# Discovery of a *Streptococcus pneumoniae* serotype 33F capsular polysaccharide locus that lacks *wcjE* and contains a *wcyO* pseudogene

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Running title: A novel pneumococcal serotype 33F capsule locus


## Abstract

Objectives:

As part of large on-going vaccine impact studies in Fiji and Mongolia, we identified 25/2750 (0.9%) of nasopharyngeal swabs that were positive for *Streptococcus pneumoniae* contained pneumococci with a divergent 33F capsular polysaccharide locus (designated '33F-1'). Here, we investigated the 33F-1 capsular polysaccharide locus to better understand the genetic variation and its impact on serotyping results.

Methods:

Whole genome sequencing was conducted on ten 33F-1 pneumococcal isolates. Initially, sequence reads were used for molecular serotyping by PneumoCaT. Phenotypic typing of 33F-1 isolates was then performed using the Quellung reaction and latex agglutination. Genome assemblies were used in phylogenetic analyses of each gene in the capsular locus to investigate genetic divergence.

Results:

All ten pneumococcal isolates with the 33F-1 *cps* locus typed as 33F by Quellung and latex agglutination. Unlike the reference 33F capsule locus sequence, DNA microarray and PneumoCaT analyses found that 33F-1 pneumococci lack the *wcjE* gene, and instead contain *wcyO* with a frameshift mutation. Phylogenetic analyses found the *cpsA, cpsB, cpsC, cpsD, wchA, wciG* and *glf* genes in the 33F-1 *cps* locus had higher DNA sequence similarity to homologues from other serotypes than to the 33F reference sequence.

Conclusions:

We have discovered a novel genetic variant of serotype 33F, which lacks *wcjE* and contains a *wcyO* pseudogene. This has important implications for molecular approaches measuring vaccine impact and disease surveillance, which continue to increase in popularity, particularly in low- and middle-income countries, where pneumococcal serotype diversity is poorly understood.

## Introduction

*Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive pathogenic bacterium and a leading cause of community-acquired pneumonia in young children and the elderly [1]. Pneumococci are classified by serotype, defined by an antigenically-distinct polysaccharide capsule. Capsule biosynthesis is encoded by the capsular polysaccharide (*cps*) locus within the pneumococcal genome. High levels of genetic diversity within this locus has resulted in over 90 pneumococcal serotypes described to date.

The pneumococcal capsule is the target for currently licensed vaccines, which only include a subset of serotypes. Although pneumococcal conjugate vaccines (PCVs) have been successful in reducing carriage and disease caused by the targeted serotypes, a rise in carriage and disease caused by serotypes not included in these vaccines is commonly observed (serotype replacement) [2,3]. To precisely monitor vaccine impact and disease surveillance, accurate tools for pneumococcal serotyping are required.

Molecular approaches to serotyping pneumococci rely on existing knowledge of *cps* loci. Data on pneumococcal *cps* loci from low- and middle-income countries (LMICs) are

relatively limited, which can impact serotyping results. For example, we recently described a novel genetic variant of pneumococcal serotype 11A in Fiji. Genetically, the *cps* locus of these isolates is most closely related to the 11F *cps* locus, with only a few minor nucleotide changes resulting in the production of 11A capsule [4].

Among the replacing serotypes post-PCV introduction, serotype 33F has become a concern world-wide. Serotype 33F is commonly reported among the predominant serotypes not included in PCVs causing invasive disease following vaccine introduction [5–7]. The increased invasive disease caused by serotype 33F has warranted its inclusion in two new vaccine formulations, which are in development by Merck [8]. In this study, we describe a novel 33F *cps* locus identified in Fiji and Mongolia by investigating the genetic basis of the variation in this locus and the potential impact this may have on serotyping results.

