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Delineating the key virulence factors and intra-species divergence of *Vibrio harveyi* by the use of whole genome sequencing

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Running title: The intra-species divergence of *V. harveyi*

Abstract

Vibrio harveyi is one of the major pathogens in aquaculture. To identify the key virulence factors affecting pathogenesis of *V. harveyi* towards fish, a field investigation was conducted for three representative fish farms infected with *V. harveyi*. Multilocus sequence typing (MLST) and whole-genome sequencing were conducted to delineate phylogenetic relationship and genetic divergence of *V. harveyi*. A total of 25 *V. harveyi* strains were isolated from the diseased fish and groundwater, which can be subtyped into 12 sequence types (STs) by MLST. Five virulence genes including *mshB*, *pilA*, *hutR*, *ureB* and *ureG* were variably presented in sequenced strains. The virulence gene profiles strongly correlated with the distinct pathogenicity of *V. harveyi* strains, in which a strain harbored all five genes exhibited the highest virulence towards fish. Phenotype assay confirmed that reduced virulence correlated with decreased motility and biofilm formation ability. Additionally, three types of type VI secretion system (T6SS), namely T6SS1, T6SS2 and T6SS3 were identified in *V. harveyi* strains, which can be classified into six, four and 12 subtypes, respectively. In conclusion, results indicated that the virulence level of *V. harveyi* is mainly determined by above virulence genes, which may play vital roles in environmental adaptation for *V. harveyi.*

Keywords: *Vibrio harveyi*; Tiger puffer; genetic divergence; type VI secretion system; virulence factors

Introduction

China is becoming the major producer and exporter of puffer fish in the world. The economic value of puffer fish in China has grown to US$2 million in 2016 (FMBA, 2017). However, due to the high-density aquaculture and inadequate management of disease, the outbreaks of the fish disease frequently occurred.

Vibrio harveyi has been considered to be amongst the most significant pathogens in the aquaculture industry (Austin et al. 2006). *V. harveyi* also has been recognized as a major bacterium capable of infecting a great variety of fish including *Lates calcarifer* (Ransangan et al. 2012), *Paralichthys olivaceu*s (Song et al. 2004), *Takifugu rubripe*s (Wang et al. 2008; Mohi et al. 2010), *Lateolabrax japonicas* (Wang et al. 2000), *Larimichth scrocea* (Ge et al. 2014), *Epinephlus awoara* and *Epinephlus coioides* (Chen et al. 2006). It has also caused significant diseases for invertebrate such as corals, oysters, prawns, lobsters, and abalone with the symptoms including eye-lesions, gastro-enteritis, and vasculitis (Austin et al. 2006).

V. harveyi can induce high levels of pathogenicity to Tiger puffer (*Takifugu rubripes*) and Turbot (*Scophthalmus maximus*) (Li et al. 2014; Wang et al. 2018). Infected *Takifugu rubripes* showed the darkened body, nodular lesions in the branchial chamber and the inner surface of the operculum. Histopathology manifested necrotized, suppurative foci, and granulomatous tissue (Mohi et al. 2010). The main clinical symptoms of diseased turbot were exophthalmia and chronic skin ulceration,

and sometimes can also cause acute sepsis, and the histopathology studies have not been reported.

The previous study showed that the pathogenicity of *V. harveyi* is a strain characteristic rather than a species characteristic as some strains can be highly virulent, whereas other strains are avirulent (Ruwandeepika et al. 2012). These observations might largely associate with the various presence of virulence factors in *V. harveyi*.

With the extensive research on the pathogenesis of *V. harveyi*, virulence was reported to be associated with the presence of extracellular products (ECPs) (Liu et al. 1996a), including proteases and haemolysins (Lee et al. 1997; Liu et al. 1996b; Liu et al. 1997), which can promote bacteria invasion into the host and survive and reproduce within their tissues (Ellis et al. 1991). Haemolysin is one of the major virulence factors in *V. harveyi*, which is known to be pathogenic towards salmonids. It was found to produce extracellular products with high hemolytic activity towards fish erythrocytes. Two closely related haemolysin genes (designated *vhh*A and *vhh*B) were identified (Zhang et al. 2001). Capsular polysaccharides (CPS) can form a capsule surrounding the bacterial cells, which is involved in attachment to host cells and play an important role in immune evasion (Hsieh et al. 2003).

Secretion systems also potentially contributed to the virulence of *V. harveyi.* The Type III secretion system (T3SS) is a conserved needle-like bacterial machinery

present in many Gram-negative bacteria that can directly inject bacterial effectors into the membrane and cytoplasm of eukaryotic cells, without releasing the effectors to the extracellular space (Shen et al. 2011; Galan et al. 2006). T3SS1 is induced by growing liquid cultures at 37 ℃ in low calcium media and can cause cytotoxicity in various cultured eukaryotic cells (Ono et al. 2006; Burdette et al. 2008). Li et al. (2017) suggested that a highly conserved T3SS1 that has been discovered in all sequenced isolates of *V. parahaemolyticus*. T3SS of *V. harveyi* is activated at low cell density and repressed at high cell density by quorum sensing, which translocates toxic effector proteins into eukaryotic cells and has a pivotal role in pathogenesis during the infection (Waters et al. 2010). But it is yet unknown whether T3SS is also conversed in *V. harveyi.*

Type VI secretion systems (T6SS), which are predicted to be involved in inter-bacterial competition in various *Vibrio* spp. under nutrient-poor conditions, are also a remarkable indicator contributing to the virulence (Dong et al. 2013; Russell et al. 2011; Russell et al. 2014). At least one main gene cluster and two auxiliary gene clusters are required to form a functional T6SS apparatus capable of conferring virulence toward hosts. T6SS is highly diverse among the *Vibrio* spp. For instance, *V. cholerae* uses the versatile T6SS to secrete anti-prokaryotic and anti-eukaryotic effectors, which is induced by growth on chitinous surfaces (Kostiuk et al. 2017). There are two T6SSs in *V. parahaemolyticus* and they are differentially regulated by externalcues such as temperature, salinity, and surface sensing (Boyd et

al. 2008). T6SS1 in *V. parahaemolyticus* was previously shown to possess antibacterial activities against a number of bacterial competitors, which is genetically diverse (Yu et al. 2012). T6SS2, like T3SS1, is present in all *V. parahaemolyticus* strains, including both environmental and clinical strains (Salomon et al. 2013), but its role in the life cycle of *V. parahaemolyticus* remains unclear. Li et al. (2017) found that the distinct antibacterial activities of T6SS1 were associated with the site variations in the genetic structure of T6SS in a bacterial competition assay. Three variation sites were identified which divided 12 *V. parahaemolyticus* strains into six types. Yang et al. (2019) further investigated the site variations of 15 *V. parahaemolyticus* strains and found the genetic structure of T6SS1 is genotype-specific. Three T6SSs (i.e. T6SS1, T6SS2 and T6SS3) have also been currently described in *V. harveyi* (Tu et al. 2017).

However, the intra-species divergence of T3SS and T6SS among *V. harveyi* strains remains largely unclear, which limited our understanding regarding the virulence difference for *V. harveyi*. Pizzutto and Hirst (1995) found *V. harveyi* is a genetically diverse species by genetic profiling and protein profiling (Pizzutto and Hirst 1995). Therefore, it is essential to distinguish between virulent strains and avirulent strains and confirm the correlation of virulence with genetically element.

Recently, whole-genome sequencing (WGS) is emerging as a high-resolution tool to prospectively confirm the evolutionary origins of *Vibrio* spp. and to identify their

virulence determinants (Martinez-Urtaza et al. 2017). With the increased application of WGS, more *V. harveyi* genomes were available for analyzing the virulence factors towards fish. For instance, virulence factors, including IlpA, OmpU, Flagellin, Cya, and Hemolysin were detected in the genome QT520, which are suggested to be responsible for the virulence of QT520 (Tu et al. 2017). Genomic analysis of pathogenic *V. harveyi* strains RT-6, ZJ0603 and CAIM 1792 were also performed, which revealed the potential virulence determinates associated with aquatic animal infection (Huang et al. 2012; Espinoza-Valles et al. 2012; Thirugnanasambandam et al. 2019). WGS of the virulence determinants in *V. harveyi* with distinct virulence level is needed which would possibly provide insights into the impacts of genetic composition on the virulence level.

The fish farms selected in our study were the top three producers of Turbot and Tiger puffer in China. From 2014 to 2015, massive outbreaks of fish disease occurred in several turbot and Tiger puffer farms in Liaoning province with typical symptoms of vibriosis (darkness body and fins lesion). In this study, we first employed Multilocus sequence typing (MLST) for the initial typing of 24 isolated *V. harveyi* from three fish farms. Afterward, WGS was performed for representative *V. harveyi* strains. The comparative genomic analysis was then performed to confirm the evolutionary relationship between the fish and environmental strains to provide novel insights into the key virulence factors affecting the pathogenesis of *V. harveyi*.

Materials and methods

Isolation and identification of *Vibrio* species in groundwater and diseased fish

A microbiological investigation was conducted in two Tiger puffer farms and one turbot farm. An epidemiological investigation showed that all the sampled fish farms bred their own brood fish, without importing of parent fish from other sources, which excluded the possible input of pathogens from external sources. Groundwater was the only source of the water used for aquaculture which was pumped into the fish tank from 20m below the ground with the salinity of 20-25 ppt. A total of 60 fish feed samples (large type Artemia (Hailin Aquatic Feed Co., Ltd, Hailin, China)) and 49 moribund fish with the symptoms of defected fins, hepatohemia and intestinal hydrops were collected from three fish farms (namely fish farm A, B, and C) between March 2014 and December 2015. The collected fish samples were aseptically and immediately transported in the sterilized bag to the laboratory and processed within 8 h. The intestinal swabs were inoculated on the thiosulfate–citrate–bile salts–sucrose (TCBS) agar (Oxoid, England) and incubated at 30°C for 24h under aerobic condition. Additionally, 24 water samples from groundwater in fish farms were collected in sterilized glass bottles. Water samples were filtered through 0.45 μm sterile filters (Millipore). Filters were placed on the TCBS agar and incubated at 30°C for 24 h under aerobic condition. Suspected yellow around colonies were subcultured on Luria broth (LB) agar to obtain pure cultures. Suspected *V. harveyi* isolates were further identified by PCR amplification of 16S rRNA (by primers 27F and 1492R) and *V. harveyi*-specific *toxR* (Pang et al. 2006). The amplicon was sequenced by Sanger

sequencing technology at Beijing Genomics Institute. The similarity of the 16S rRNA gene sequence was examined by using the BLASTn program in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Total of 25 *V. harveyi* strains were obtained in this study (Table S1). Prior to molecular typing, strains were grown aerobically on nutrient agar medium (NA) and in nutrient broth (NB) with 2% NaCl overnight at 28 ℃.

