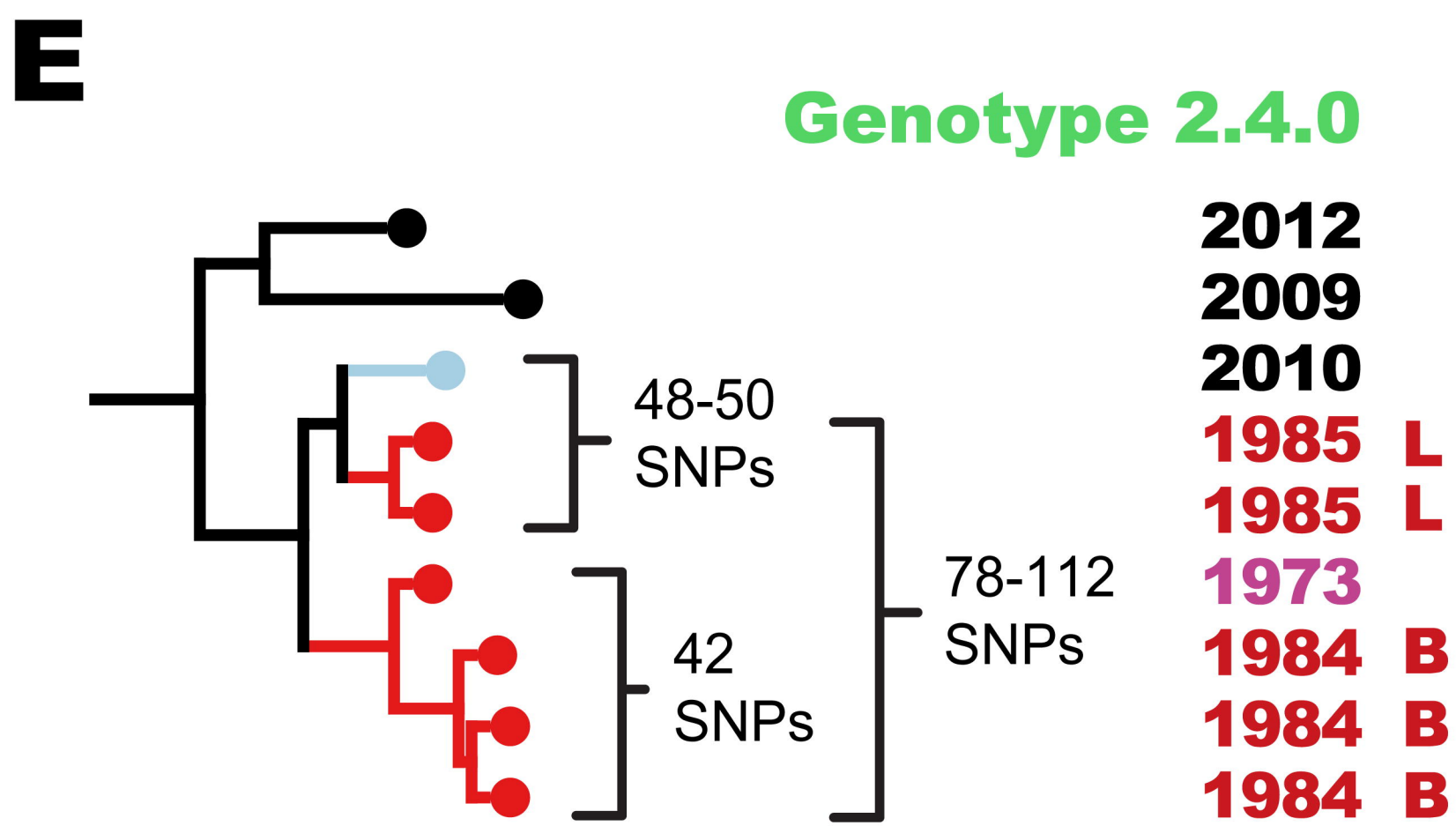
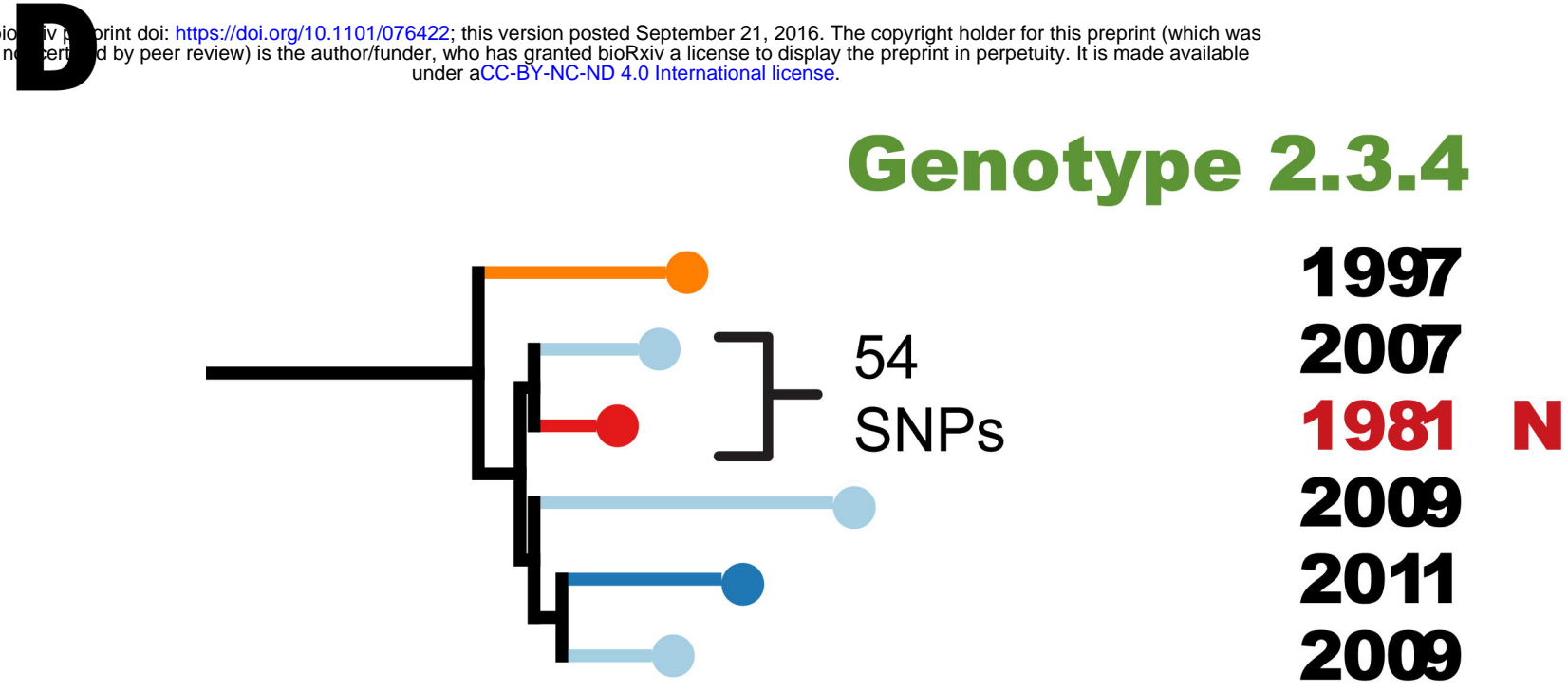