## Materials and Methods

### Nasopharyngeal swab collection and screening for pneumococci

Nasopharyngeal swabs were collected from either healthy participants (Fiji) or children with pneumonia (Mongolia) in accordance with WHO recommendations [9]. Ethical approval for the study in Fiji was granted from the Fiji National Research ethics review committee, as well as The University of Melbourne Human research ethics committee. Ethical approval for the study in Mongolia was granted from the ethics committee associated with The Ministry of Health in Mongolia and the Royal Children's Hospital. Written consent for those who participated in these studies was provided by

parents/guardians. Following collection, the swabs were placed in 1 ml skim milk, tryptone, glucose, and glycerol media [10] and stored at -80°C. Swabs were stored at -80°C until required. Samples were screened for the presence of pneumococci by conducting real-time quantitative PCR (qPCR) on DNA extracted from 100 µl aliquots of the swabs using the pneumococcal *lytA* gene as a target as previously described [11].

**Molecular typing by microarray**

Molecular typing of pneumococci was performed by DNA microarray. An aliquot of the nasopharyngeal swab was inoculated onto Horse Blood Agar supplemented with gentamicin (5 µg/ml), to select for pneumococci, and incubated overnight at 37°C with 5% $CO_2$. For plates with α-hemolytic growth, the bacterial growth was collected using 1 ml PBS, pelleted by centrifugation and stored at -30°C until required for DNA extraction. DNA was extracted from thawed bacterial pellets using the QIAcube HT with the QIAamp 96 DNA QIAcube HT Kit (Qiagen) with the inclusion of a pre-treatment lysis step consisting of the addition of 180 µl lysis buffer (20 mM TrisHCl, 2 mM EDTA, 1% Triton X-100, 2 mg/ml RNase A, 20 mg/ml lysozyme) to the bacterial pellet and incubated at 37ºC for 60 min. The rest of the extraction procedure was as per the kit instructions. This DNA was then used for microarray as described previously [12]. In brief, 200 ng of DNA was labelled with Cy3 or Cy5 using the Genomic DNA ULS Labeling Kit (Agilent Technologies) and incubated at 85°C for 30 min. The labelled pneumococcal DNA was incubated with Senti-SPv1.5 microarray slides (BUGS Bioscience) overnight at 65°C rotating at 20 rpm. Microarray slides were washed,

scanned, and analyzed using Agilent microarray scanner and feature extraction software. Serotype calls were analyzed by Senti-NET software.

**Bacterial isolates**

The *S. pneumoniae* isolates used in this study were purified from ten nasopharyngeal swabs containing 33F-1 from Fiji and Mongolia on selective media as described above. Isolates were confirmed as *S. pneumoniae* with microarray and whole genome sequencing.

**Whole genome sequencing and molecular typing**

For whole genome sequencing, DNA was extracted from pure cultures using the Wizard SV genomic DNA purification system (Promega) with some modifications. Briefly, pneumococcal cultures were pre-treated with a lysis solution containing 5 mM EDTA, 3 mg/ml lysozyme and 37.5 µg/ml mutanolysin in TE buffer and incubated at 37°C for 2 h. Proteinase K was added to a final concentration of 1 mg/ml and samples were incubated at 55°C for 1 h. Following incubation, 200 µl of nuclear lysis buffer and 5 µl of RNase (final concentration of 40 µg/ml) were added and samples were incubated at 80°C for 10 min. The rest of the extraction procedure was performed as per the manufacturer's instructions. Eluted DNA was sequenced in 2 x 300 bp paired end reads on the MiSeq platform. Using the Geneious 11.0.4 software package [13], sequence reads were trimmed with BBDuk and *de novo* assembled using SPAdes. The capsule loci were annotated within Geneious using a database consisting of capsule loci from the 90

serotypes described by Bentley et al. [14]. Sequence reads were also used for molecular typing with PneumoCaT [15].

**Sequence analysis**

Pairwise alignments were performed in Geneious 11.04 using MUSCLE. Additional alignments examining the *wcyO* gene for insertions/deletions were conducted using Clustal Omega. Phylogenetic analyses were performed for each 33F-1 *cps* gene using MEGA 7 [16]. For each gene, the phylogenetic analysis included a representative 33F-1 sequence as well as homologues from all other serotypes containing that gene as described by Bentley et al. [14], where GenBank accession numbers are provided. DNA sequences were aligned using MUSCLE and the alignments were used to generate maximum likelihood trees based on the Tamura-Nei model. Phylogenetic relationships were statistically analyzed by bootstrapping (1000 replicates). The 33F-1 *cps* loci have been deposited in Genbank (accession no. MH256127, MH256128, MH256129, MH256130, MH256131, MH256132, MH256133, MH256134, MH256135, MH256136).

