GENOME ANALYSIS OF PREDOMINANT *AEROMONAS* SPECIES ISOLATED FROM DISEASED FRESHWATER FISHES AND THEIR VIRULENCE IN STRIPED SNAKEHEAD FISH (*CHANNA STRIATA*)

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การวิเคราะห์จีโนมเชื้อแอโรโมนาสสปีชีส์เด่นที่แยกได้จากปลาน้ำจืดป่วยในประเทศไทยและความรุนแรงของเชื้อในปลาช่อนนา

นายมูห์ ฟัดลุลละห์ มูร์ซาลิม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Mr. Muhammad Fadhlullah Mursalim
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มูห์ ฟัดลุลละห์ มูร์ซาลิม :

การวิเคราะห์จีโนมเชื้อแอโรโมนาสสปีชีส์เด่นที่แยกได้จากปลาน้ำจืดป่วยในประเทศไทยและความรุนแรงของเชื้อในปลาช่อนนา. (GENOME ANALYSIS OF PREDOMINANT *AEROMONAS* SPECIES ISOLATED FROM DISEASED FRESHWATER FISHES AND THEIR VIRULENCE IN STRIPED SNAKEHEAD FISH (*CHANNA STRIATA*))

อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ

เชื้อแอโรโมแนส เป็นแบคทีเรียก่อโรคที่สำคัญในปลาน้ำจืด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการกระจายตัวและความหลากหลายของเชื้อแอโรโมแนส รวมถึงรูปแบบการดื้อยาต้านจุลชีพ ตัวอย่างเชื้อแอโรโมแนสจำนวน 86 ตัวอย่างจากปลาป่วยในฟาร์มเลี้ยงปลา 13 แห่งทั่วประเทศไทย ได้ถูกทำการทดสอบทางชีวเคมี, เครื่องจำแนกชนิดแบคทีเรียอัตโนมัติในระบบแมสสเปคโตรเมตรี (MALDI-TOF MS), การทดสอบลูกโซ่โพลิเมอเรส (PCR) และการวิเคราะห์ลำดับยีน *gyrB* เพื่อจำแนกเชื้อตัวอย่างทั้งหมด

เครื่องจำแนกชนิดแบคทีเรียอัตโนมัติในระบบแมสสเปคโตรเมตรี (MALDI-TOF MS) ทดสอบและยืนยัน 100% (86 ตัวอย่าง) ที่ระดับสกุลและ 88.4% (76 ตัวอย่าง) ที่ระดับสปีชีส์ ขณะที่การวิเคราะห์ลำดับยีน *gyrB* จำแนกได้ 6 สปีชีส์ ได้แก่ *A. veronii* (72.1%), *A. jandaei* (11.6), *A. schubertii* (9.3%), *A. diversa* (3.5%), *A. hydrophila* (2.3%) และ *A. caviae* (1.2%) ตัวอย่างเชื้อแอโรโมแนสมีความดื้อต่อยาต้านจุลชีพ อะม็อกซีซิลลิน (99%) แอมพิซิลลิน (98%) ออกโซลินิกแอซิด (81.4%) ออกซีเตตราไซคลิน (77%) ไตรเมโธพริม-ซัลฟาเมทอกซาโซล (24%) และเอนโรฟลอกซาซิน (21%)