Multilocus sequence typing (MLST) and *in silico* MLST

Genomic DNA was extracted using a bacterial genome extraction kit (TIANGEN, China) according to the manufacturers' instructions. Five housekeeping genes including *recA*, *pyrH*, *rpoD*, *gyrB* and *rctB* were sequenced with the primers and procedures previously described (Pascual et al. 2010) (Table S2). *In silico* MLST of 25 publicly available *V. harveyi* genomes were performed with the MLST2.0 web server (https://cge.cbs.dtu.dk//services/MLST/) (Larsen et al. 2012). The sequences of the housekeeping genes were then concatenated. A phylogenetic tree of *V. harveyi* was constructed by the neighbor-joining method with MEGA (version 5.0) (Tamura et al. 2011). Kimura's two-parameter model was used as the substitution model for the construction of phylogenetic tree.

Whole-genome sequencing and *de novo* assembly

Nine strains were selected and used for genome sequencing. The genomic DNA was extracted from overnight bacterial cultures grown in LB by genome extraction kit

(TAINGEN, China). Genomic DNA was fragmented and tagged for multiplexing with the Nextera XT DNA Sample Preparation Kit (Illumina). The tagged DNAs were paired-end sequenced with the Illumina HiSeq 2500 platform at Beijing Novogene Bioinformatics Technology Co., Ltd. The FASTQ reads were quality trimmed with Trimmomatic (v0.36) (Bolger et al. 2014), and bases with a PHRED score of < 30 were removed from the trailing end. To obtain the draft genomes, the contigs from the chromosomes were assembled *de novo* with SPAdes version 3.0 (Bankevich et al. 2012). Gap closure was conducted to obtain the plasmid sequence by using program Pilon (Walker et al. 2014). Genome information for nine strains was deposited in GenBank under BioProject No.PRJNA503785.

Gene content analysis

RAST server (http://rast.nmpdr.org/) was used to annotate the sequences from each draft genome (Aziz et al. 2008). The genomes were uploaded into the Virulence Factors of Pathogenic Bacteria Database (VFDB), genes encoding virulence factors were identified through BLASTn searches. (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) (Chen et al. 2011). Antimicrobial resistance genes were identified using AMRFinder (Feldgarden et al. 2019). Finally, the secretion systems in the sequenced strains were predicted by T346Hunter server (http://bacterial-virulence-factors.cbgp.upm.es/T346Hunter (Martínez-García et al. 2015).

Identification of SNPs and phylogenetic inferences

A total of 34 *V. harveyi* isolates, including 25 publicly available genomes (Table S1) and 9 sequenced strains were selected and analyzed. The strains represented a diverse geographical distribution of *V. harveyi* collected between 1935 and 2014. The genome of QT520 was used as the reference genome to call the Single-nucleotide polymorphisms (SNPs) for *V. harveyi*. SNP calling was performed with a previously developed pipeline to guarantee that only genuine SNPs were included in the analysis (Chan et al. 2016). Briefly, the Burrows-Wheeler alignment (BWA) tool (version 0.7.5) was used to map the reads against the reference genome. SAMtools version 0.1.19 was used to further filter the SNPs identified from BWA mapping by SNP quality. SNPs with low sequence quality (quality score < 30 or was covered by < 10 reads) were filtered out. After filtering, 32,372 SNPs in total were identified from the 40 genomes, of which SNPs located in the repetitive regions were removed. The phylogenetic trees were constructed with the maximum likelihood method using RAxML 7.2.8 (Stamatakis, 2006). Generalized Time Reversible (GTR) with Gamma rates (G) and Invariant sites (I) (GTR+ G + I) model was used.

Challenge test with different *V. harveyi* isolates towards Tiger puffer

Before the challenge test, healthy Tiger puffer (*Takifugu Rubripes*, 21±2.6g) were transferred to 50 L seawater in plastic tanks, and were acclimated for one week under the following conditions: 40 fishes/tank, natural photoperiod, seawater at salinity 30ppt, pH 7.8, constant aeration and controlled temperature at 22-25 °C. The immersion challenge tests were conducted according to Zhu et al. (2018) with some

modifications. Based on the results of MLST, one or two strains were selected from each lineage. Different *V. harveyi* strains were cultured in tryptic soy broth (TSB) (Thermo Fisher Oxoid, Basingstoke, England) at 28 °C under constant agitation (120 min^{-1}) until the OD_{600} reached 0.8, afterwards, the bacterial suspension was adjusted to 1×10^8 cfu·mL^{-1}. The immersion procedure was carried out by immersing 40 fish in a 50L-tank for 4 days with a final bacterial density of 1×10^5 cfu·mL^{-1}. Fish in the negative control group were immersed in seawater without inoculums. Following the immersion, mortality was recorded daily for 72h. Results were presented as mean ± SD (standard deviation). All experiments were conducted in triplicates.

LDH cytotoxicity assay

HeLa cells were routinely cultured in DMEM with 10% (v/v) fetal bovine serum at 37 °C. For LDH assays, HeLa cells were plated in a 24-well tissue culture plate at 8×10^4 cells per well and grown for 24h. Nine *V. harveyi* strains were grown in TSB at 28°C overnight. *Vibrio parahaemolyticus* ATCC17082 was used as positive control, while its mutant ATCC17802-IS*Val* with a disruption of v*opS* (VP1686) by insertion sequence was used as negative control (Fu et al. 2020). Overnight bacterial cultures were diluted with DMEM to an optical density at 600nm (OD_{600}) of 0.3 and grown at 37 °C for 30min to induce T3SS1. Induced *V. harveyi* isolates were then used to infect HeLa cells at a multiplicity of infection (MOI) of 10. At 4h postinfection, lactate dehydrogenase (LDH) release into the culture medium was evaluated as a measure of cytotoxicity and host cell lysis by using a colorimetric cytotoxicity detection kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Assays were

repeated at least three times with similar results, and results of a representative experiment are shown.

Swimming motility assay

The swimming motility assay was performed on soft TSB as described by Mey and Payne (2011). *V. harveyi* strain was grown overnight in TSB broth, and 5 µl aliquots (OD_{600} = 1.0) were spotted in the center of the molten L agar containing 100 µg ml^{-1} EDDA. Iron sources were spotted onto the solidified agar as follows: 20 µl of 10 mM $FeSO_4$; 5 µl of marine invertebrate haemoglobin preparation (50 µM haem content). Plates were incubated for 24 h, after which the diameters of the motility halos were measured. All assays were repeated at three times independently. The values were expressed as mean ± standard deviation (SD).

Biofilm formation assay

Briefly, an overnight culture of *V. harveyi* was diluted to an OD_{600} of 0.1 in TSB broth and 200 µl aliquots of these suspensions were pipetted into the wells of a 96 well plate. Then the bacteria were allowed to adhere and grow without agitation for 24 h at 28°C. Biofilm formation assay was then quantified by crystal violet staining, as described previously by Yang and Defoirdt (2015). The experiments were repeated at three times independently. The values were expressed as mean ±SD.

Co-culture assay

To validate the antibacterial function of T6SS in *V. harveyi*, co-culture assay was conducted as described by Li et al. (2017). Briefly, nine sequenced *V. harveyi* strains and *V. parahaemolyticus* strain ATCC17802 were pre-cultured separately in TSB at 30 °C for 18 h and then were diluted at an initial cell density of approximately 10^5

CFU ml^{-1}. Each *V. harveyi* strain was mixed with *V. parahaemolyticus* strain ATCC17802 at a 4:1 (*V. harveyi* to *V. parahaemolyticus*) ratio, respectively. Above co-cultures and single-culture of *V. parahaemolyticus* strain ATCC17802 were incubated in 1/10 diluted TSB at 25 °C for 72 h. Serial dilutions of samples were inoculated onto TCBS agar to determinate the density of *V. harveyi*. The plates were incubated at 37 °C for 24 h. Experiments were repeated three times.

Antibiotic susceptibility test

Antibiotic susceptibility was assessed using the disc diffusion method as described elsewhere (Jorgensen et al. 2009). A total of 12 commercially-prepared, the chemotherapeutic discs (BD) were used in the test were chloramphenicol (CHL, 30μg), kanamycin (KAN， 30μg), neomycin (NEO, 30μg), doxycycline (DOX， 30μg), florfenicol (FLO, 30μg), TMP (5μg), compound sulfamethoxazole (SMZ23.75μg, TMP1.25μg), norfloxacin (NOR, 10μg), ciprofloxacin (CIP, 5μg) amoxicillin (AMO, 10μg), gentamicin (GEN, 10μg), erythrocin (ERY, 15μg). After 24h of incubation at 28°C, the diameters of the inhibition zone were measured by vernier caliper. Breakpoints of the antibiotics were interpreted using the Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (ninth edition) published by the Clinical and Laboratory Standards Institute (CLSI) (Cockerill et al. 2012).

Statistical Analysis

Data were shown as mean ± standard deviation (n = 3), and were compared with one-way ANOVA, followed by Tukey's *post hoc* test using the SPSS statistical software (version 20.0). Differences between each sample were recognized to be statistically significant at the $p < 0.05$ level.

Results

Isolation and MLST of *V. harveyi* and selection of strains for WGS

A total of 25 *V. harveyi* strains were isolated from the groundwater and diseased fish in three different fish farms (Figure 1, Table S3). In addition, a total of 60 fish feed samples from three fish farms were analyzed microbiologically but no *Vibrio* sp. was isolated. Together with 25 publicly available *V. harveyi* genomes, MLST typing subtyped 50 *V. harveyi* strains into nine Lineages and 32 sequence types (STs). MLST typing revealed that *V. harveyi* obtained from diseased fish in three fish farms can be sub-typed into 17 STs. MLST typing also showed that four STs (ST9, ST13, ST21 and ST32) obtained from diseased fish were also identified in the groundwater.

For each Cluster, one or two strains were selected. Then we performed WGS for nine strains; 2HWH0020 (Lineage I), 2HWH015 (Lineage II), 2HWH013 (Lineage III), 2HWH021 (Lineage IV), WHWH006 and 2HWH008 (Lineage V), DLP1171 (Lineage VII), and WHWH007 and 2HWH009 (Lineage VIII) (Figure 2A).

General statistics of the genomes and phylogenetic analysis of *V. harveyi* genomes

General statistics of the sequencing data are shown in **Table 1**. The strain 2HWH009 shows the largest genome size (6.24 Mb), whereas the strain WHWH006 displays the smallest one (5.97 Mb). The total number of coding sequences (CDS) ranged from 5,372 to 5,657 with an average G+C content of 44.8%.

Sequencing results also showed that plasmids were also present in eight out of nine strains with size ranging from 52.5 to 66.9 kb (Table 2). Five strains harbored a plasmid which have 71%-85% coverage to QT520 plasmid p3 (CP018684.2), suggesting they originated from a common ancestor. Meanwhile, strain 2HWH020 harbored a plasmid with 85%/96.3% coverage/identity to plasmid p345-67 (CP025540.1). Notably, strain 2HWH013, 2HWH015, and 2HWH008 all contained a 52.5 kb novel plasmid with only 15% coverage to QT520 plasmid p1 (CP018682.2)

Phylogenetic analysis based on core genome SNPs of *V. harveyi* by Maximum-likelihood (ML) method identified five Clusters (Cluster I to V). Strains from farms A and C were located at Cluster V, while strains from farm B located in Cluster III and IV (Figure 1B). In agreement with MLST results, strains belonged to the same ST were also clustered together in the genome tree. Specifically, WHWH007 and 2HWH009 from ST29, 2HWH015 and 2HWH013 from ST11, and WHWH006 and 2HWH008 from ST17 all belonged to three different clones.