**Quellung and latex agglutination serotyping**

Quellung serotyping was performed as described previously [17]. A saline suspension of pneumococci was prepared from an overnight culture on non-selective agar. Using an inoculation loop, 1 µl was placed on a microscope slide and mixed with 1 µl of antisera from the Statens Serum Institut (SSI) (http://www.ssi.dk/ssidiagnostica). The sample was then viewed under the microscope (x400 magnification). A positive reaction was defined as an enlargement or ‘swelling’ of cells, with serotype call based on the reaction profile

with each typing sera. For latex agglutination, latex reagents were prepared with SSI typing sera [18] and testing performed as previously described [19]. The bacterial suspension and latex reagent (10 µl of each) were mixed on a glass slide. The slide was then incubated on an orbital shaker for 2 min at ~140 rpm. A positive reaction was defined by the presence of visible agglutination.

## Results

As part of ongoing programs in the Asia-Pacific region measuring pneumococcal vaccine impact, we have collected nasopharyngeal swabs from healthy participants in Fiji, and children diagnosed with pneumonia in Mongolia. In these studies, we have used DNA microarray to serotype pneumococci contained within these swabs. DNA microarray uses 15,000 oligonucleotides that are spotted onto glass slides and recognize each capsule gene from the 90+ serotypes. Labelled pneumococcal DNA is allowed to hybridize to the oligonucleotides so that pneumococcal serotype can be inferred. From 2750 swabs that contained pneumococci 25 (0.9%) contained pneumococci that typed as ‘33F-like’ (hereby referred to as ‘33F-1’). Ten of these samples were selected and the pneumococci from these swabs were isolated for further analysis (Table 1).

Microarray reported the *wciG, glf* and *wcjE* genes in the nasopharyngeal swabs containing these isolates as ‘absent/divergent’. The *wcyO* gene was also detected, which has not been reported in the serotype 33F *cps* locus previously. To investigate the impact of the divergent 33F-1 *cps* locus on other molecular approaches to serotyping, we sequenced the genomes of all ten isolates and ran the sequence reads through the

PneumoCaT pipeline [15]. PneumoCaT uses *wcjE* to differentiate 33A from 33F, as this gene contains a frameshift mutation in 33F, resulting in a lack of WcjE-mediated O-acetylation of the 33F capsular polysaccharide [20]. Consistent with microarray, PneumoCaT typed all isolates as 33F and was unable to detect *wcjE*. Phenotypic serotyping methods (Quellung and latex agglutination) also typed these isolates as 33F.

Following investigation of the 33F-1 *cps* locus, it was evident that not only did all ten isolates lack *wcjE,* the locus contained *wcyO* at this position. The *wcyO* gene encodes an acetyltransferase and mediates the same modification as *wcjE* (6-O-acetylation of galactose) [21]. The *wcyO* open reading frame from all 33F-1 isolates contained a frameshift mutation. The *wcyO* gene in 33F-1 pneumococci from Fiji had a single T insertion (position 313) whereas this gene in isolates from Mongolia contained a single A deletion (position 246) (Fig. 1). These frameshift mutations were also confirmed by Sanger sequencing and were not present in traditional *wcyO*-containing isolates (serotypes 34 and 39) from Fiji (Supplementary Fig. S1).