จากการศึกษานี้ระบุว่า *A. veronii* เป็นสปีชีส์หลักของเชื้อแอโรโมแนสที่พบในปลาน้ำจืดที่เลี้ยงในประเทศไทย การวิเคราะห์ความหลากหลายทางพันธุกรรมที่แสดงโดยยีน *gyrB* แสดงให้เห็นว่า *A. veronii* ที่แยกได้จากปลาชนิดต่างชนิดกันได้ถูกจัดให้อยู่ในคลัสเตอร์เดียวกัน บ่งชี้ว่า *A. veronii* ที่มีความใกล้ชิดทางพันธุกรรมสามารถแพร่เชื้อในปลาชนิดอื่นได้ การทดสอบ ERIC-PCR จาก 62 ตัวอย่างของ *A. veronii* ที่แยกจากตัวอย่างปลาป่วย มีเพียง 33 ตัวอย่างเท่านั้นที่แสดงแถบ(Band)มากกว่า 1 แถบ และตัวอย่างที่เหลือไม่สามารถจำแนกได้โดยใช้ ERIC-PCR อย่างไรก็ตาม รูปแบบ ERIC-PCR 33ตัวอย่างสามารถจำแนกเชื้อแอโรโมแนสได้เป็นสี่กลุ่ม ที่มีความคล้ายคลึงกัน 95% การทดสอบเชื้อในปลาช่อนพบว่าตัวอย่างเชื้อ *A. veronii* ทั้งหมดที่ทำการแยกเชื้อจากปลาต่างๆ สามารถก่อให้เกิดโรคได้ และตัวอย่างเชื้อ *A. veronii* NBCF28 (แยกได้จากปลาดุก) เป็นสายพันธุ์ที่รุนแรงที่สุดโดยมีค่า LD_{50} ที่ 1.2×10^5 CFU/มล. การวิเคราะห์เปรียบเทียบจีโนมระหว่างสายพันธุ์ *A. veronii* 5 สายพันธุ์ที่มีแหล่งที่มาแตกต่างกันในประเทศไทย เผยให้เห็นปัจจัยหลักที่ก่อให้เกิดความรุนแรงของเชื้อ *A. veronii* ได้แก่ fimbriae, flagella, toxins, type II, type III, and type VI secretion systems, CRISPR/Cas system, และจำนวนของ prophages

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Muhammad Fadhlullah Mursalim : GENOME ANALYSIS OF PREDOMINANT *AEROMONAS* SPECIES ISOLATED FROM DISEASED FRESHWATER FISHES AND THEIR VIRULENCE IN STRIPED SNAKEHEAD FISH (*CHANNA STRIATA*). Advisor: Assoc. Prof. CHANNARONG RODKHUM, D.V.M., Ph.D., D.T.B.V.P.

Aeromonas is an important bacterial pathogen in freshwater fishes. This study aimed to investigate the distribution and diversity of *Aeromonas* spp. including their antimicrobial resistance patterns. 86 *Aeromonas* isolates were collected from diseased fish in 13 fish farms around Thailand. Biochemical tests, MALDI-TOF MS, PCR assays, and the *gyrB* gene sequence analysis were used to identify all isolates. MALDI-TOF MS confirmed 100% (86 isolates) at the genus level and 88.4% (76 isolates) at the species level. Six *Aeromonas species* were confirmed based on sequences of *gyrB* gene, including *A. veronii* (72.1%), *A. jandaei* (11.6%), *A. schubertii* (9.3%), *A. diversa* (3.5%), *A. hydrophila* (2.3%), and *A. caviae* (1.2%). Antimicrobial susceptibility test for all *Aeromonas* isolates exhibited resistance against amoxicillin (99%), ampicillin (98%), oxolinic acid (81.4%), oxytetracycline (77%), trimethoprim-sulfamethoxazole (24%), and enrofloxacin (21%). Our findings indicated that *A. veronii* is the predominant species in cultured freshwater fishes in Thailand. The genetic diversity expressed by the *gyrB* gene exhibited *A. veronii* isolates from different fish species in the same cluster. It indicates that *A. veronii,* which has genetic closeness, can infect other fish species. Out of 62 *A. veronii* isolates, only 33 isolates showed more than one band, and the others were untypable when typing using ERIC-PCR. However, 33 ERIC profiles were observed, and the isolates were categorized into four clusters with 95% similarity. Experimental challenge evaluation using striped snakehead fish revealed that all *A. veronii* strains isolated from different fishes are susceptible (mortality ≥50%), and put *A. veronii* strains NBCF28 (walking catfish) is the most virulent strain with LD_{50} 1.2 $x10^5$ CFU/ml. The genomic comparisons analysis among five *A. veronii* strains of distinct origins in Thailand revealed the main factors contributing to *A. veronii* virulence, including fimbriae, flagella, toxins, type II, type III, and type VI secretion systems, CRISPR-Cas system, and the number of prophages. These results confirm the genetic information of *A. veronii* strains that recently caused high mortality in cultured freshwater fishes.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

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Muhammad Fadhlullah Mursalim