Virulence difference of *V. harveyi* towards fish and its correlation with virulence gene profile

The challenged fish with nine *V. harveyi* isolates all began to develop typical signs for Vibriosis within 24 h. Disease fish showed a slightly darkened body with slow swam. Mass mortalities occurred from 36h. Fish necropsy showed clinical signs including an increase of bleeding and mucus; a large area of hemorrhage in the liver; a swollen and deeper-colored spleen; a slightly bleeding and swollen kidney, a yellow-colored intestinal wall, as well as some undigested food in the intestinal tract. However, the gill filaments showed no significant signs. The final mortality was distinct for different strains.

To identify the key virulence factors accounting for the virulence difference in the *V. harveyi* genome, we considered the gene sequences of two hundred forty putative prokaryotic virulence-related genes to identify possible similarity in nine strains that we sequenced. These genes encoded proteins involved in antiphagocytosis (*cpsABCD*); chemotaxis (*cheWBAZY*); colonization (*pilABCD*, Type IV-A pilus); flagellar biosynthesis (*flgABCDEFGHIJKLMN*, *fliADEFGHIJKLMNOPQRS*, *flhABF*); motility regulation (*motABXY)*; iron uptake (*hutR* and *hutA*) and toxin genes including *tlh* (encoding for Thermolabile hemolysin), and *ast* (Heat-stable cytotonic enterotoxin) (Table S4).

We found that strains harbored different sets of virulence genes (Table 3). Five genes including *mshB*, *pilA*, *hutR*, *ureB* and *ureG* were variably presented in nine sequenced strains which divided them into four groups with different virulence genes profiles.

The results revealed distinct virulence gene profiles strongly correlated with the

pathogenicity of *V. harveyi* towards fish. Strain DLP1171 harbored all five virulence genes exhibited the highest virulence towards fish. In contrast, lower virulence was observed in the strains from Profile 1 and 2, which is likely associated with the absence of *pilA*, *hutR*, and/or *mshB*. Likewise, Strains from Profile 3 also have relatively low virulence (73.3% to 75.0%) with the absence of *ureB* and *ureG*.

Other virulence-associated genes including *vhh*, *hcp*, and *chiA* were also detected in all sequenced *V. harveyi* genomes.

Genetic diversity of T3SS secretion systems

Next, we also analyzed if genetic diversity of T3SS also contributed to the distinct virulence level. Twenty-six of the *V. harveyi* strains analyzed in our study contain highly conserved T3SS1 gene. The $T3SS1_{vh}$ in *V. harveyi* strain QT520 contains 44 genes (locus tag BG259-RS01345 to BG259-RS01550 and BG259-RS06840 to BG259-RS06845) located on chromosome I. Comparative analysis of the $T3SS1_{vh}$ revealed the $T3SS1_{vh}$ cluster of *V. harveyi* is highly conservative. We observed variation in three sites in few strains (site one, site two and site three) as shown in Figure 3 in the QT520 $T3SS1_{vh}$ cluster.

The sequenced *V. harveyi* strains were used to infect HeLa cells to investigate the function of T3SS1*vh*. The *V. parahaemolyticus* strain ATCC17802 was used as a positive control with 85% cell lysis, while its T3SS1 mutant ATCC17802-IS*Val* was used as negative control with only 34% cell lysis (Figure 3B). By using the

LDH cytotoxicity assay, we registered similar cytotoxicity toward HeLa cells with around 80% of cell lysis among sequenced strains (P> 0.05).

Genetic diversity of T6SS

Next, we further identified three T6SSs in various *V. harveyi* strains, which was named T6SS1*vh*, T6SS2*vh* and T6SS3*vh*, respectively.

Twenty-nine strains carry T6SS1$_{vh}$ on chromosome I. T6SS1$_{vh}$ in strain QT520 contains 28 genes (BG259-RS03180 to BG259-RS03315). The majority of these strains harbored a conservative T6SS1$_{vh}$ Cluster except for two variation sites (Figure 4A). Based on the variations at two sites, T6SS1$_{vh}$ in *V. harveyi* can be divided into five genotypes (A to E), of which nine sequenced strains belonged to Genotype B. Additionally, CAIM1075 not only had tunicate site one and two, but also lost five genes at the end of T6SS1 cluster (Table S5).

T6SS2$_{vh}$ was identified in twenty-five strains *V. harveyi* and located on chromosome II. The QT520 T6SS2$_{vh}$ cluster contains 23 genes (locus tags from BG259-RS20355 to BG259-RS20465). T6SS2$_{vh}$ was intact in QT520 as well as four sequenced strains (2HWH008, 2HWH009, 2HWH021 and DLP1171), while deletion of BG259-RS20460 or BG259-RS20430 were found in the strain WHWH006 and remaining four strains, respectively (Figure 4B).

Thirty-four genes constitute T6SS3$_{vh}$ in QT520 from BG259-RS21945 to BG259-RS22115, in which BG259-RS22105 is a MIX effector, and BG259-RS22100 is its cognate immunity protein. BG259-RS22095 is a protein with unknown function that is secreted in a T6SS1$_{vh}$-dependent manner and contains a predicted peptidoglycan binding domain like the C-terminal domain of outer membrane protein *OmpA*. T6SS3$_{vh}$ was distributed in 13 strains of *V. harveyi* in this study. Comparative analysis of the T6SS3$_{vh}$ of *V. harveyi* revealed significant variations at two different sites. Site one encompasses genes BG259-RS22115 to BG259-RS22085, which the main variation was due to the deletion or insertion of genes. Site two is the highly variable region in T6SS3$_{vh}$ cluster, which is highly variable and distinct in almost every strain (Figure 4C). Among nine sequenced strains, only strains 2HWH009 and WHWH007 harbored T6SS3.

Phenotype difference among *V. harveyi* strains

Next, we tested the phenotypic difference among the nine sequenced strains to determine whether the virulence gene profile resulted in distinct phenotype. Resulted shown that there are significant differences in the motility among *V. harveyi* strains in the presence of haem content, of which the strain DLP1171 had the largest swimming halo and was significantly higher than other strains ($P < 0.01$) (Figure 5A), following by strains 2HWH008 and WHWH006. Strains 2HWH020, 2HWH013 and 2HWH015 have weakest the motility compared with other strains ($P<0.01$).

Biofilm formation assays showed that the biomass of biofilms for strains DLP1171, WHWH006 and 2HWH008 was significantly greater than other strains, indicating they have higher colonization ability ($P<0.01$). The biofilm formation ability of strain 2HWH013 is the weakest (Figure 5B). In addition, strains 2HWH020, 2HWH015 and ATCC33852 exhibited similar biofilm formation ($P>0.05$).

To validate whether the variations in T6SS affect the antibacterial function, we conducted a co-culture assay for each of sequence strain with *V. parahaemolyticus* strain ATCC17802. The co-culture assay showed that the growth rate of single-culture ATCC17802 were significantly higher than those co-cultured with various *V. harveyi* strain, indicating the antibacterial activities is functional for all sequenced strains. However, there was no significant difference among the nine strains in terms of growth rate, indicating the genetic variations in T6SS have no significant impact on the antibacterial function (Figure 5C).

Antibiotic resistance (AR) profiles and antibiotic resistance genes in the sequenced strains

Overall, the *V. harveyi* strains isolated from *Takifugu rubripes* and Turbot showed similar AR profiles. All of the *V. harveyi* strains were sensitive to compound sulfamethoxazole, chloramphenicol, and florfenicol, and resistant to norfloxacin and amoxicillin (Table S6). Genomic analysis revealed that sequenced strains 2HWH009 carried *blaTEM-B* (penicillinase) which was not identified in other sequenced strains.

Discussion

In this study, we undertook a systemic survey on the genetic diversity and virulence profiles of *V. harveyi* in three fish farms in Liaoning province. *V. harveyi* was predominantly found in both diseased fish and nearby groundwater. We first performed MLST to delineate the origins of the pathogens in the three fish farms. We demonstrated that concatenated sequences of five *Vibrio* housekeeping genes subtyped 54 *V. harveyi* strains into 37 STs. To better understand the genetic diversity of *V. harveyi*, we selected nine *V. harveyi* strains for genomic sequencing. Maximum likelihood method was employed to infer the phylogenetic tree using the core genome SNPs of *V. harveyi*. (**Figure 2B**). Phylogenomic analysis revealed a clear divergence among the nine sequenced strains, suggesting *V. harveyi* in three farms is genetically diverse. Another interesting finding is that several *V. harveyi* strains from different countries belonged to the same clone, indicating possible transmission of *V. harveyi* between countries by seafood trade. For example, strain CAIM464 and CAIM463 differing by 23 SNPs come from Greece and Spain, respectively. Likewise, DLP1112 (China) and ATCC14126 (USA) also possibly came from the same source.

Many advances have been made in the knowledge of environmental persistence of *V. harveyi* and other pathogenic *Vibrio* spp. in the aquatic reservoirs (Zhu et al. 2018), Numerous studies also revealed the potential virulence factors of *V. harveyi* that involved in fish infection through WGS (Espinoza-Valles et al. 2012; Tu et al. 2017). However, these studies do not demonstrate how these virulence-related genes

contribute to the virulence level. The lack of clarity about key virulence factors affecting the pathogenesis of *V. harveyi* is one of the main problems facing researchers. This work analyzed the key virulence factors affecting the virulence of *V. harveyi* by both phenotypic and genotypic analysis. Genomic analysis revealed that the difference in the genes associated with Type IV-A pilus and iron acquisition might contribute to the subtle variations in pathogenicity for nine sequenced strains. The *pilA* gene from the *pilABCD* gene cluster was also absent from strains in Profile 1 and 2. This gene cluster encodes proteins for the Type IV-A pilus, which is essential for the secretion of Hap and colonization of infant mice or adherence to HEp-2 cells (Fullner and Mekalanos 1999). Type IV pilus is found on the surface of a variety of Gram-negative bacteria and have been demonstrated to be important host colonization factors. Previous research showed that inactivation of *pilA* reduced the ability of *V. vulnificus* to form biofilms and significantly decreased adherence to human epithelial cells (HEp-2) and virulence in a mouse model (Paranjpye and Strom, 2005). In addition, PilA also was found to contribute to persistence of the bacterium in oysters (Paranjpye et al., 2007). Biofilm formation assay confirmed that the strains with the deletion of *pilA* have significant lower biofilm. Thus, the absence of *pilA* gene might significantly reduce the attachment to the host, resulting in a low infection rate. Likewise, the absence of *mshB* (encoding Mannose-sensitive hemagglutinin (MSHA)) would also affect the functionality of MSHA type IV pilus.

hutR encoding haem receptors is absent in most of strains except for DLP1171. Mey and Payne (2011) suggested that a *hutA* mutant in *V. cholerae* only had a slight defect

504 in growth using haemin as the iron source. *V. cholerae* encodes two haem receptors,
505 *HutR* and *HasR*, while in this study we found *V. harveyi* harbored HutR and HutA
506 instead. As *HutR* has significant homology to *HutA* as well as to other outer
507 membrane haem receptors, the absence of *hutR* might not have significant impact on
508 their virulence. Motility assay confirmed that the strains with the deletion of *hutR*
509 have significant lower swimming halo, suggesting it is the major contributors in the
510 determination of pathogenicity in *V. harveyi* strains.