In addition to the differences in *wcjE* and *wcyO,* microarray detected some divergence in other genes in the 33F-1 *cps* locus compared to the reference 33F sequence. To gain a better understanding of the relationships of the 33F-1 *cps* genes to homologues from other serotypes we performed phylogenetic analyses for each gene. In support of the pairwise alignments (Supplementary Table S1), the 33F-1 *wciB, wciD, wciE, wciF, wzy* and *wzx* genes clustered with 37/33A/33F sequences (Fig. 2F-K). In contrast, 33F-1 *cpsA, cpsB, cpsC, cpsD* (also known as *wzg, wzh, wze* and *wzd*, respectively) and *wchA*

clustered with serotype 33B sequences (Figures 2A-D), *wciG* with serotype 37 (Fig. 2L), *glf* with serotypes 34 and 39 (Figure 2M) and *wcyO* with 33C, 34 and 39 (Fig. 2N). All branches had strong statistical support (>85% bootstrap score from 1000 replicates for all genes, except *cpsD* with a 67% bootstrap score for the 33F-1/33B branch).

## Discussion

Pneumococcus is a highly successful pathogen in part due to the high level of capsule diversity, resulting in over 90 serotypes each with unique antigenic properties. Even small genetic differences in the *cps* locus can have biologically relevant consequences, impacting opsonophagocytosis efficiency and immune recognition. Serotypes 33F and 33A have the same *cps* locus, except that 33F has a *wcjE* gene containing a frameshift mutation [22]. Using DNA microarray, we identified a high degree of genetic divergence in the capsule DNA sequence of some serotypes in Fiji and Mongolia. Here, we characterized a serotype 33F variant (33F-1) that has the same genes as the canonical 33F and 33A *cps* loci, except it possesses *wcyO* instead of *wcjE*. Interestingly, in the 33F-1 variants *wcyO* is predicted to encode a truncated protein due to a frameshift mutation. These frameshift mutations suggest a loss of 6-O-acetylation in 33F-1 capsular polysaccharide as the truncated protein (110 amino acids and 91 amino acids long in the Fiji and Mongolia isolates, respectively compared to the full length WcyO protein of 352 amino acids) would unlikely be functional.

The frameshift mutations in isolates from Fiji and Mongolia have both occurred within homopolymeric regions (Fig. 1, Supplementary Fig. S1). Such regions are prone to

slipped-strand mispairing, whereby errors made during DNA replication can result in the insertion or deletion of a nucleotide [23]. Therefore, we postulate that the frameshift mutations in the 33F-1 *wcyO* genes are the result of slipped-strand mispairing events.

This the first report of the *wcyO* acetyltransferase gene in the 33F *cps* locus, and it is also the first report of a naturally occurring frameshifted allele of *wcyO*. The fact that the mutation type and location differ between isolates from Fiji and Mongolia demonstrates this mutation event has occurred on at least two independent occasions. Whether the mutation of *wcyO* is due to selective pressure to inactivate a disadvantageous gene or due to a lack of selective advantage conferred by WcyO-mediated acetylation remains to be investigated. Previously, mutations have been identified in other pneumococcal capsule acetyltransferase genes including *wciG* [24] and *wcjE* [22,25,26]. Serotype 11E, which lacks WcjE-mediated acetylation can evade opsonophagocytosis more efficiently compared to 11A (which possess WcjE-mediated acetylation) [25]. Pneumococci expressing 33F capsules, which lack WcjE-mediated acetylation, exhibit enhanced survival during drying compared to serotype 33A (with intact WcjE-mediated acetylation) [27]. Laboratory constructed *wciG* mutants in serogroup 33 isolates were more susceptible to opsonophagocytosis, and displayed increased adherence and biofilm formation, which has yet to be evaluated in wildtype strains [27]. It is plausible that mutation of *wcyO* in the 33F-1 pneumococci may serve a similar purpose, however this requires further investigation.

Within the 33F-1 *cps* locus we identified 7/15 genes that exhibit higher DNA sequence similarity to homologues from other serotypes rather than 33F. Both *glf* and *wcyO* are similar to sequences from serotypes 34 and 39 (and 33C for *wcyO*) (Fig. 2M and 2N) and *cpsA, cpsB, cpsC, cpsD* and *wchA* similar to sequences from 33B (Fig. 2A-E). Recombination of the pneumococcal capsule genes resulting in mosaic *cps* loci such as that of 33F-1 have been reported previously [28,29]. Although it is difficult to infer the direction of horizontal transfer of these genes, the mosaic nature of the 33F-1 *cps* locus would suggest an ancestral 33F-1 *cps* locus was the recipient of these genes.