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LIST OF ABBREVIATIONS

AMC	Amoxicillin
AMP	Ampicillin
BLAST	Basic Local Alignment Search Tool
CDSs	Coding Sequences
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ENR	Enrofloxacin
FFC	Florfenicol
NGS	Next Generation Sequencing
MHA	Mueller-Hinton Agar
OA	Oxolinic acid
OT	Oxytetracycline
RAST	Rapid Annotation Subsystem Technologies
SXT	sulfamethoxazole-trimethoprim
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VFDB	Virulence Factor Database

CHAPTER I. INTRODUCTION

1.1 Importance and Rationale

Global Aquaculture production peaked at approximately 179 million tonnes in 2018, with aquaculture representing 47% of the total and 53% if non-food uses (including a reduction to fish meal and fish oil) are excluded. Consequently, aquaculture is expected to play a significant role in ensuring food security and alleviating poverty. Fish consumption is part of many cultural traditions and health benefits as a good source of protein, fatty acids, vitamins, minerals, and essential micronutrients (FAO, 2020). Globally, Thailand is one of the most aquaculture production countries, where freshwater fish production is mainly for consumption and has become one of the most influential industrial activities (FAO, 2020). Freshwater fish production in Thailand was assessed at 471,238 tons in 2020, with revenue valued at USD 802 million. In addition, tilapia, walking catfish, Asian sea bass, and snakehead contributed to 80% of total production (DOF, 2020). However, the disease outbreak causes many problems in the aquaculture sector, including economic losses. The fish are cultivated with high stocking densities, making fish under stress and easily infected by bacterial pathogens. According to the Department of Fisheries (DOF), total economic losses due to diseases for cultured freshwater fish in Thailand were approximately USD 300 million/year (DOF, 2020).

The significant problem in commercial fish farms is bacterial pathogens. One of them is the genus *Aeromonas*, the common troublesome pathogen in humans and fish (Janda and Abbott, 2010). They are virtually ubiquitous bacteria that play several roles in the aquatic environment (Martino et al., 2014; Khor et al., 2015). *Aeromonas* are Gram-negative, rod-shaped, oxidase-positive, catalase-positive, motile by polar flagellation, and facultative anaerobic (Nordmann and Poirel, 2002; Carnahan and Joseph, 2005).

Aeromonas has been associated with motile *Aeromonas* septicemia (MAS), which produces hemorrhagic, ulcerative, and septicemia in fish (Rahman et al., 2002). These bacteria have an important role in massive damage, leading to high mortality rates and severe economic losses in aquaculture. Several *Aeromonas* spp. with septicemia were reported in different freshwater fish: *A. hydrophila* from tilapia (Soto-Rodriguez et al., 2013); *A. veronii* from channel catfish, tilapia (Zhang et al., 2016; Dong et al., 2017; Hassan et al., 2017); *A. schubertii* from snakehead fish (Chen et al., 2012); *A. sobria* from tilapia (Li and Cai, 2011); and, *A. jandaei* from tilapia (Dong et al., 2017).

Nowadays, the genus of *Aeromonas* comprises more than 30 genospecies (Erdem et al., 2011). The molecular approach has become the leading choice for precise identification, revealing some inconsistencies in the biochemical test (Beaz-Hidalgo et al., 2010). DNA-DNA hybridization was often used to identify *Aeromonas* (Janda and Abbott, 2010). Molecular characterization using housekeeping gene sequences has been reliable marker for differentiating the genus *Aeromonas* taxonomy (Yáñez et al., 2003; Nhung et al., 2007; Beaz-Hidalgo et al., 2015). The *gyrB* (DNA gyrase subunit-B), one of the housekeeping genes that play an essential role in the DNA replication process, has been effectively proven to study phylogenetic of the genus *Aeromonas* (Yamamoto and Harayama, 1996; Yáñez et al., 2003). Furthermore, in distinguishing closely related *Aeromonas* species, housekeeping genes are more accurate than 16S rDNA (Martinez-Murcia et al., 2005; Beaz-Hidalgo et al., 2010). However, these techniques are expensive, labor-intensive processes and need trained personnel. Recently, several researchers reported and recommended that Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), a typing based on protein fingerprints analysis of whole-cell, is an effective method for identifying *Aeromonas* (Soler et al., 2004; Benagli et al., 2012;

Latif-Eugenin, 2015; Shin et al., 2015) in the aquaculture sector, particularly at the genus level (Perez-Sancho et al., 2018; Piamsomboon et al., 2020)