511

512 Berutti et al. (2014) suggested that urease in *V. parahaemolyticus* strains enhanced
513 their ability to grow in seawater and thus create alkaline microhabitats. This function
514 could enhance survival of *V. harveyi* in the environment. However, whether the
515 presence of *ureB* and *ureG* is vital to the infection of fish remains unclear.

516

517 The further comparative genomic analysis suggested that secretion system is highly
518 diverse among *V. harveyi* isolates. We noticed that the T3SS1 were genetically
519 conserved among genomes: 80% of *V. harveyi* isolated included in this study. It
520 mainly includes regulatory proteins LcrF and LscZ, molecular chaperone YopN and
521 YopD, Type III secretion spans bacterial envelope protein YscO and YscG, secretion
522 protein YscP and YscE, type III effector protein YopR and YopB.

523

524 The gene content and structure of T3SS1 in *V. harveyi* ($T3SS1_{vh}$) and *V.*
525 *parahaemolyticus* ($T3SS1_{vp}$) are very similar and conserved. However, *V. harveyi*

526 T3SS1$_{vh}$ contains 42 genes located on chromosome 1. The loci harbour three variable
527 sites able to discriminate four subtypes of strains. T3SS1$_{vp}$ contains 51 genes, 49
528 genes located on chromosome 1 and 2 genes on chromosome 2, including the variable
529 site (*vp1676-vp1679*) that discriminate into two subtypes strain.
530
531 Aside from conserved T3SS, we analyzed the fine structure T6SS1 in sequenced
532 genomes. Consistently with the previous observation, all the three sites contain genes
533 encoding the RIMD2210633 T6SS1 effectors. The T6SS1 effector repertoire varies
534 between the different *V. parahaemolyticus* isolated (Li et al. 2017).
535
536 In this study, we discovered 3 types of T6SSs *V. harveyi* strains, named T6SS1$_{vh}$,
537 T6SS2$_{vh}$, and T6SS3$_{vh}$. T6SS1$_{vh}$ and T6SS2$_{vh}$ are located on chromosome 1, while
538 T6SS3$_{vh}$ is located on chromosome 2. Compared with other *Vibrio* sp. genome,
539 T6SS1$_{vh}$ is exclusively presented in *V. harveyi* and contains three variable sites.
540 Genetic content and structure of T6SS2$_{vh}$ were highly similar to T6SS2 in *V.*
541 *parahaemolyticus* (T6SS2$_{vp}$), indicating they shared a common ancestor. Nevertheless,
542 T6SS2$_{vh}$ contains two additional variable genes. T6SS3$_{vh}$ corresponds to T6SS1$_{vp}$.
543 T6SS3$_{vh}$ were located on chromosome 2 and contain two variable sites. Likewise, Li
544 et al. (2017) also suggested that T6SS1$_{vp}$ was highly variable among *V.*
545 *parahaemolyticus* strains and identified two variable sites in T6SS1$_{vp}$ among *V.*
546 *parahaemolyticus* strains, which were designated Site one and Site two, respectively.
547 In *V. parahaemolyticus* reference strain RIMD221063, Site one encompasses genes

548 vp1388 to vp1390. *vp1388* encodes a major T6SS1$_{vp}$ effector with a MIX domain,
549 while vp1389 is its cognate immunity protein. VP1390 is a T6SS1$_{vp}$ secreted protein
550 containing a peptidoglycan-binding domain. Site two spans *vp1415* to *vp1420*.
551 VP1415 is another important T6SS1$_{vp}$ effectors, while VP1416 is its cognate
552 immunity protein. Two variable sites in T6SS3$_{vh}$ are exactly corresponded to Site one
553 and Site two in T6SS1$_{vp}$, indicating these two sites both were prone to genetic
554 exchange.
555
556 The type VI secretion system (T6SS) is recognized as an important defense
557 mechanism in several Gram-negative pathogens. For instance, Miyata et al. (2011)
558 reported that VasX, secreted by type VI secretion system to kill a protozoa
559 *Dictyostelium discoideum*. Sheng et al. (2012) found that the expression of a
560 hemolysin coregulated protein (Hcp1) from T6SS, was found to be strictly regulated
561 in *Vibrio alginolyticus*. Likewise, Sun et al. (2019) also found TssJ from the T6SS of
562 *V. harveyi* is a putative antigen, which has the potential as a vaccine against infection.
563 Our study found T6SS1 and T6SS2 were highly conserved among different *V. harveyi*
564 strains and suggested that the all of the regulatory genes were highly conserved
565 among the *V. harveyi* genomes, which also served as a potential target for disease
566 control of Vibriosis. Therefore, it is of significant interest to further search for
567 anti-virulence agents targeting the virulence genes associated with colonization and
568 attachment as a novel biocontrol strategy for animal production.

569 **Conclusion**

570 This study conducted a genomic and phenotypic analysis to investigate the genetic
571 diversity of *V. harveyi* in Tiger puffer and Turbot fish farms and to delineate the key
572 virulent determinant of *V. harveyi*. Comparative genomic analysis indicated that the
573 virulence difference of *V. harveyi* is mainly determined by the presence of *mshB*, *pilA*,
574 *ureB* and *ureG*, implying that these genes may play vital roles in environmental
575 adaptation for *V. harveyi.* In particular, our discovery provides novel insights into the
576 genetic divergence of T6SS in *V. harveyi*. Further research is needed to quest for
577 novel antivirulence agents, i.e. inhibitors of either natural or synthetic origin to
578 interfere with the expression of virulence genes.

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584 **Conflict of interest**

585 The authors declare that there is no conflict of interest.

586 **Ethical approval**

587 All applicable international, national, and/or institutional guidelines for the care and
588 use of animals were followed.

589

590 **Reference**

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839 **Figure Legend**

840

841 **Figure 1** Sampling sites in this study. The sampling positions in Dalian, Huludao, and
842 Zhuanghe are indicated in the square. The distribution of the isolates in different
843 seasons was shown in pie chart. The sampling sites were mapped by the ArcGIS
844 Desktop 10.2 software.

845

846 **Figure 2. Phylogenetic relationship of *V. harveyi* strains. Phylogenetic**
847 **relationship of 50 *V. harveyi* strains based on the sequences of six concatenated**
848 **house-keeping genes (A).** The neighbor-joining method was used to infer the
849 evolutionary relationships of the isolates. The bootstrap was performed with 1000
850 replicates. Distribution of polymorphic nucleotide sites among 50 concatenated
851 sequences of *V. harveyi* strains were showed in the right side. The five housekeeping
852 genes of each strain correspond to one of the five bands, each vertical line in the band
853 represents a mutation site (relative to ATCC 14126), and the density of the vertical
854 line in the band clearly reflects the relative position of the mutation site. Strains with
855 pentagram are selected for whole genome sequencing. **Phylogenomic relationship of**
856 **34 *V. harveyi* strains (B).** The maximum likelihood method was used to infer the
857 evolutionary relationships of the isolates based on their SNPs obtained from *V.*
858 *harveyi* core genome. The bootstrap was performed with 1000 replicates. The unit of
859 the scale bar indicates the evolutionary distance in substitutions per nucleotide.
860 Genomic analysis divided 34 *V. harveyi* into five Clusters designated as Cluster I to V.
861 Strains from farm A and C were located at Cluster V, while strains from farm B
862 located in Cluster III and IV.

863

864 **Figure 3. Genetic variation and cytotoxicity of T3SS1.** Schematic representations
865 comparison of T3SS1 clusters of *V. harveyi*. T3SS1 in QT520 strain were used as

reference **(A)**. QT520 genome locus numbers are shown above and gene names below. Genes with variations between the strains analyzed are highlighted with different colors. Thirty-one *V. harvryi* strains analyzed in this study harbored a highly conserved T3SS1. Cytotoxicity assay of *V. harveyi* strains contain a conserved T3SS1 **(B)**. HeLa cells were infected with the indicated *V. parahaemolyticus* strains for 4 h at a MOI of 10. Lactate dehydrogenase (LDH) release was evaluated as the measure of cytotoxicity against host cells. Error bars indicate the standard deviations. Data are representative of three independent experiments.

Figure 4. Schematic representations comparison of T6SS1 (A), T6SS2 (B), and T6SS3(C) clusters. T6SS1, T6SS2, and T6SS3 in QT520 strain were used as reference. QT520 genome locus numbers are shown above and gene names below. Genes with variations between the strains analyzed are highlighted with different colors. Genes in QT520 strain are labeled in white when they are the same or highly conserved but in other colors when otherwise. The same color indicates conserved genes (90% amino acid identity). The missing genes are indicated by dash lines. The size of triangle represented the length of genes.

Figure 5 Phenotype assays of sequenced *V. harveyi* strains. A: Swimming motility assay; B: Biofilms formation in 24-well plates; C: Growth curve of *Vibrio parahaemolyticus* with or without each *V. harveyi* strain. VP-2HWH021 to VP-DLP1171: growth curve of *V. harveyi* strain co-cultured with *V. parahaemolyticus* strain ATCC17802. VP: single culture of *V. parahaemolyticus* strain ATCC17802 was used as control. Data are representative of three independent experiments. Error bar was omitted for clarity. Colony-forming units (CFU) of *V. parahaemolyticus* were determined in each interval.