Interestingly, serogroup 33 related *cps* genes have been identified in two non-pneumococcal streptococcal strains (*Streptococcus oralis* subsp. *tigurinus* Az_3a and *Streptococcus mitis* SK269) [30]. The 33D-like *cps* locus in *S. mitis* SK269 exhibited low similarity to the 33F-1 *cps* locus, including the absence of *wchA* and *wciC* and all other *cps* gene exhibiting <76% DNA identity (Supplementary Table S1). The *S. oralis* subsp. *tigurinus* Az_3a *cps* locus possessed the same genes as the 33F-1 locus with variable DNA identity across the locus (<77% with the 33F-1 *cpsA, cpsB, cpsC, cpsD, wchA* and *wciB* genes, >96% for *wciC, wciD, wciE, wciF* and *wzy* genes, and 85-90% for *wzx, wciG* and *glf* genes, Supplementary Table S1). The higher DNA identity of 33F-1 *cps* genes with homologues from other pneumococcal serotypes suggests the Az_3a *cps* locus may have evolved independently of the 33F-1 locus. In contrast to 33F-1, the *wcyO* gene in Az_3a has an intact open reading frame and is most similar to the *wcyO* gene from pneumococcal serotype 21 (DNA identity score 86.8% with serotype 21 *wcyO* compared to 74.5% with 33F-1 *wcyO*). The existence of a divergent 33F-1 *cps* locus with a

functional *wcyO* raises interesting questions around why this gene has been inactivated in 33F-1 pneumococci but remains intact in a non-pneumococcal streptococcal species.

This study describes a novel genetic basis for pneumococcal serotype 33F, which we discovered from nasopharyngeal isolates from Fiji and Mongolia. Serotype 33F is a replacing serotype in invasive disease following vaccine introduction [5–7]. The public health importance of 33F is reflected in that it has been included in two upcoming vaccine formulations (PCV15 and PCV24) [8]. In addition, there is increasing popularity in molecular serotyping approaches and it is therefore important to identify genetic variants, which have the potential to impact serotyping results. This is particularly important for the implementation of these methods in LMICs, where there is limited understanding of the pneumococcal *cps* loci. The data gained from this study will be used to update genetic typing tools for more accurate typing of serotype 33F in LMICs.

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## Tables and figures

Table 1. Pneumococcal 33F-1 isolates used in this study.

| Isolate | Source | Country of isolation |
|---|---|---|
| PMP1348 | Nasopharynx of healthy child (2-7 years old) | Fiji |
| PMP1349 | Nasopharynx of healthy child (5-8 weeks old) | Fiji |
| PMP1351 | Nasopharynx of healthy child (12-23 months old) | Fiji |
| PMP1352 | Nasopharynx of healthy child (12-23 months old) | Fiji |
| PMP1353 | Nasopharynx of healthy child (5-8 weeks old) | Fiji |
| PMP1379 | Nasopharynx of healthy child (12-23 months old) | Fiji |
| PMP1380 | Nasopharynx of healthy child (12-23 months old) | Fiji |
| PMP1383 | Nasopharynx of healthy adult | Fiji |
| PMP1386 | Nasopharynx of child with pneumonia | Mongolia |
| PMP1387 | Nasopharynx of child with pneumonia | Mongolia |

Figure 1. Comparison of the *wcyO* open reading frames of 33F-1 sequences to a representative serotype 34 sequence. Only a selected portion of the DNA sequence is shown. Numbers refer to the position number in the serotype 34 sequence with an in-frame *wcyO* gene.

Figure 2. Maximum likelihood phylogenetic trees of 33F-1 *cps* genes with homologues from all other serotypes. As all genes except *wchA* and *wcyO* were identical in all 33F-1 isolates only one sequence is included as a representative. Un-collapsed trees are provided in Supplementary Figure S2. Tree for *wciC* is not shown as this gene is only present in serotypes 33F, 33A and 37, which all have over 98% DNA sequence identity to the 33F-1 sequence. DNA sequences were aligned using MUSCLE and trees were constructed using the Tamura-Nei model in MEGA 7. Only bootstrap values above 50% are shown.