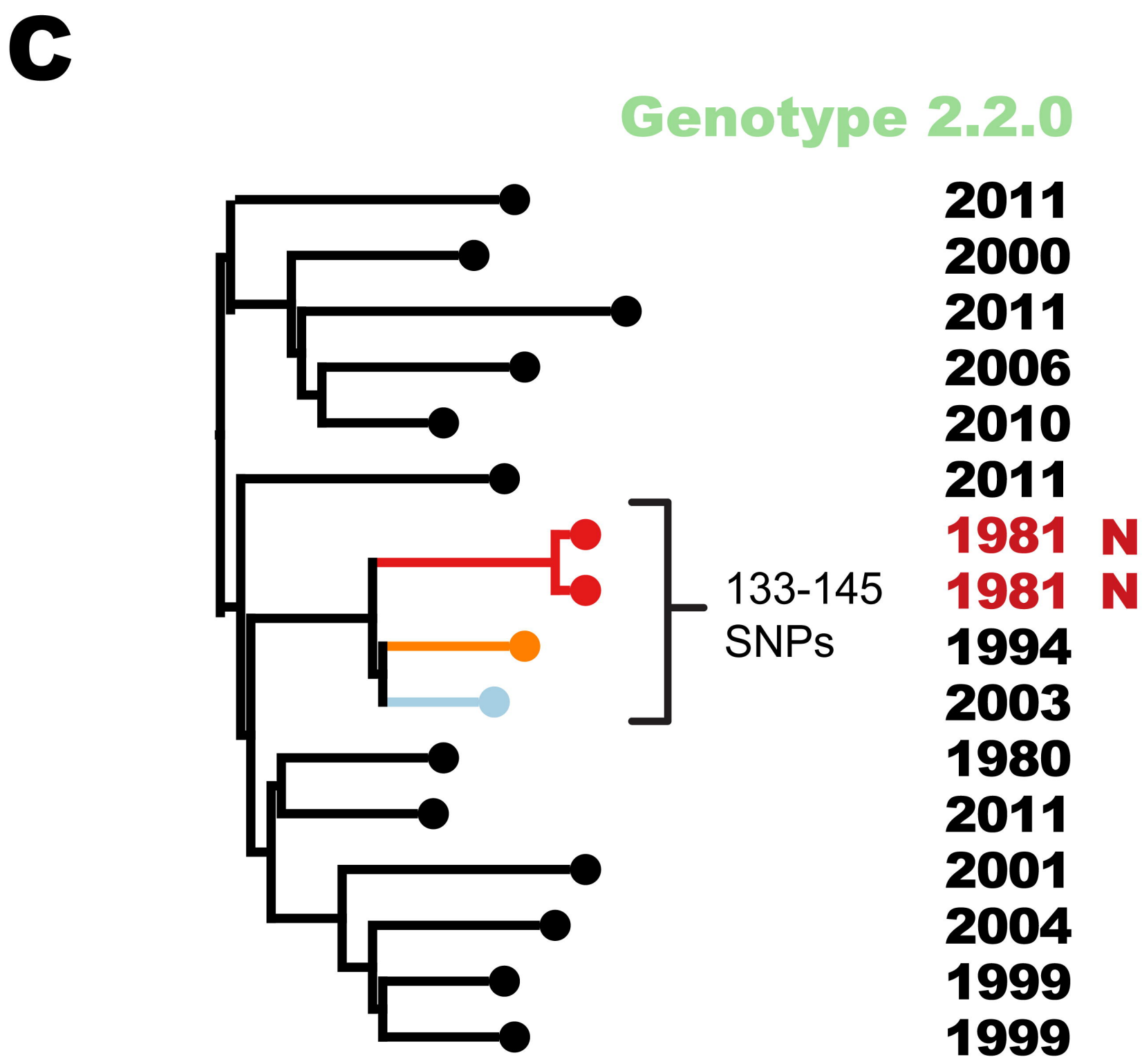