892

Table 1. General features of sequenced *V. harveyi* genomes

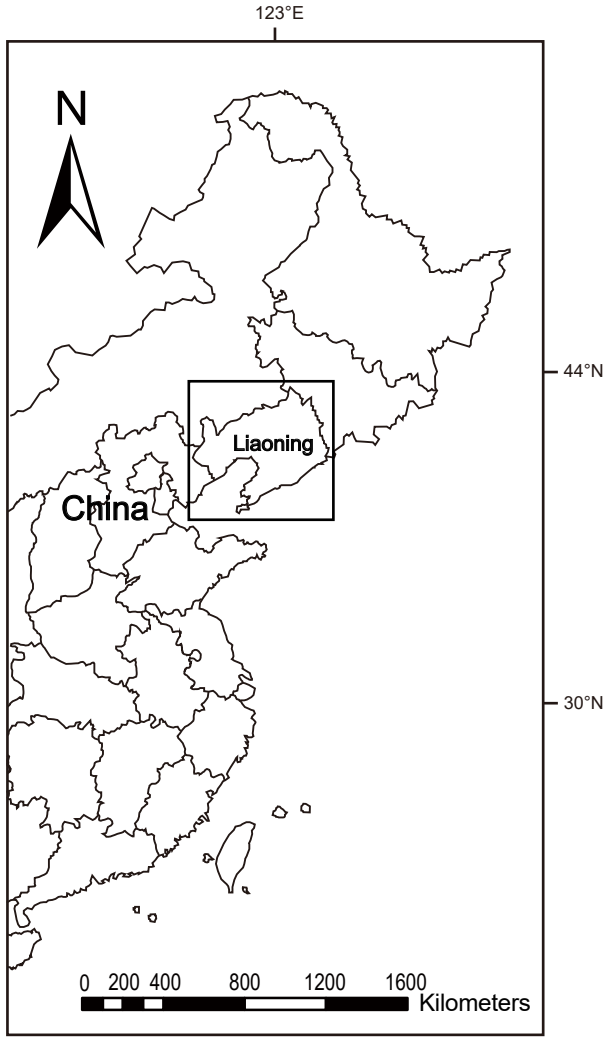
Strain Name	Total Length (bp)	Coverage (X)	GC content	N50	Number of Contigs	Number of Coding Sequences	Number of RNAs
2HWH021	5, 996, 903	46	44.8	455, 428	49	5, 406	116
2HWH020	6, 122, 059	53	44.8	501, 224	89	5, 561	127
2HWH015	6, 048, 939	40	44.8	297, 567	67	5, 432	111
2HWH013	6, 043, 515	40	44.8	297, 567	66	5,431	111
2HWH009	6, 245, 879	45	44.8	428, 775	72	5, 657	113
2HWH008	6, 060, 798	47	45	474, 109	17	5, 418	113
WHWH007	6, 127, 018	50	44.8	482, 813	84	5, 526	114
WHWH006	5, 973, 839	49	44.8	479, 193	47	5, 372	118
DLP1171	6, 056, 611	55	44.8	516, 227	64	5, 496	117

Table 2. General information of plasmids identified in nine strains.

Strain	plasmid name	Size(kb)	Highest Coverage %	Highest Identity %	Similar plasmid	Accession No.
2HWH009	pVH09-1	54.5	71	98.3	QT520 plasmid p3	CP018684.2
WHWH007	pVH07-1	54.5	71	98.3	QT520 plasmid p3	CP018684.2
2HWH021	pVH21-1	66.9	77	96.9	QT520 plasmid p3	CP018684.2
2HWH013	pVH13-1	52.5	15	98.5	QT520 plasmid p1	CP018682.2
2HWH015	pVH15-1	52.5	15	98.5	QT520 plasmid p1	CP018682.2
WHWH006	pVH06-1	59.8	85	94.6	QT520 plasmid p3	CP018684.2
2HWH008	pVH08-1	59.8	85	94.6	QT520 plasmid p3	CP018684.2
	pVH08-2	52.5	15	98.5	QT520 plasmid p1	CP018682.2
2HWH020	pVH20-1	54.1	85	96.5	p345-67	CP025540.1

Table 3. Virulence difference of *V. harveyi* towards fish and its correlation with virulence gene profile

Strain	Sequence Type	Mortality rate (%)		Mannose-sensitive hemagglutinin (MSHA type IV pilus)			Type IV pilus				Heme receptors		Thermolabile hemolysin	Heat-stable cytotonic enterotoxin	Urease		Profile
		36 h	72 h	*mshA*	*mshB*	*mshD*	*pilA*	*pilB*	*pilC*	*pilD*	*hutA*	*hutR*	*tlh*	*ast*	*ureB*	*ureG*	
2HWH020	ST9	35.0±0.0	43.3±1.5	+	-	+	-	+	+	+	+	-	+	-	+	+	1
2HWH013	ST14	23.3±1.15	30.8±2.5	+	-	+	-	+	+	+	+	-	+	+	+	+	
2HWH015	ST12	33.3±0.0	40.8±2.5	+	-	+	-	+	+	+	+	-	+	+	+	+	
2HWH021	ST17	46.6±0.58	55.5±3.0	+	+	+	-	+	+	+	+	-	+	+	+	+	2
2HWH009	ST36	40.0±1.0	51.7±3.0	+	+	+	-	+	+	+	+	-	+	+	+	+	
WHWH007	ST36	36.7±0.58	57.5±3.0	+	+	+	-	+	+	+	+	-	+	+	+	+	
2HWH008	ST21	63.3±0.58	73.3±3.0	+	+	+	+	+	+	+	+	+	+	+	-	-	3
WHWH006	ST21	70.0±1.0	75.0±2.0	+	+	+	+	+	+	+	+	+	+	+	-	-	
DLP1171	ST32	78.3±0.58	92.5±1.0	+	+	+	+	+	+	+	+	+	+	+	+	+	4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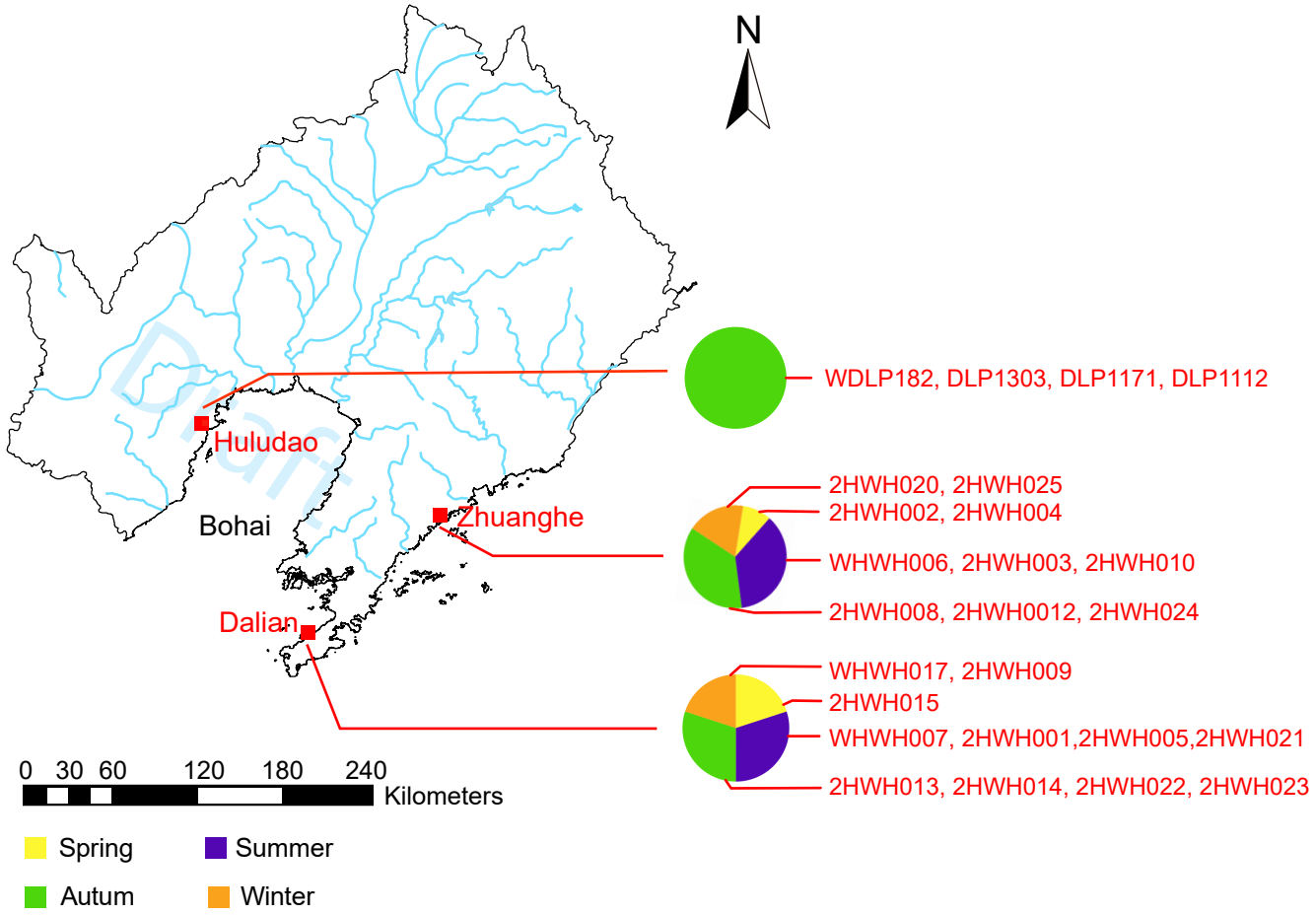
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Dalian
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WHWH006, 2HWH003, 2HWH010
2HWH008, 2HWH0012, 2HWH024
WHWH017, 2HWH009
2HWH015
WHWH007, 2HWH001,2HWH005,2HWH021
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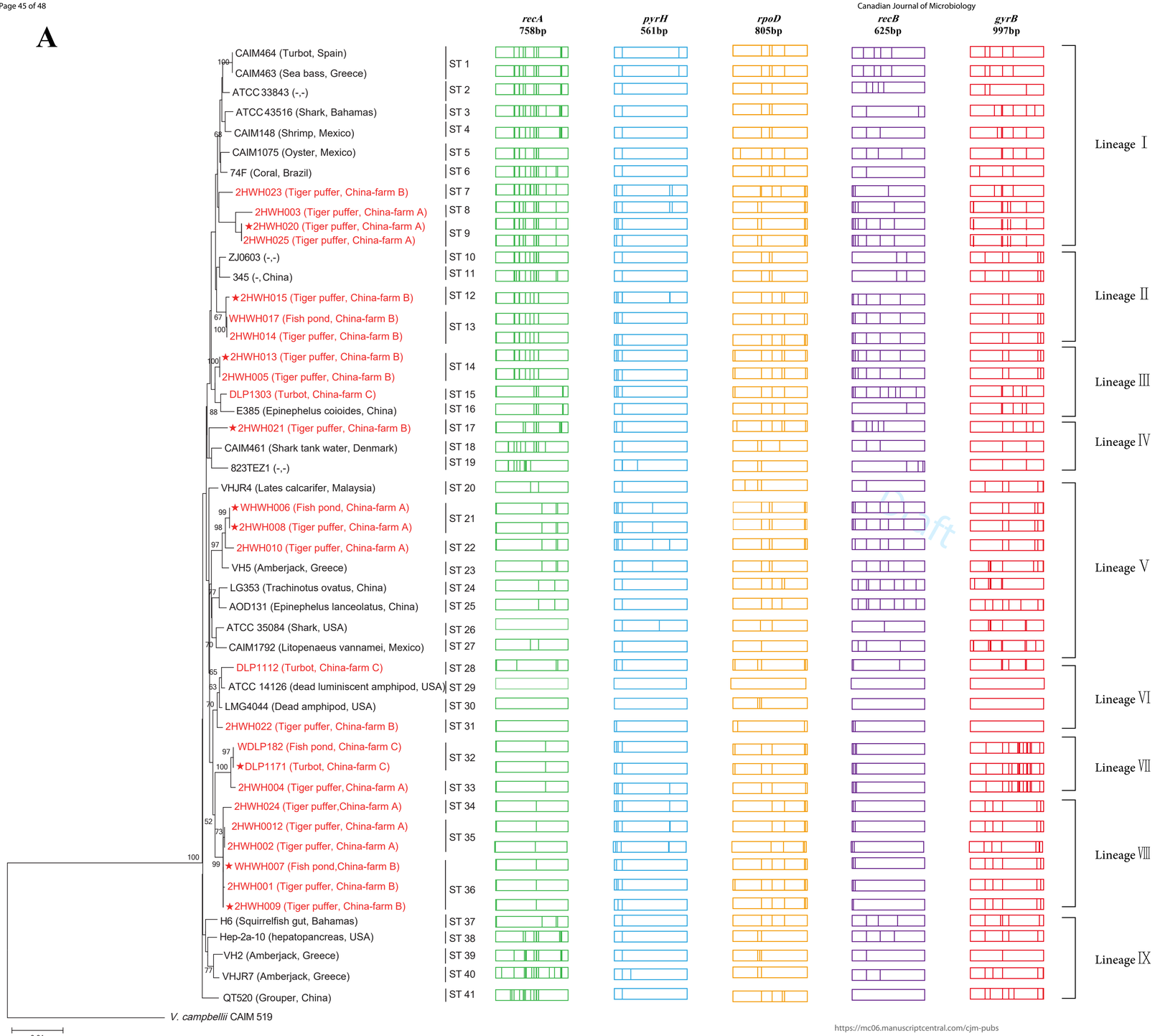
Kilometers
Spring
Summer
Autum
Winter