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wcyO(34)              CTA TAT AAG ATA CTT GGT AAA TTT ATG GAA ATT ATC TAT TAT TTT CAT   192
                      L   Y   K   I   L   G   K   F   M   E   I   I   Y   Y   F   H
wcyO(33F-1_Fiji)      CTA TAT GAG ATA CTT GGT AAA TTT ATG GAA ATT ATC TAT TAT TTT CAT
                      L   Y   E   I   L   G   K   F   M   E   I   I   Y   Y   F   H
wcyO(33F-1_Mongolia)  CTA TAT GAG ATA CTT GGT AAA TTT ATG GAA ATT ATC TAT TAT TTT CAT
                      L   Y   E   I   L   G   K   F   M   E   I   I   Y   Y   F   H
                            150           160           170           180           190

wcyO(34)              ATG CCA TTA TTT ATG GCT ATA TCG GGT GTA TTT TTC TCT ATT CAA ATA   240
                      M   P   L   F   M   A   I   S   G   V   F   F   S   I   Q   I
wcyO(33F-1_Fiji)      ATA CCA TTA TTT ATG GCT ATA TCG GGT GTA TTT TTC TCT ATT CAA ATA
                      I   P   L   F   M   A   I   S   G   V   F   F   S   I   Q   I
wcyO(33F-1_Mongolia)  ATA CCA TTA TTT ATG GCT ATA TCG GGT GTA TTT TTC TCT ATT CAA ATA
                      I   P   L   F   M   A   I   S   G   V   F   F   S   I   Q   I
                              200           210           220           230

wcyO(34)              AAA AAA GAT CGA TGG AAT AAG ATT GAG AAA TTA TTA ACT AGT AAG TTT   288
                      K   K   D   R   W   N   K   I   E   K   L   L   T   S   K   F
wcyO(33F-1_Fiji)      AAA AAA GAT CGA TGG AAT AAG ATT GAG AAA TTA TTA ACT AGT AAG TTT
                      K   K   D   R   W   N   K   I   E   K   L   L   T   S   K   F
wcyO(33F-1_Mongolia)  AAA AAG ATC GAT GGA ATA AGA TTG AGA AAT TAT TAA CTA GTA AGT TTA
                      K   K   I   D   G   I   R   L   R   N   Y   *
                                  250           260           270           280

wcyO(34)              AAA AGA TTA ATA TTG CCA TTT TTT GTT TTT ACT TTA TTA TAT AGT TTG   336
                      K   R   L   I   L   P   F   F   V   F   T   L   L   Y   S   L
wcyO(33F-1_Fiji)      AAA AGA TTA ATA TTG CCA TTT TTT TGT TTT TAC TTT ATT ATA TAG TTT
                      K   R   L   I   L   P   F   F   C   F   Y   F   I   I   *
wcyO(33F-1_Mongolia)  AAA GAT TAA TAT TGC CAT TTT TTG TTT TTA CTT TAT TAT ATA GTT TGC

                       290           300           310           320           330

wcyO(34)              CCA TTA AAA TAT ATA TCA AAC TAC TAC AAT GGT GTT TCG TTT TGG AGA   384
                      P   L   K   Y   I   S   N   Y   Y   N   G   V   S   F   W   R
wcyO(33F-1_Fiji)      GCC ATT AAA ATA TAT ATC AAA CTA CTA CAA TGG TGT TTC ATT TTG GAG