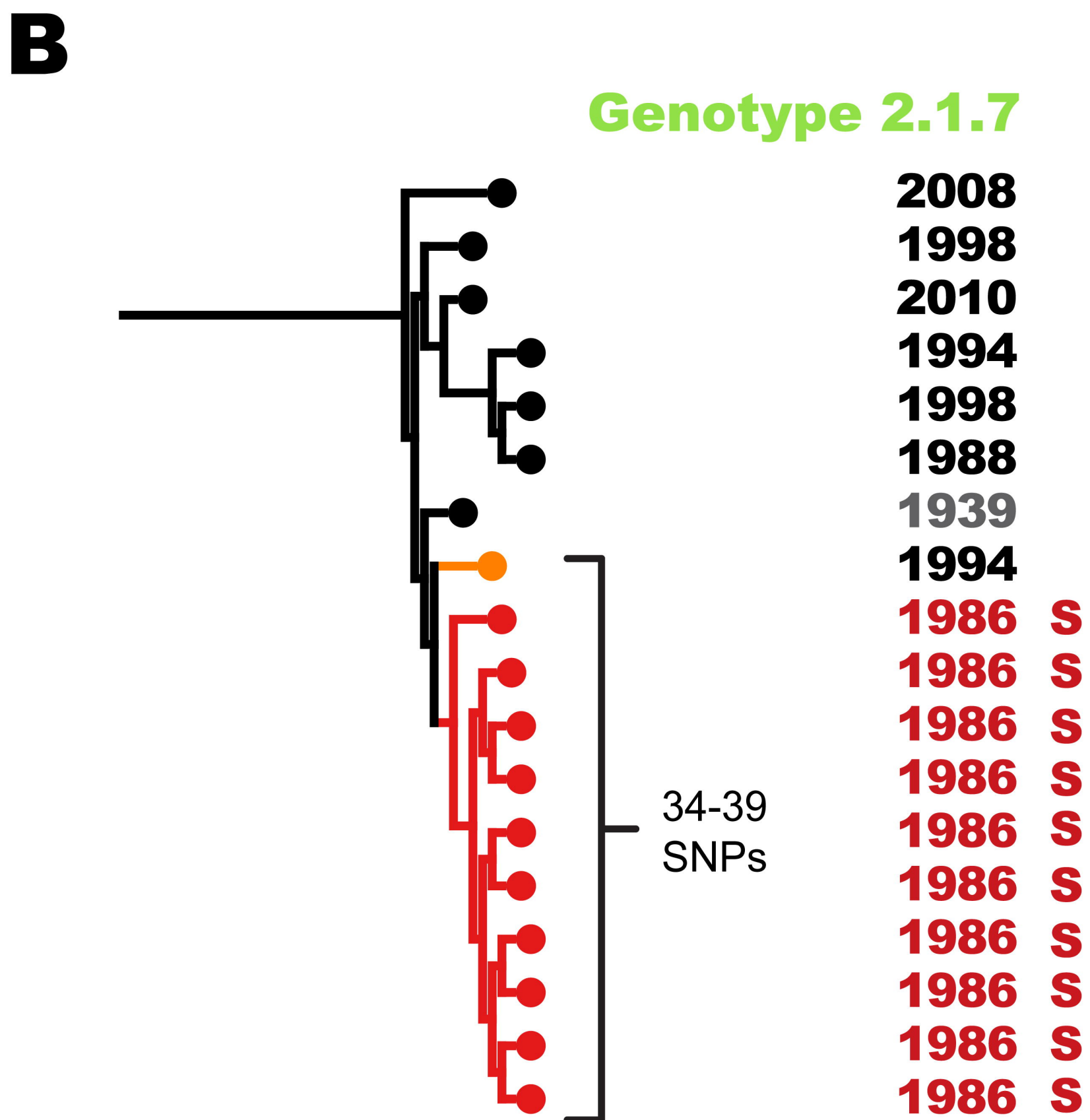
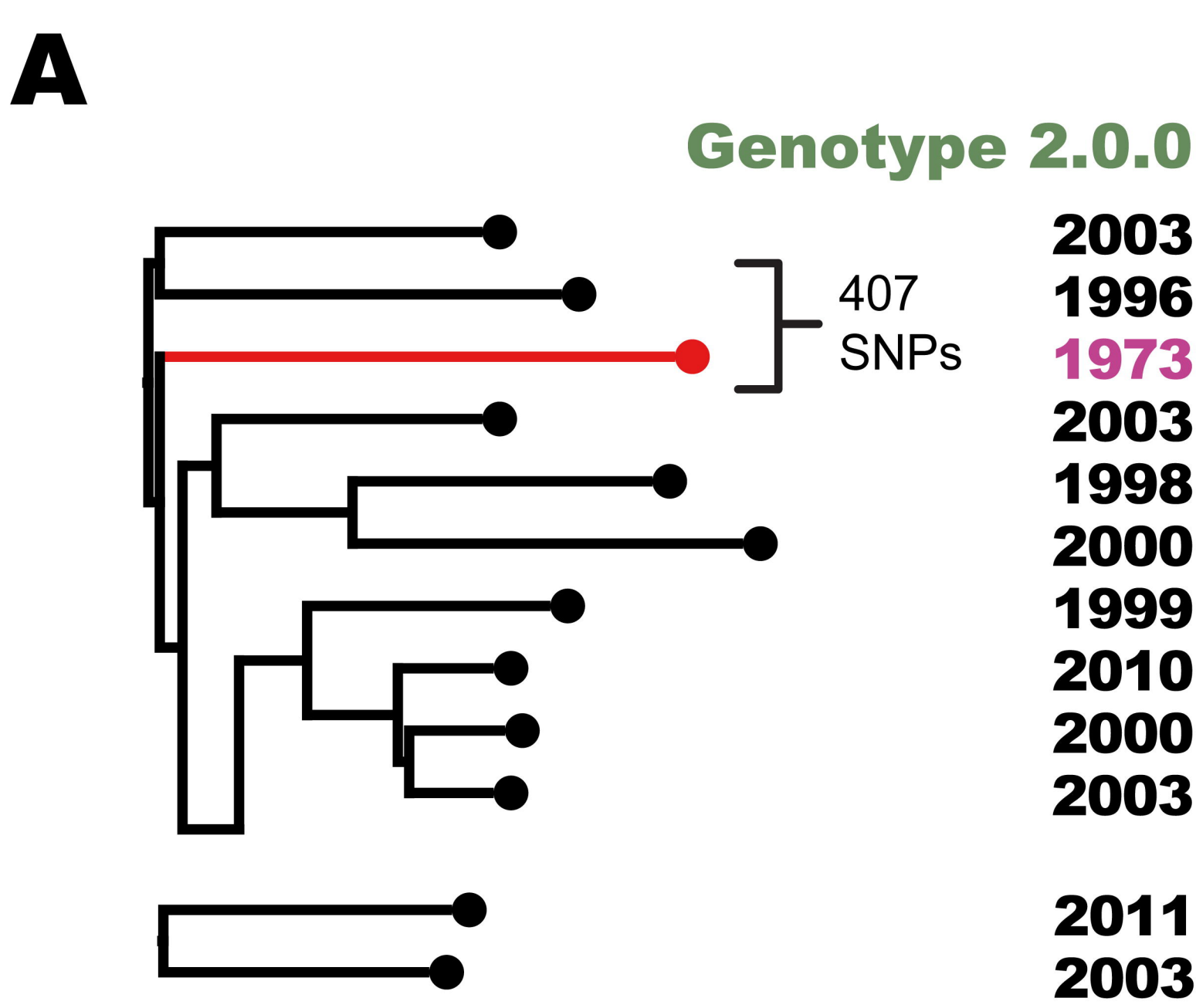