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR), a 127-bp imperfect palindrome, is easy to implement, not require any costly tools, and is reproducible, which occurs in multiple copies in the genomes of gram negatives Enterobacteriaceae (Versalovic et al., 1991; Soler et al., 2003; Figueras et al., 2005). This method has been determined as an efficient tool for tracking *Aeromonas* strains to their sources, which is essential for an epidemiological study (Ye et al., 2012; Shao-wu et al., 2013). Whole Genome Sequencing (WGS) is a high throughput DNA sequencing methodology with powerful technology over the past 15 years, and new methods are continually being commercialized (Barton et al., 2018). The virulence output from the virulome analysis is essential to determine virulence factors. However, information on the virulence factor of *Aeromonas* spp. isolated from different fish species can cause diseases in striped snakehead fish (*Channa striata*) based on genomic approach is lacking. Stripped snakehead fish, a carnivorous freshwater fish, are native to South Asian countries, one of valuable fish in Asia due to containing high protein that is useful for food, pharmaceutical, and traditional medicine (Ambak et al., 2006; Aliyu-Paiko et al., 2010).

Hence the aims of this study are 1) to detect and identify *Aeromonas* spp. isolated from diseased freshwater fish, which showed symptoms of motile *Aeromonas* septicemia; 2) to characterize predominant *Aeromonas* spp. isolated from diseased freshwater fish (red tilapia, Nile tilapia, striped snakehead fish, catfish, and Asian sea bass) and other epidemiological locations in Thailand; 3) To evaluate the virulence of predominant *Aeromonas* spp. in striped snakehead fish (*Channa striata*); 4) to analyze the virulence genes profile of predominant *Aeromonas* spp. using genomic approach.

1.2 Objectives of the Study

The Objectives of the study are as follows :

1. To detect and identify *Aeromonas* spp. isolated from diseased freshwater fish, which showed symptoms of motile *Aeromonas* septicemia.
2. To characterize the predominant *Aeromonas* spp. isolated from the diseased freshwater fish (red tilapia, Nile tilapia, striped snakehead fish, catfish, and Asian sea bass) and other epidemiological locations in Thailand.
3. To evaluate the virulence of predominant *Aeromonas* spp. in striped snakehead fish.
4. To analyze the virulence profile of predominant *Aeromonas* spp. using genomic approach.

1.3 Hypotheses

1. *Aeromonas* spp. could be detected and identified from different diseased freshwater fish (red tilapia, Nile tilapia, striped snakehead fish, catfish, and Asian sea bass).
2. Some genetic diversities could be found from predominant *Aeromonas* spp. isolated from diseased freshwater fish (red tilapia, Nile tilapia, striped snakehead fish, catfish, and Asian sea bass) from different epidemiological locations in Thailand.
3. Several virulence genes could affect the pathogenic potential of predominant *Aeromonas* spp.