wcyO(33F-1_Mongolia)  CAT TAA AAT ATA TAT CAA ACT ACT ACA ATG GTG TTT CAT TTT GGA GAG

                           340           350           360           370           380
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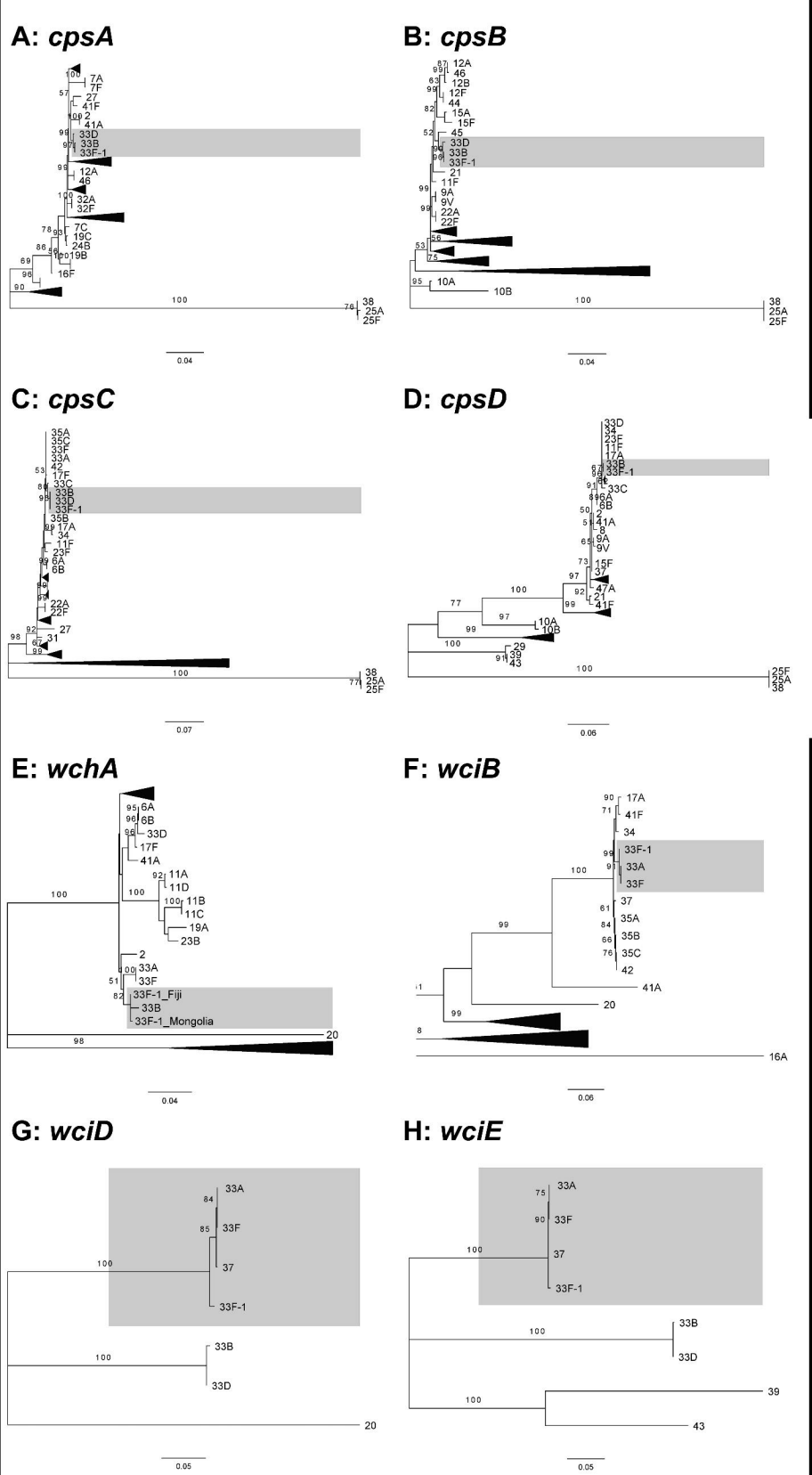
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7F
27
41F
2
41A
33D
33B
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46
32A
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19C
24B
19B
16F
38
25A
25F
0.04
B: cpsB
12A
46
12B
12F
44
15A
15F
45
33D
33B
33F-1
21
11F
9A
9V
22A
22F
10A
10B
38
25A
25F
0.04
C: cpsC
35A
35C
33F
33A
42
17F
33C
33B
33D
33F-1
35B
17A
34
11F
23F
6A
6B
22A
22F
27
31
38
25A
25F
0.07
D: cpsD
33D
34
23F
11F
17A
33B
33F-1
33C
6A
6B
2
41A
8
9A
9V
15F
37
47A
21
41F
10A
10B
29
39
43
25F
25A
38
0.06
E: wchA
6A
6B
33D
17F
41A
11A
11D
11B
11C
19A
23B
2
33A
33F
33F-1_Fiji
33B
33F-1_Mongolia
20
0.04
F: wciB
17A
41F
34
33F-1
33A
33F
37
35A
35B
35C
42
41A
20
16A
0.06
G: wciD
33A
33F
37
33F-1
33B
33D
20
0.05
H: wciE
33A
33F
37
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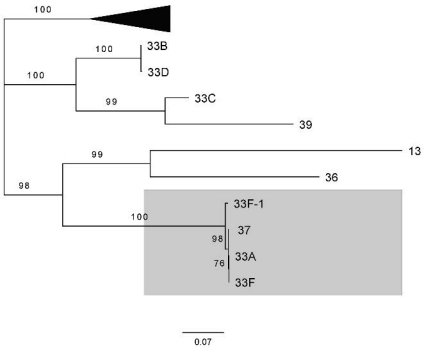
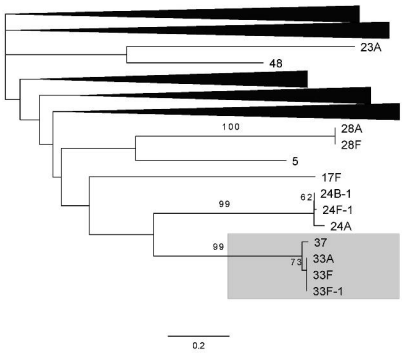
33B
33D
39
43
0.05