**B**



**C**

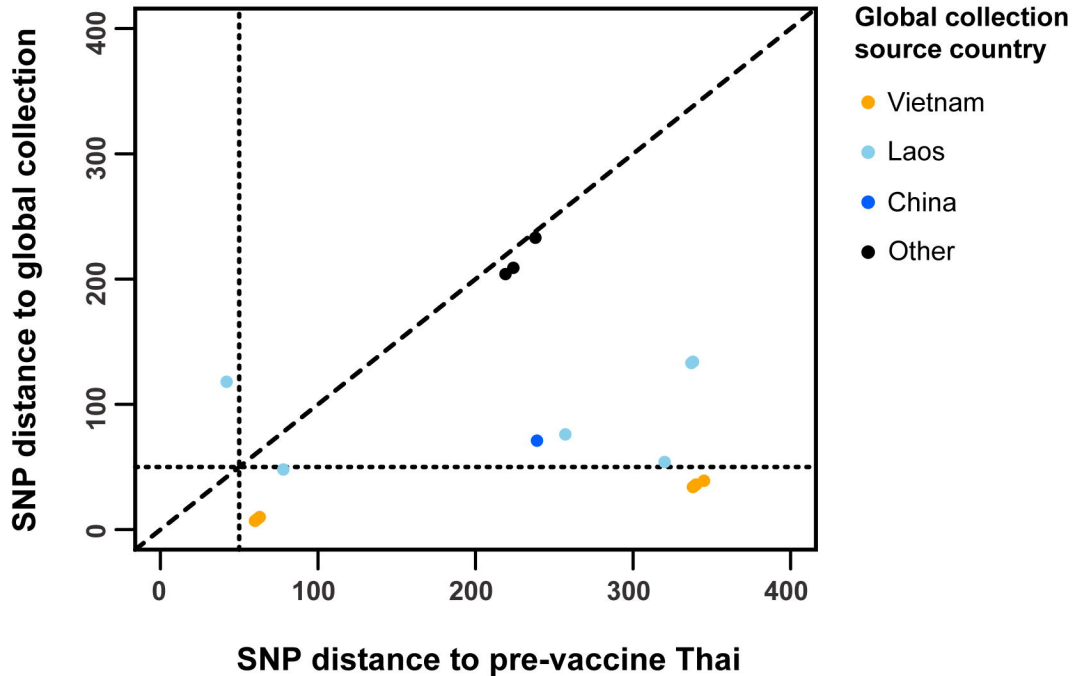


