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Research paper

Comparative genome analysis of fish pathogen *Flavobacterium columnare* reveals extensive sequence diversity within the species

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ABSTRACT

Flavobacterium columnare is one of the deadliest fish pathogens causing devastating mortality in various freshwater fish species globally. To gain an insight into bacterial genomic contents and structures, comparative genome analyses were performed using the reference and newly sequenced genomes of *F. columnare* including genomovar I, II and I/II strains isolated from Thailand, Europe and the USA. Bacterial genomes varied in size from 3.09 to 3.39 Mb (2714 to 3101 CDSs). The pan-genome analysis revealed open pan-genome nature of *F. columnare* strains, which possessed at least 4953 genes and tended to increase progressively with the addition of a new genome. Genomic islands (GIs) present in bacterial genomes were diverse, in which 65% (39 out of 60) of possible GIs were strain-specific. A CRISPR/cas investigation indicated at least two different CRISPR systems with varied spacer profiles. On the other hand, putative virulence genes, including those related to gliding motility, type IX secretion system (T9SS), outer membrane proteins (Omp), were equally distributed among *F. columnare* strains. The MLSA scheme categorized bacterial strains into nine different sequence types (ST 9–17). Phylogenetic analyses based on either 16S rRNA, MLSA and concatenated SNPs of core genome revealed the diversity of *F. columnare* strains. DNA homology analysis indicated that the estimated digital DNA-DNA hybridization (dDDH) between strains of genomovar I and II can be as low as 42.6%, while the three uniquely tilapia-originated strains from Thailand (1214, NK01 and 1215) were clearly dissimilar to other *F. columnare* strains as the dDDH values were only 27.7–30.4%. Collectively, this extensive diversity among bacterial strains suggested that species designation of *F. columnare* would potentially require re-emendation.

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1. Introduction

Flavobacterium columnare, a Gram-negative, filamentous bacterium, ubiquitously found in aquatic environments including soil and water, is commonly known as an aetiological agent of columnaris disease in freshwater fishes (Declercq et al., 2013). Columnaris, also recognized as saddleback disease, is a notorious infectious disease in freshwater aquaculture species due to its high severity which can cause acute mortality up to 100% within 24 h (Declercq et al., 2013). To this day, *F. columnare* has been distributed worldwide and the outbreaks of acute mortality associated with columnaris have also been reported in several cold and warm aquaculture freshwater fish species, such as channel catfish (*Ictalurus punctatus*), red tilapia (*Oreochromis* sp.), Nile tilapia (*Oreochromis niloticus*), common carp, (*Cyprinus carpio*), striped catfish (*Pangasianodon hypophthalmus*) and rainbow trout (*Oncorhynchus mykiss*), leading to tremendous losses in the aquaculture industry annually (Dong et al., 2015a; LaFrentz et al., 2012; Rahman et al., 2010; Shoemaker et al., 2008; Tien et al., 2012).

Despite diverse host and geographical milieus, *F. columnare* isolates have been recognized for their phenotypic homogeneity which leads to a limitation of subspecies classification using phenotypic markers such as biochemical profiles (Figueiredo et al., 2005). On the contrary, genetic diversity was commonly observed among the isolates and the term 'genomovar' was later coined by Triyanto and Wakabayashi to describe the genotypically distinct isolates of *F. columnare* (Triyanto and Wakabayashi, 1999b). Classification of genomovar relies on the restriction fragment length polymorphism of the bacterial rRNA gene (16S–

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RFLP) and has been regarded as the most practical genotypic system for *F. columnare* (LaFrentz et al., 2014; Olivares-Fuster et al., 2007). Presently, *F. columnare* can be categorized in to five genomovars (I, II, II-B, III and I/II) and epidemiolocal studies indicate that distribution of bacterial genomovar is likely geographical-dependent (LaFrentz et al., 2014). In Thailand, genomovar II has been identified as the predominant strain, while genomovar I/II rarely occurs (Dong et al., 2015a; LaFrentz et al., 2016). Apart from genomovar typing system, genetic diversity of *F. columnare* isolates on local and global scales have been described extensively by other molecular genotyping approaches, such as 16-23S intergenic spacer region (ISR) sequencing, amplified fragment length polymorphism (AFLP) fingerprinting, and single-strand conformation polymorphism (SSCP) (Arias et al., 2004; Darwish and Ismaiel, 2005; Olivares-Fuster et al., 2007). Recently, the multilocus sequence analysis (MLSA) based on DNA sequence variation within six housekeeping genes was proposed to genetically assess population structure of *F. columnare* (Ashrafi et al., 2015). Unfortunately, although the MLSA scheme can indeed confer highly discriminatory and unambiguous data, the samples included in the previous MLSA study were limited only to the genomovar I strains isolated in Finland (Ashrafi et al., 2015).