1.4 Conceptual framework

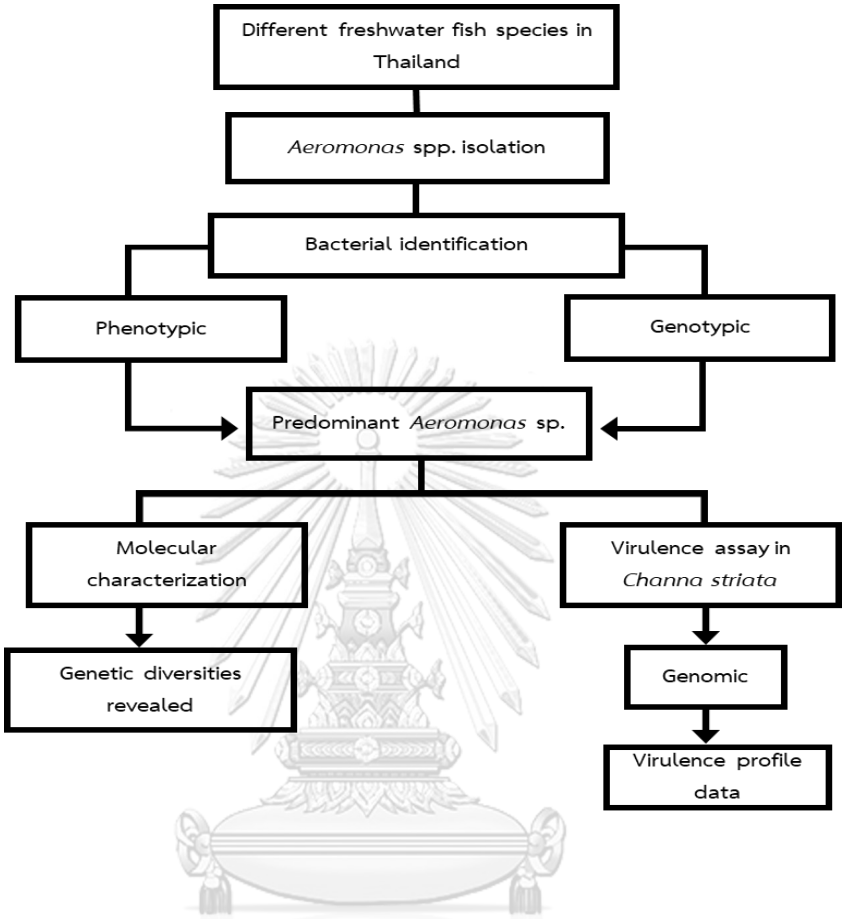

Figure 1 Conceptual framework

CHAPTER II. LITERATURE REVIEW

2.1 Freshwater aquaculture

Freshwater aquaculture is still becoming a promising sector worldwide for human consumption and non-food utilization. Most people work in fish production, which becomes one source of nutrition with high-quality protein and essential amino acids, minerals, vitamins, and fats (FAO, 2016).

Thailand has become one of the reputable exporters of fish products for the past few years. Geographical conditions support this sector, where Thailand is a peninsula country with water over 319 thousand kilometres. Thailand's freshwater fish production was assessed at 471,238 tons in 2020, estimated at USD 802 million. In addition, tilapia, walking catfish, Asian sea bass, and snakehead contributed to 80% of total production (DOF, 2020).

2.2 Striped snakehead fish culturing

The striped snakehead fish (*Channa striata*), a member of the *Channidae* family, is commercial fish in Thailand for its delicacy and albumin content, widely used in pharmaceuticals and traditional medicine (Boonyaratpalin et al., 1985; Qin et al., 1997; Mollah et al., 2009). This species is native to South Asian countries and widely distributed from China to India, Ceylon, and Southeast Asia, in rivers, lakes, swamps, marshes, canals, and ponds. This species also has a high ability to adapt to low environments due to its ability to survive in water with high ammonia content and low dissolved oxygen levels due to air-breathing skill, which is assisted by a suprabranchial chamber (Qin et al., 1997; Chandra and Banerjee, 2004; Mollah et al., 2009). In Thailand, it is found almost all over the country, except in mountainous areas. It is one of the leading fish food in other parts of Southeast Asia and is considered by the Chinese as a food fish, especially for enhancing wound healing. In the last ten years, snakehead fish cultivation has started in Thailand's eastern and

central parts. Cultivation has overgrown in the previous five years, replacing catfish cultivation due to recent disease problems and fluctuating prices (Boonyaratpalin et al., 1985).

Snakehead is cultured in a monoculture system. The culture operation is intensive with continuous water flow if the farm is adjacent to an irrigation canal or daily water exchange by pumping from a trench or river into the pond, 2–4 hours a day. Usually, pond size ranges from 800–1000 m^2 and 200–400 m^2 in Suphanburi and Samut Songkhram. The optimum pond size is 800 m^2 since the culture system is very intensive. The 200–400 m^2 ponds are the right size for a farmer with limited land and capital (Boonyaratpalin et al., 1985).

2.3 *Aeromonas* sp. and disease

The waterborne bacteria*, Aeromonas,* are Gram-negative bacteria broadly placed in diverse water ecosystems, with various species causing infection in humans, fish, and other marine species (Tomas, 2012; Fernández-Bravo and Figueras, 2020). Most fish species could be infected by Motile *Aeromonas* septicemia (MAS). *Aeromonas* infection can occur either as primary or secondary infection under stress conditions, leading to massive mortality and financial losses. Unsuitable environmental conditions become factors that cause stress on fish (Kozinska, 2007). The typical clinical symptoms showed abnormal swimming, swollen abdomen, and enteritis. Generally, infected fish will show hemorrhagic lesions on the skin and internal organs (Dong et al., 2017). Commonly, mesophilic *Aeromonas* are found in the aquatic environment under stress conditions*. Aeromonas* have been identified from different freshwater fish species, including *A. hydrophila* from tilapia (Soto-Rodriguez et al., 2013); *A. veronii* from channel catfish, tilapia (Zhang et al., 2016; Dong et al., 2017; Hassan et al., 2017); *A. schubertii* from snakehead fish (Chen et al., 2012); *A. sobria* from tilapia (Li & Cai 2011); *A. hydrophila*, *A. veronii*, *A. schubertii*