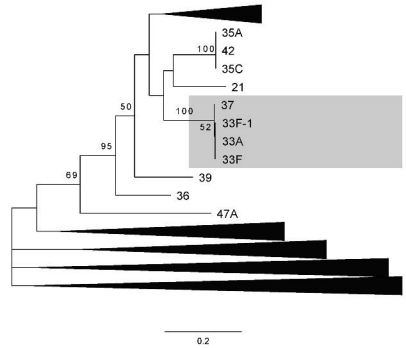
## I: *wciF*



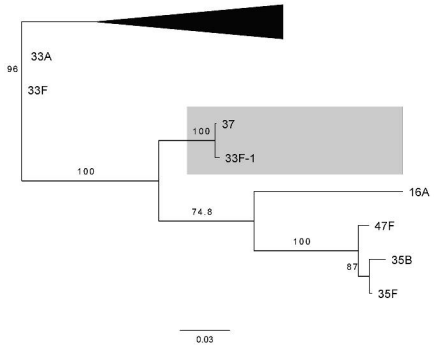

## J: *wzy*



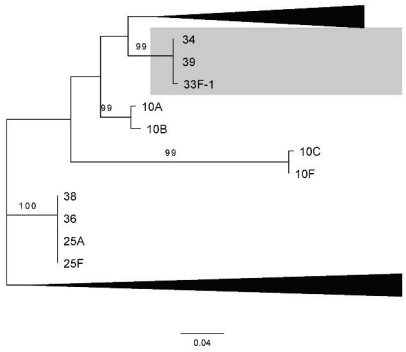

## K: *wzx*

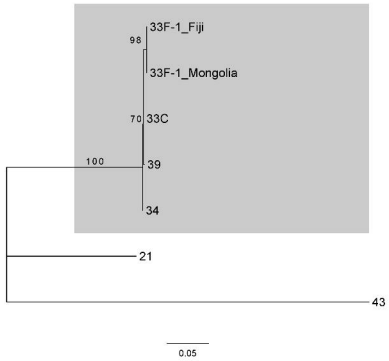



## L: *wciG*




## M: *glf*




## N: *wcyO*