A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pyrH 561bp
rpoD 805bp
recB 625bp
gyrB 997bp
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ATCC 43516 (Shark, Bahamas)
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CAIM1075 (Oyster, Mexico)
74F (Coral, Brazil)
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ZJ0603 (-,-)
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2HWH005 (Tiger puffer, China-farm B)
DLP1303 (Turbot, China-farm C)
E385 (Epinephelus coioides, China)
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CAIM461 (Shark tank water, Denmark)
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VHJR4 (Lates calcarifer, Malaysia)
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★2HWH008 (Tiger puffer, China-farm A)
2HWH010 (Tiger puffer, China-farm A)
VH5 (Amberjack, Greece)
LG353 (Trachinotus ovatus, China)
AOD131 (Epinephelus lanceolatus, China)
ATCC 35084 (Shark, USA)
CAIM1792 (Litopenaeus vannamei, Mexico)
DLP1112 (Turbot, China-farm C)
ATCC 14126 (dead luminiscent amphipod, USA)
LMG4044 (Dead amphipod, USA)
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★DLP1171 (Turbot, China-farm C)
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2HWH0012 (Tiger puffer, China-farm A)
2HWH002 (Tiger puffer, China-farm A)
★WHWH007 (Fish pond,China-farm B)
2HWH001 (Tiger puffer, China-farm B)
★2HWH009 (Tiger puffer, China-farm B)
H6 (Squirrelfish gut, Bahamas)
Hep-2a-10 (hepatopancreas, USA)
VH2 (Amberjack, Greece)
VHJR7 (Amberjack, Greece)
QT520 (Grouper, China)
V. campbellii CAIM 519
ST 1
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ST 35
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ST 39
ST 40
ST 41
Lineage Ⅰ
Lineage Ⅱ
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Lineage Ⅴ
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Lineage Ⅶ
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Lineage Ⅸ
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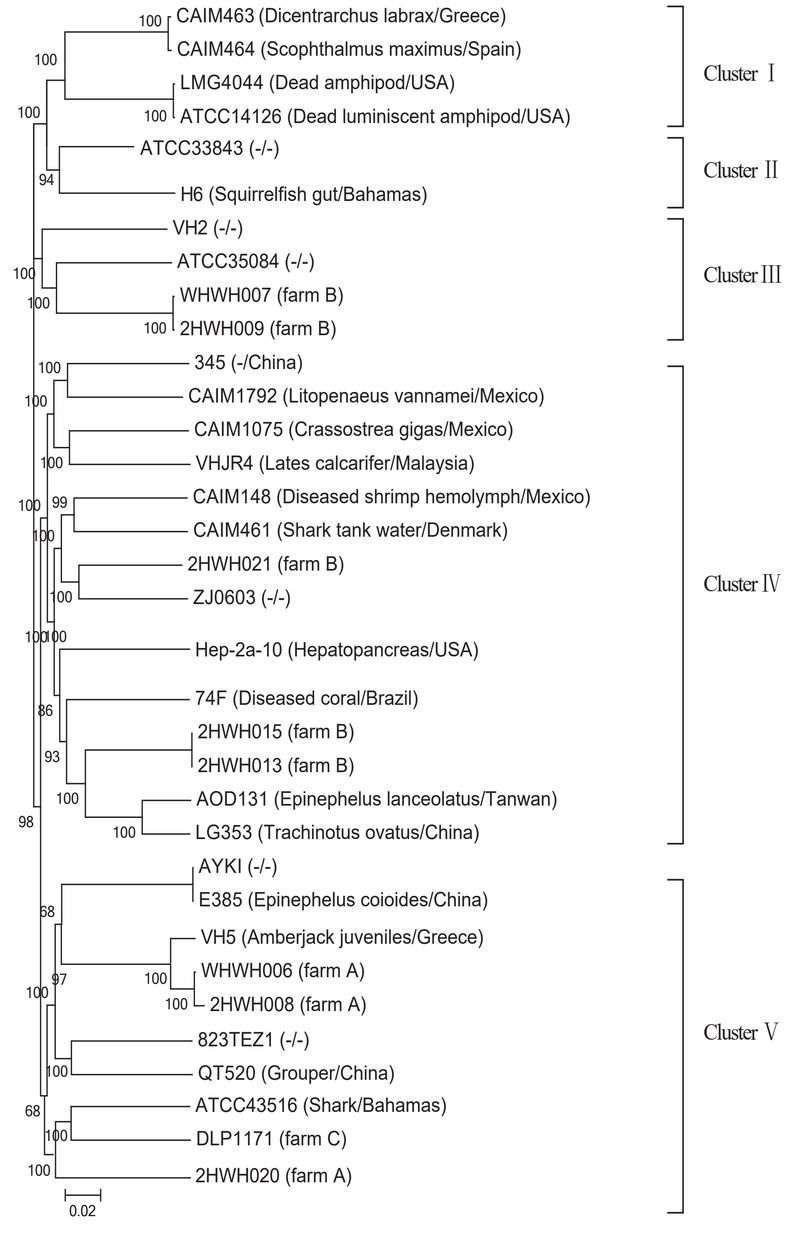
B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H6 (Squirrelfish gut/Bahamas)
VH2 (-/-)
ATCC35084 (-/-)
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2HWH009 (farm B)
345 (-/China)
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CAIM1075 (Crassostrea gigas/Mexico)
VHJR4 (Lates calcarifer/Malaysia)
CAIM148 (Diseased shrimp hemolymph/Mexico)
CAIM461 (Shark tank water/Denmark)
2HWH021 (farm B)
ZJ0603 (-/-)
Hep-2a-10 (Hepatopancreas/USA)
74F (Diseased coral/Brazil)
2HWH015 (farm B)
2HWH013 (farm B)
AOD131 (Epinephelus lanceolatus/Tanwan)
LG353 (Trachinotus ovatus/China)
AYKI (-/-)
E385 (Epinephelus coioides/China)
VH5 (Amberjack juveniles/Greece)
WHWH006 (farm A)
2HWH008 (farm A)
823TEZ1 (-/-)
QT520 (Grouper/China)
ATCC43516 (Shark/Bahamas)
DLP1171 (farm C)
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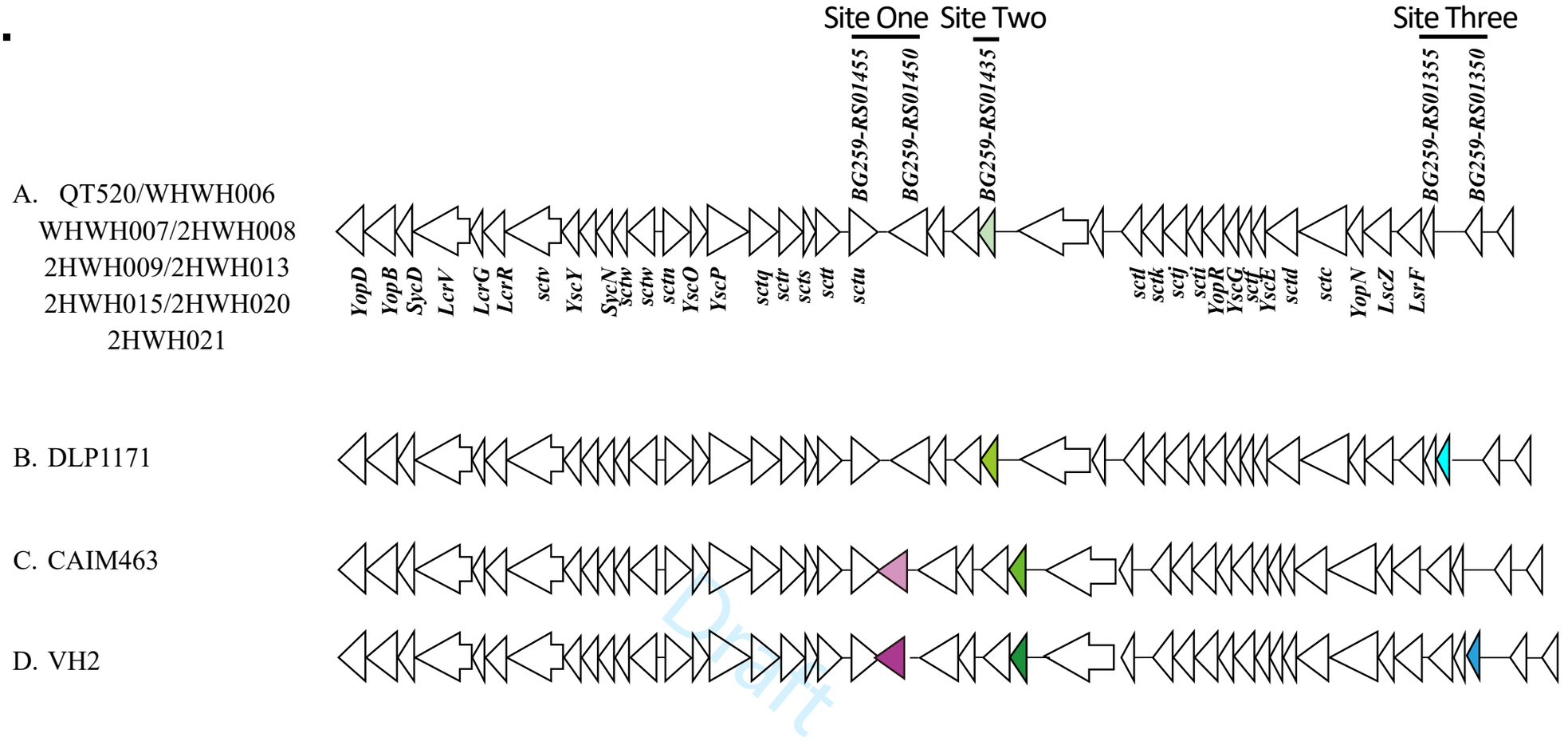
Cluster Ⅰ
Cluster Ⅱ
Cluster Ⅲ
Cluster Ⅳ
Cluster Ⅴ
0.02