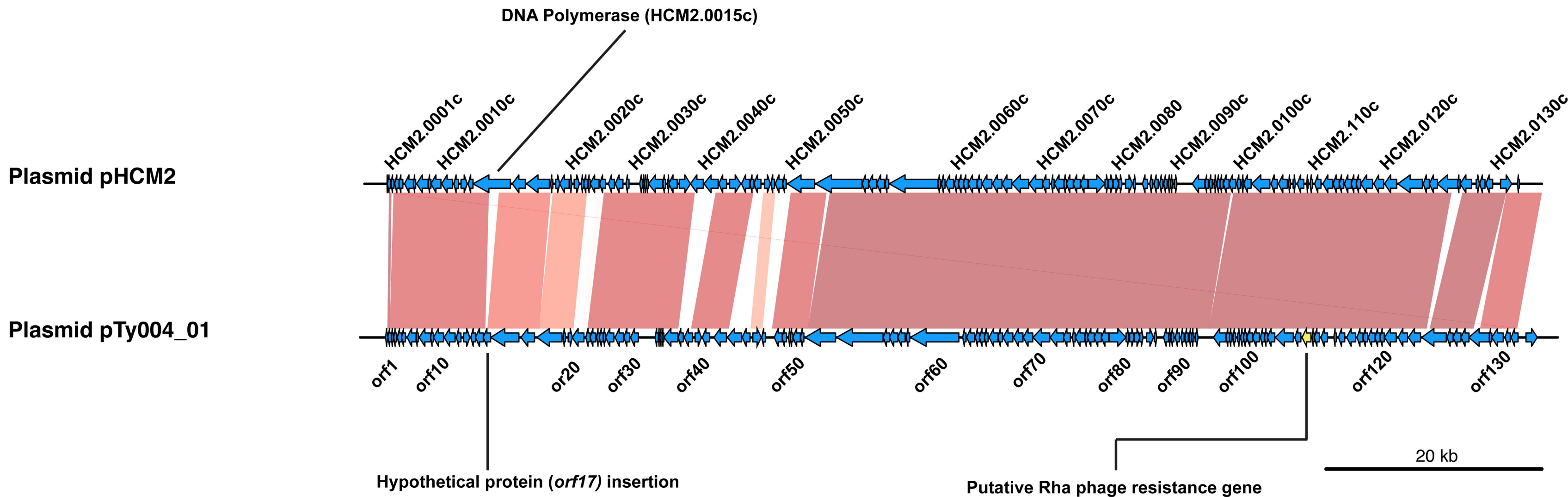




96 SNPs

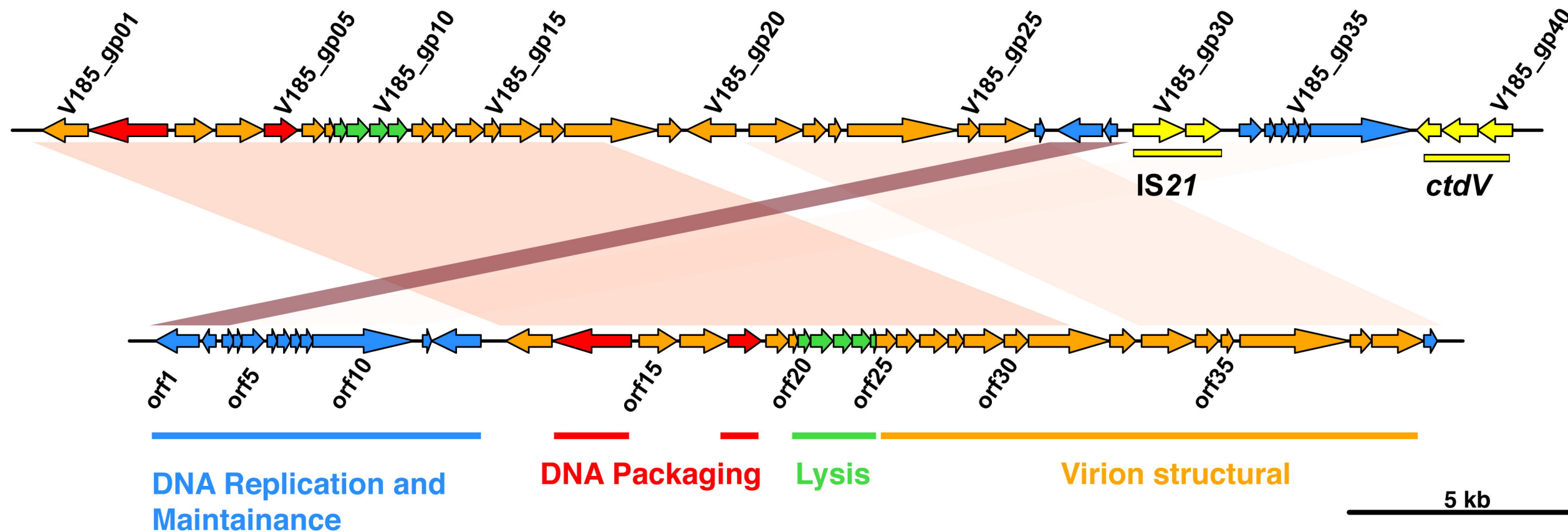






**A**

Phage fiae91-ss



**B**

Phage SflV