from Asian sea bass freshwater fish (Mursalim et al., 2020), and *A. jandaei* from tilapia (Dong et al., 2017).

2.4 Phenotypic Identification

Phenotypic identification is observed to see changes in the growth and metabolism of a microbe based on physiological, morphological, and biochemical characteristics. Primary biochemical tests identify *Aeromonas* species, including Gram-negative staining, oxidase-positive, catalase-positive, and growth on primary laboratory media such as tryptic soy agar. However, species-level identification is limited due to the inconsistent result of the strain (Janda and Abbott, 2010).

119 *Aeromonas* strains mainly isolated from diseased fish were identified phenotypically and then compared using molecular techniques (16S rRNA-RFLP and *rpoD* sequences). The results revealed that only 35.5% were correctly identified phenotypically at the species level (Beaz-Hidalgo et al., 2010). On the other hand, several commercial identification methods (API 20E, Vitek, BBL Crystal, MicroScan W/A, among others) reported that this system was inferior for identifying *Aeromonas* (Park et al., 2003; Soler et al., 2003). The accuracy of six commercial methods for *Aeromonas* identification was compared using the *rpoB* sequencing as molecular identification. The result demonstrated that commercial identification systems showed misidentification at the species level. The study also supported previous studies about difficulty distinguishing *Aeromonas* and *Vibrio* (Lamy et al., 2010).

2.5 MALDI-TOF MS identification.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is one of the latest advancements of this century in clinical microbiology, which is helpfully for bacterial identification in clinical laboratories based on protein fingerprinting with effective time-saving (Benagli et al., 2012;

Vavrova et al., 2015). It is rapid, sensitive, specific, and supported with good accuracy, making this method suitable for identifying isolates of *Aeromonas*.

Principally, MALDI-TOF MS locates the proteins of microorganisms that connect with the 16S rRNA gene. However, this gene has limitations due to the low resolution for identifying closely related *Aeromonas* species, impacting this method's resolution (Latif-Eugenin, 2015). 92 *Aeromonas* strains obtained from clinical and environmental were investigated by MALDI-TOF MS and initially confirmed by *gyrB* sequencing. This study revealed better differentiation among *Aeromonas* strains MALDI-TOF MS dendrogram. Subsequently, in 2015, two new studies were conducted (Latif-Eugenin, 2015; Shin et al., 2015). A total of 65 cases of *Aeromonas* from clinical strains were investigated and initially confirmed by *gyrB* sequencing and showed 98.5% suitability at the genus level and 92.3% at the species level using the MALDI-TOF MS (Shin et al., 2015). The results were supported by the results obtained by Latif-Eugenín (2015), who investigated 179 clinical strains from hospitals in Spanish, with 98.3% suitability at the genus level, and 91.1% at the species level using MALDI-TOF MS. The studies found that MALDI-TOF MS was a helpful tool since the identification error was <10%, while with phenotypic identification methods, mistakes frequently occur. The accuracy of this method depends on updating the database and selecting the reference of isolates. Correct identification increased after updating the database with 14 new spectra of *Aeromonas* strains isolated from the fish (Pérez-Sancho et al., 2018). As a result, updating the database is important for increasing the reliability of this method to correct the identification of the bacteria.

2.6 Identification based on housekeeping gene

Housekeeping genes are those genes that encode proteins with fundamental functions for the existence of a cell. The detection of new species described with housekeeping genes using MLPA (Multilocus Phylogenetic Analysis) approach showed

higher resolution than the 16S rRNA gene (Stackebrandt et al., 2002; Navarro and Martinez-Murcia, 2018; Zhong et al., 2019).