A.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Site Two
Site Three
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2HWH009/2HWH013
2HWH015/2HWH020
2HWH021
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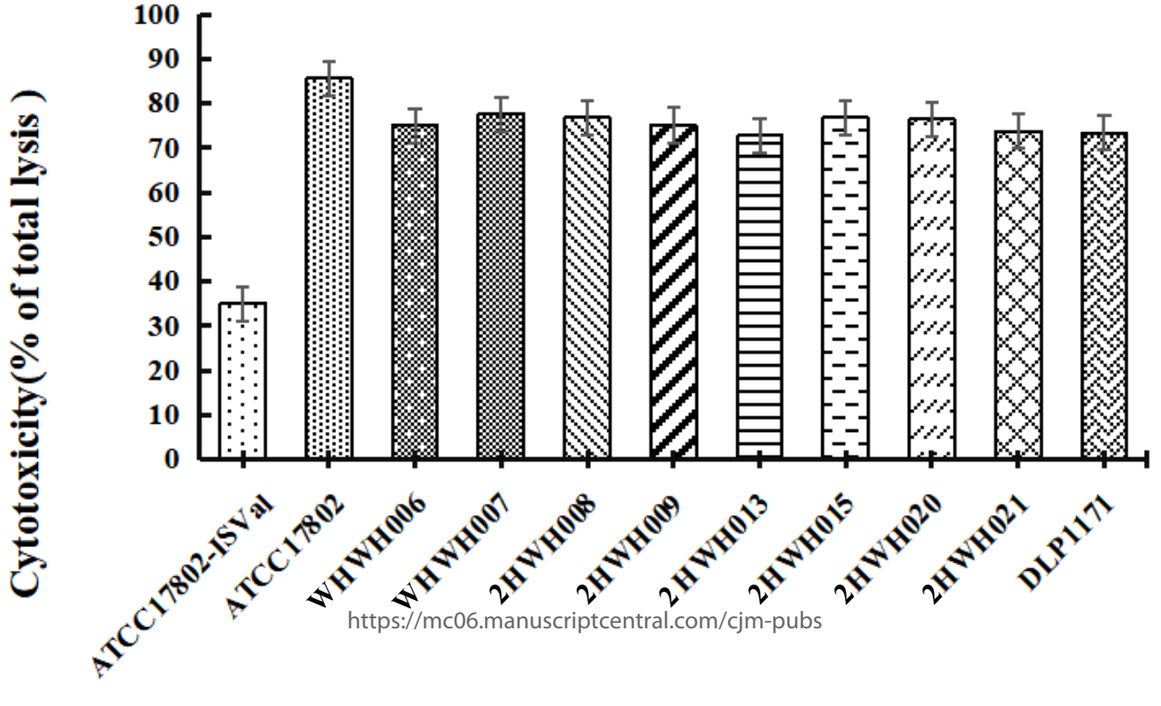
B. DLP1171
C. CAIM463
D. VH2

B.


Cytotoxicity(% of total lysis)
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90
80
70
60
50
40
30
20
10
0
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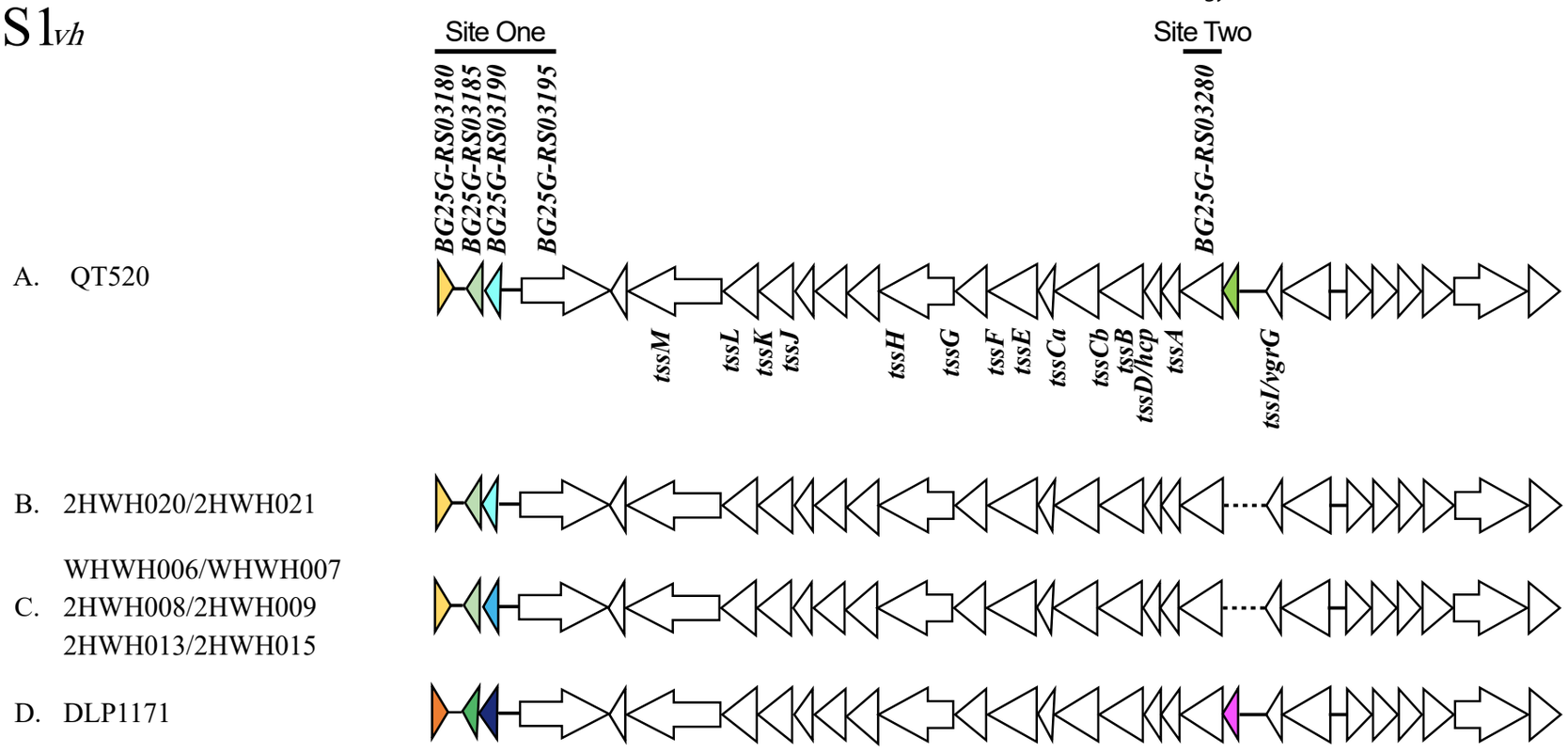
DLP1171

T6SS1*vh*



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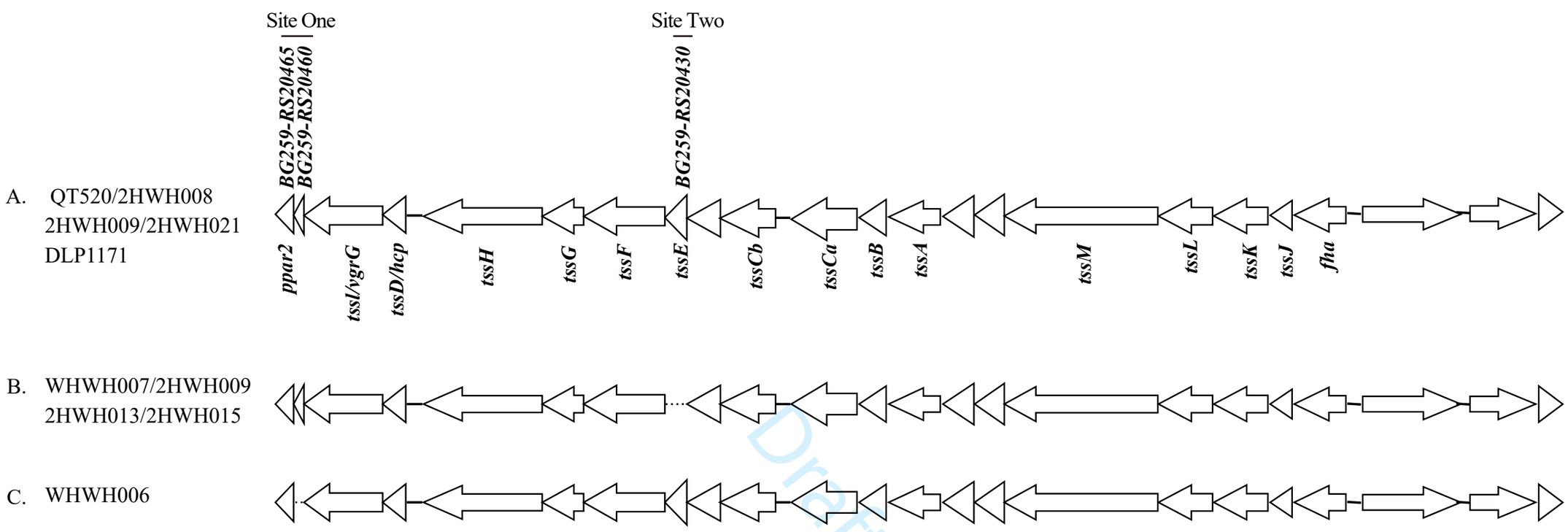