Comparative genomics simply means the study pertaining comparison of genomes of different organisms. These analyses have proved useful, lending insight into the variations of genomic features, including DNA sequence, gene repertoire, genomic structural landmarks (for instance pathogenicity island) and phylogenetic relationship. Currently, comparative genomics have been acknowledged as the essential elements in, for instance, population genetics and molecular evolution (Xia, 2013). The continual cost reduction of Next-generation sequencing (NGS) and rapid advancement of the relevant bioinformatic tools make the reconstruction of bacterial genomes more feasible, even for laboratories with fewer resources. However, despite the economic significance of *F. columnare*, relatively few pathogenic isolates have been sequenced to the genome level (Kumru et al., 2016; Tekedar et al., 2012). Presently (October 2016), only four complete genomes of *F. columnare* isolates, limited to genomovar I and II, are available in the GenBank database which is considerably too few for comparative genome analysis. In this study, genomic DNA of five *F. columnare* strains recovered from diseased fish in Thailand were sequenced using NGS-technology. Comparative analyses also included the genomes of *F. columnare* deposited in GenBank to extend the temporal and geographical scales of analysis. To gain more detailed information about pathogenic mechanisms and phylogeny of *F. columnare*, comparative genome analyses were focused specifically on the distribution of putative virulence genes, genomic islands and phylogenetic relationships among the selected bacterial strains.

2. Materials and methods

2.1. Bacterial strains

Five strains of *F. columnare* (1214, 1215, 1362, CF1 and NK01) associated with disease outbreaks were isolated from farmed fish of different species comprised of red tilapia (*Oreochromis* sp.), Nile tilpia (*Oreochromis niloticus*) and striped catfish (*Pangasianodon hypophthalmus*), from the distinct geographical locations (Table 1). Three *F. columnare* strains recovered from red tilapia were previously assigned to genomovar II and I/II (Dong et al., 2015a; LaFrentz et al., 2016), while two strains recovered from Nile tilapia (NK01) and striped catfish (CF1) were assigned to genomovar II in the present study by using the 16S–RFLP method described by LaFrentz et al. (2016). The isolates were preserved at −80 °C in Anacker and Ordal (AO) broth containing 10% glycerol and 20% bovine serum. In addition, the genome sequences *F. columanare* genomovar I (strain ATCC46512 and Pf1) and II (strain 94–081 and C#2) were also included for comparative genomic analyses in the present study.

2.2. Bacterial growth conditions and DNA extraction

To grow the bacteria, the cryopreserved stock was inoculated on AO agar at 30 °C for 24–48 h. Subsequently, a colony of *F. columnare* was subcultured in 3 mL AO broth for 24 h with 160 rpm agitation. The bacterial solution was used for genomic DNA extraction using NucleoSpin® Microbial DNA (Macherey-Nagel, Germany). The qualities and quantities of the extracted DNA were evaluated using 1-μl-spectrophotometer (NanoDrop™, Thermo Fisher Scientific, USA) and Qubit™ Fluorometric Quantitation (Thermo Fisher Scientific, USA), respectively. The extracted DNA was stored at −20 °C until used for library preparation.

2.3. Sequencing and assembly

The DNA library was constructed from the extracted genomic DNA of *F. columnare* strains using Nextera XT DNA Library Preparation Kit (Illumina, USA) according to the manufacturer's instructions. The DNA library was then subjected to genome sequencing using the Illumina MiSeq platform with 151 paired-end run length. After obtaining the

Table 1
Summarizing main characteristics of *F. columnare* isolates included in comparative genomic analysis.

Isolate	Genomovar	Geographical origin (year)	Host	Number of contigs (fold-coverage)	Assembly size (Mb)	%GC	CDS	Structural RNAs	Number of subsystems	NCBI accession no.	Reference
1214	II	Phetchaburi, Thailand (2012)	Red tilapia (*Oreochromis* sp.)	145 (28.72×)	3.38	30.0	3077	88	336	SAMN06216357	(Dong et al., 2015a)
1215	I/II	Phetchaburi, Thailand (2012)	Red tilapia	376 (25.72×)	3.34	30.7	3101	94	313	SAMN06216377	(Dong et al., 2015a)
1362	II	Kanchanaburi, Thailand (2013)	Red tilapia	166 (28.33×)	3.16	30.6	2823	75	324	SAMN06216423	(Dong et al., 2015a)
CF1	II	Ratchaburi, Thailand (2014)	Striped catfish (*Pangasianodon hypophthalmus*)	423 (19.75×)	3.09	30.8	2714	53	318	SAMN06216426	(Dong et al., 2015c)
NK01	II	Nongkhai, Thailand (2014)	Nile tilapia (*Oreochromis niloticus*)	134 (11.47×)	3.39	29.9	3031	94	334	SAMN06216427	(Dong et al., 2015b)
ATCC49512	I	France (1987)	Brown trout (*Salmo trutta*)	1	3.16	31.5	2818	84	317	NC_016510	(Tekedar et al., 2012)
Pf1	I	Wuhan, China (2014)	Yellow catfish (*Pelteobagrus fulvidraco*)	1	3.17	31.6	2805	95	314	NZ_CP016277	(Zhang et al., 2016)
94–081	II	Mississippi, US (1994)	Channel catfish (*Ictalurus punctatus*)	1	3.32	30.8	2903	82	323	NZ_CP013992	(Kumru et al., 2016)
C#2	II	Unknown (2004)	Yellow catfish (*Pelteobagrus fulvidraco*)	1	3.32	31.0	2846	120	321	NZ_CP015107	(Bartelme et al., 2016)

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