One of the housekeeping genes, *gyrB*, which specifically encodes the subunit B of DNA gyrase, is the most widely used housekeeping gene in taxonomic studies and allow more excellent reliability in the phylogenetic classification of *Aeromonas* (Gonçalves Pessoa et al., 2019). It was introduced as the first housekeeping gene used for phylogenetic analysis of *Aeromonas* and proved to have higher resolution to clarify *Aeromonas* species than 16S rRNA closely (Yáñez et al., 2003). Furthermore, because of the intraspecies sequence diversity, *gyrB* is proposed as a useful target for the simultaneous identification of species and strains (Yáñez et al., 2003). Hence, these genes were excellent molecular markers for analyzing phylogenetics from many *Aeromonas* species (Martínez-Murcia et al., 2016; Chen et al., 2019; Zhang et al., 2019).

2.7 Molecular typing of *Aeromonas* spp.

Genetic characterization provides approximate data, including scientific classification, advancement, and the genetic population of bacteria (van Belkum et al., 2001). Generally, the fingerprinting approach is still widely used to identify genotypic characterization of bacteria (Emerson et al., 2008). Several fingerprinting methods were found to clarify the clonal relatedness of aeromonads for epidemiological investigation. Different techniques have been developed to determine the molecular epidemiology among *Aeromonas* strains. These methods are Pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), randomly amplified polymorphic DNA-PCR (RAPD-PCR), and amplified fragment length polymorphism (AFLP) (Martínez-Murcia and Lamy, 2015; Fernández-Bravo, 2019).

ERIC-PCR is an imperfect palindromic sequence of 127 bp that occurs in multiple copies in the genomes of *Enterobacteriaceae*, *Vibrio*, and other bacterial genera. ERIC elements in enterobacterial genomes vary between different species and have been used as a genetic marker to characterize isolates within a bacterial species. This technique is simple to implement, does not require any costly tools, and is exceptionally reproducible (Hulton et al., 1991; Versalovic et al., 1991; Son et al., 2002; Soler et al., 2003; Figueras et al., 2005). The ability to distinguish from the *Enterobacteriaceae* group, both phylogenetic and clone, still becomes one of the excesses of this method (Khor et al., 2015).

The outcome of this method is a band pattern obtained by amplification using PCR with ERIC primers comprised of ERIC1R and ERIC2. Different band sizes are formed during amplification (Versalovic et al., 1991). ERIC-PCR has demonstrated several previous studies to differentiate among *Aeromonas* strains. This method confirmed that *Aeromonas* strains collected from the clinical and environmental sources have different clusters (Sechi et al., 2002; Szczuka and Kaznowsky, 2004). On the other hand, 85 genotypes observed using *A. hydrophila, A. caviae,* and *A. veronii* biovar *sobria* were performed using RAPD and ERIC-PCR, which indicated that the strains were highly diverse (Yousr et al., 2010). 59 *A. hydrophila* strains isolated from common carp were successfully clustered into three branches based on ERIC-PCR patterns (Shao-wu et al., 2013). In 2014, the same characterization was obtained from 25 *A. veronii* isolated from diseased Trionix by ERIC-PCR (Ye et al., 2012). Therefore, ERIC-PCR is sufficiently reliable to determine genetic relatedness among *Aeromonas* species and identify strains to the species level based on fingerprinting band patterns.

2.8 *Aeromonas* virulence factors

Bacterial Pathogens use weapons provided by their bodies to spread infection, referred to as virulence properties. *Aeromonas* infections consist of several factors that contribute to their capability to cause various clinical symptoms (Pablos et al., 2011). Several virulence factors allow *Aeromonas* to control the host immune response and cause disease. Structural components, toxins, and extracellular products, acting jointly or individually, enable these microorganisms to colonize and infect hosts (Beaz-Hidalgo and Figueras, 2013; Hoel et al., 2017). The ability of bacteria to adhere to host tissues is crucial in many microorganisms' early phases of infection. Bacteria attach to host tissues and cells and change their defense mechanisms, initiating colonization. *Aeromonas* has structural components that facilitate its attachment mechanism in the colonization process, such as flagella, pili, capsule, Slayer, and lipopolysaccharide (LPS) (Gonçalves Pessoa et al., 2019).