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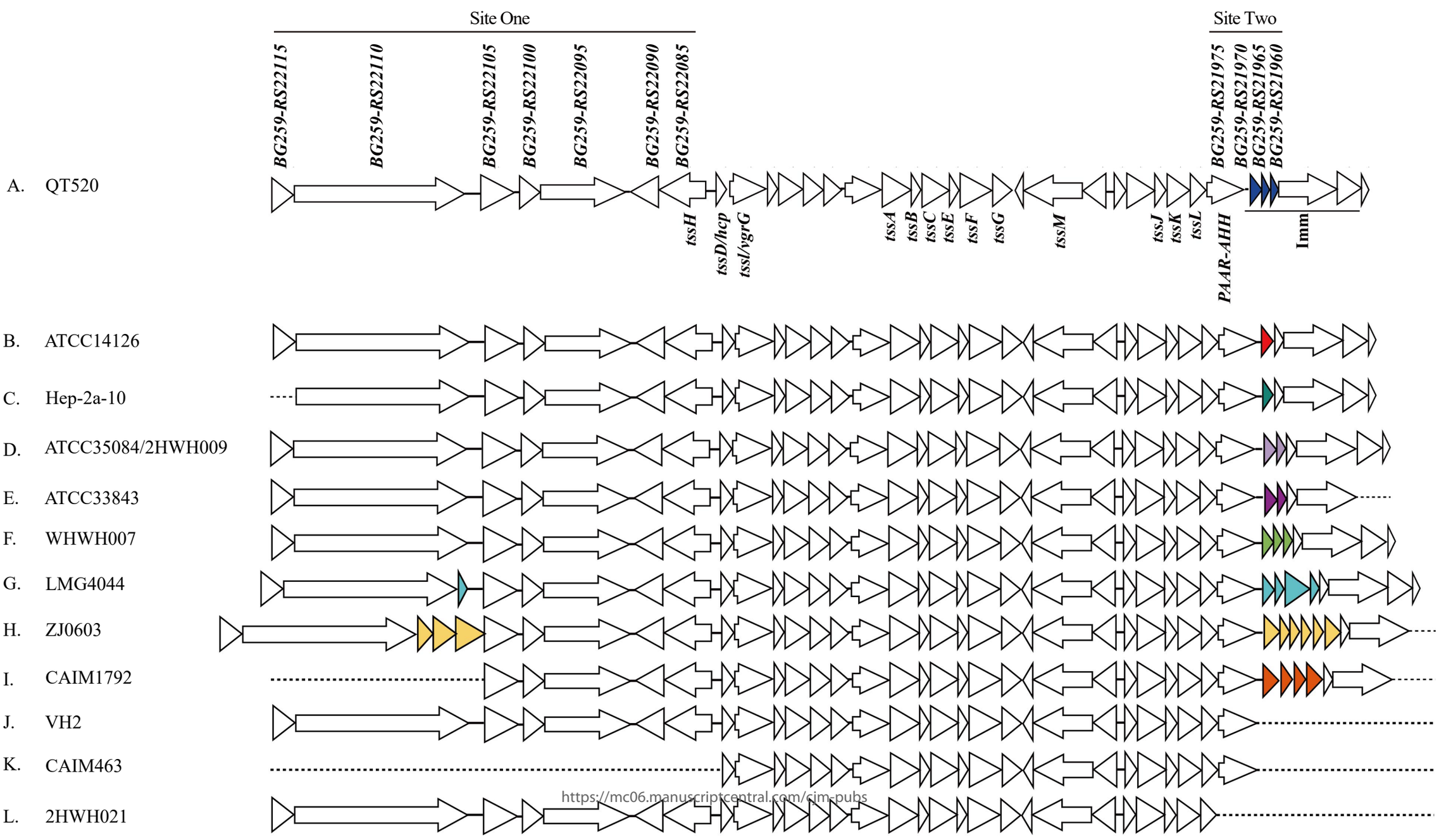

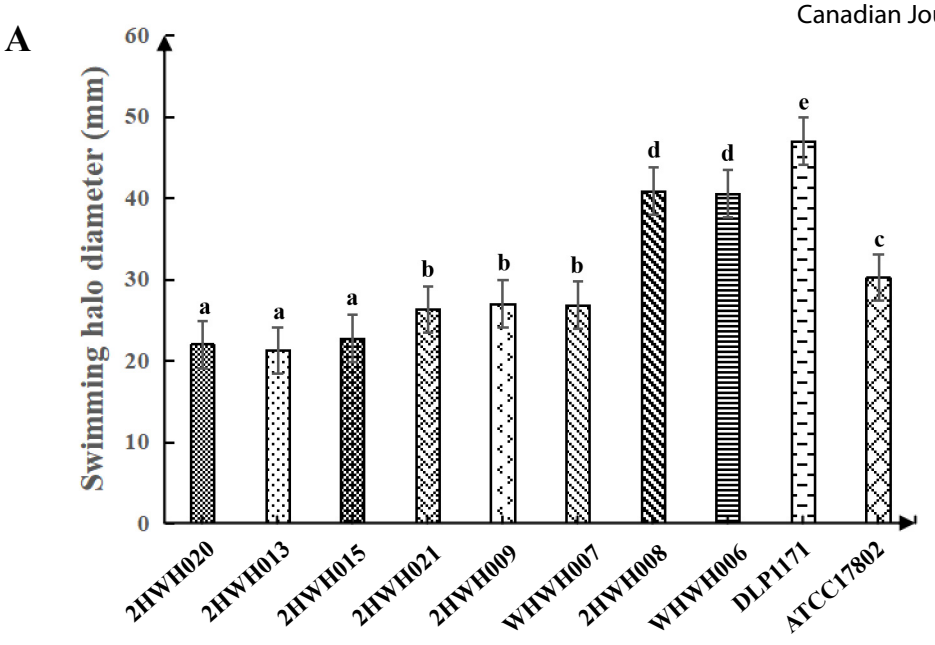
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10
20
30
40
50
60
a
a
a
b
b
b
d
d
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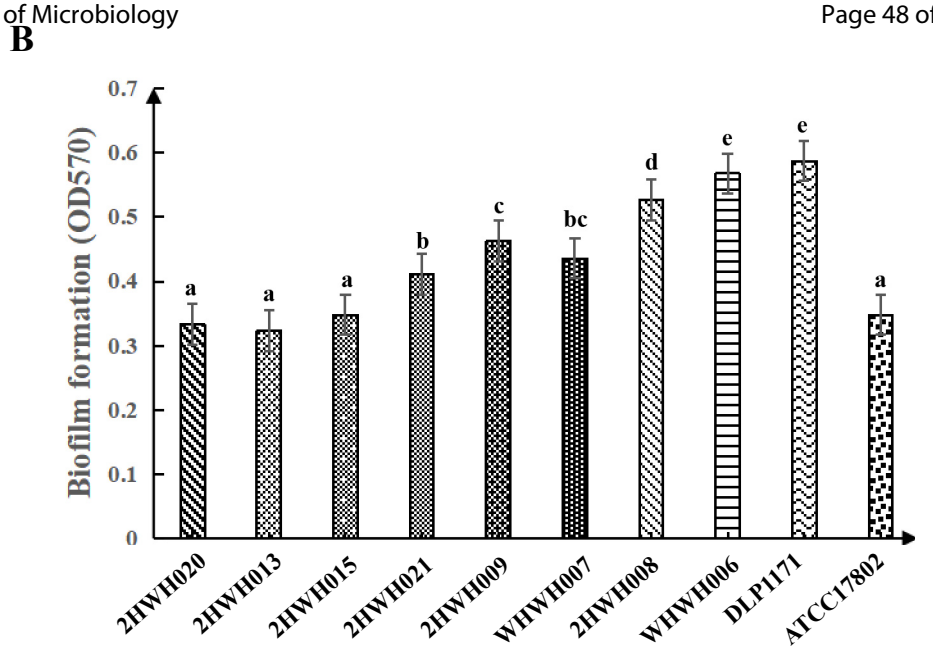
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0.7
a
a
a
b
c
bc
d
e
e
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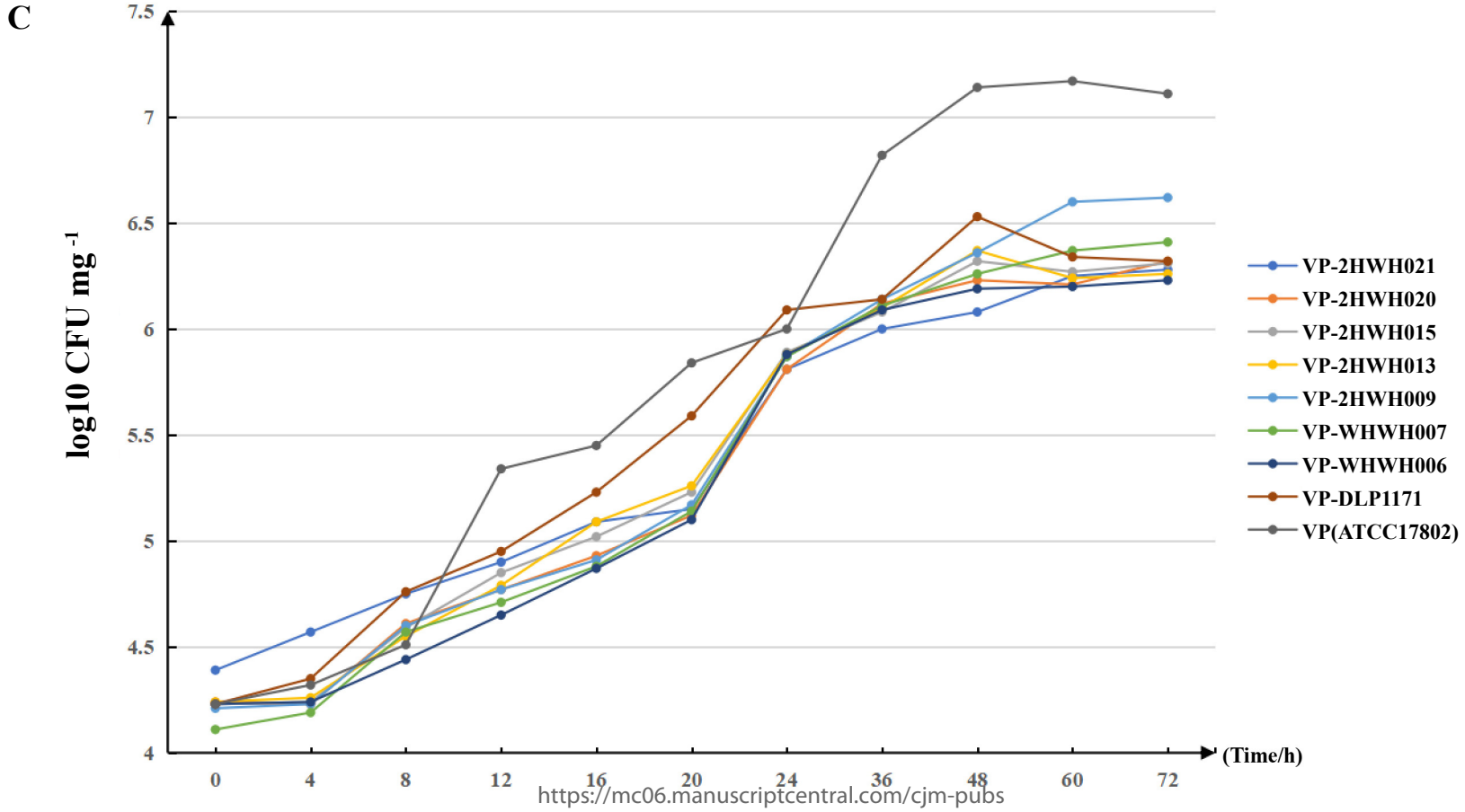
ATCC17802


C
log10 CFU mg-1
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4.5
5
5.5
6
6.5
7
7.5
0
4
8
12
16
20
24
36
48
60
72
(Time/h)
VP-2HWH021
VP-2HWH020
VP-2HWH015
VP-2HWH013
VP-2HWH009
VP-WHWH007
VP-WHWH006
VP-DLP1171
VP(ATCC17802)