Extracellular proteins and toxins are produced by extracellular components, manifesting from *Aeromonas* species and host cell interaction. Several extracellular proteins and toxins are secreted by *Aeromonas*, such as protease, lipase, enterotoxin, hemolysin, and shiga toxin (Janda and Abbott, 2010).

Six different secretion systems have been revealed in Gram-negative bacteria; there are T1SS, T2SS, T3SS, T4SS, T5SS, and T6SS. Particularly in *Aeromonas,* four of them have been reported, including T2SS, T3SS, T4SS, and T6SS and play an important role in transporting virulence factors to the extracellular medium or directly to the host cell (Beaz-Hidalgo and Figueras, 2013). T2SS is a secretion pathway that transports substances to the extracellular environment, primarily toxins and enzymes. T3SS, also known as injectisome, is the most common system involved in *Aeromonas* virulence. Its structure and sensory proteins (AexU and AexT) that work together to form a eukaryotic cell pore T6SS gene cluster have been identified in non-pathogenic organisms. It is present in 25% of Gram-negative

bacteria. As structural components of this secretion system, phage mechanisms are capable of injecting protein effectors directly into the cytosol of the target cell. It was said to be in charge of producing the hemolysin-coregulated protein (hcp) and VgrG (valine-glicyne repeat G) family, with eukaryotic and bacterial cells being the primary targets (Gonçalves Pessoa et al., 2019).

2.9 Whole-genome and virulome analysis

WGS (whole-genome sequencing), known as complete genome sequencing, is a comprehensive method of analyzing the entire genomic DNA of a cell at a single time by using sequencing techniques such as Sanger sequencing, shotgun approach, or high throughput NGS sequencing (Behjati and Tarpey, 2013). More than a thousand genes and genomes have been submitted into the genome database, while sequencing technology has been progressed rapidly and cheaper. Nowadays, the company has many high throughput sequencing tools, such as Illumina, Thermo Fisher, Oxford Nanopore, and Pacific Biosciences (Loman et al., 2012; Deurenberg et al., 2017). One of the significant advantages of NGS is that one protocol can be used for all pathogens in both identification and typing applications. Hence, this approach has been helpful in medical microbiology and infection prevention measures (Zhou et al., 2016).

Several available in silico tools are currently used to reveal the virulence properties of the bacterial pathogen, such as Basic Local Alignment Search Tools or BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Rapid Annotation using subsystem Technology (http://rast.nmpdr.org), Virulence Factor of Pathogenic Bacteria (http://www.mgc.ac.cn/VFs/), and many more. Mobile genetic elements (MGEs) such as prophage can be detected using *in silico* program platform provided by the genetic research center. Bacteriophages (Phages) can enter and persist in the bacterial genome and be searched using PHASTER (Zhou et al., 2011; Arndt et al.,

2016). It is an important upgrade version from PHAST with rapid identification and annotation for finding prophage sequences inside bacterial genomes and Plasmids (Arndt et al., 2016). Another genomic element such as CRISPR (Clustered regularly interspaced short palindromic repeats) can be detected by submitting contigs of the bacterial genome at https://crisprcas.i2bc.paris-saclay.fr, an improved webtool service (Couvin et al., 2018). The genomic approach has revealed several previous studies to examine the virulence properties of bacterial pathogens. A comparison of the genomes of *A. veronii* strains ML09-123 confirmed that the distribution of mobile elements is dependent on host and geographic origin, implying that this species has undergone significant genetic exchange. The information presented here provides insight into the genomic variation of *A. veronii* and identifies a pathotype that has a global impact on aquaculture (Tekedar et al., 2019). In 2020, Genomic analysis successfully revealed multiple virulence factors that impact virulent strain *A. veronii* strain TH0426 genomes, such as those related to fimbriae, flagella, toxins, iron ion uptake systems, T2SS, T3SS, and T6SS (Yang et al., 2021).

CHAPTER III. MATERIALS AND METHODS

According to the proposed objectives, the experiment will be divided into three parts; 1) Distribution of *Aeromonas* spp. from diseased freshwater fishes in Thailand part; 2) Molecular characterization of predominant *Aeromonas* spp. isolated from diseased freshwater fishes; Part 3) Comparative pathogenicity *A. veronii* in striped snakehead fish (*Channa striata*), and Part 4) Virulome analysis of the fish pathogen *A. veronii* reveals reason for increased virulence within the species. The research design is shown in figure 2 below.

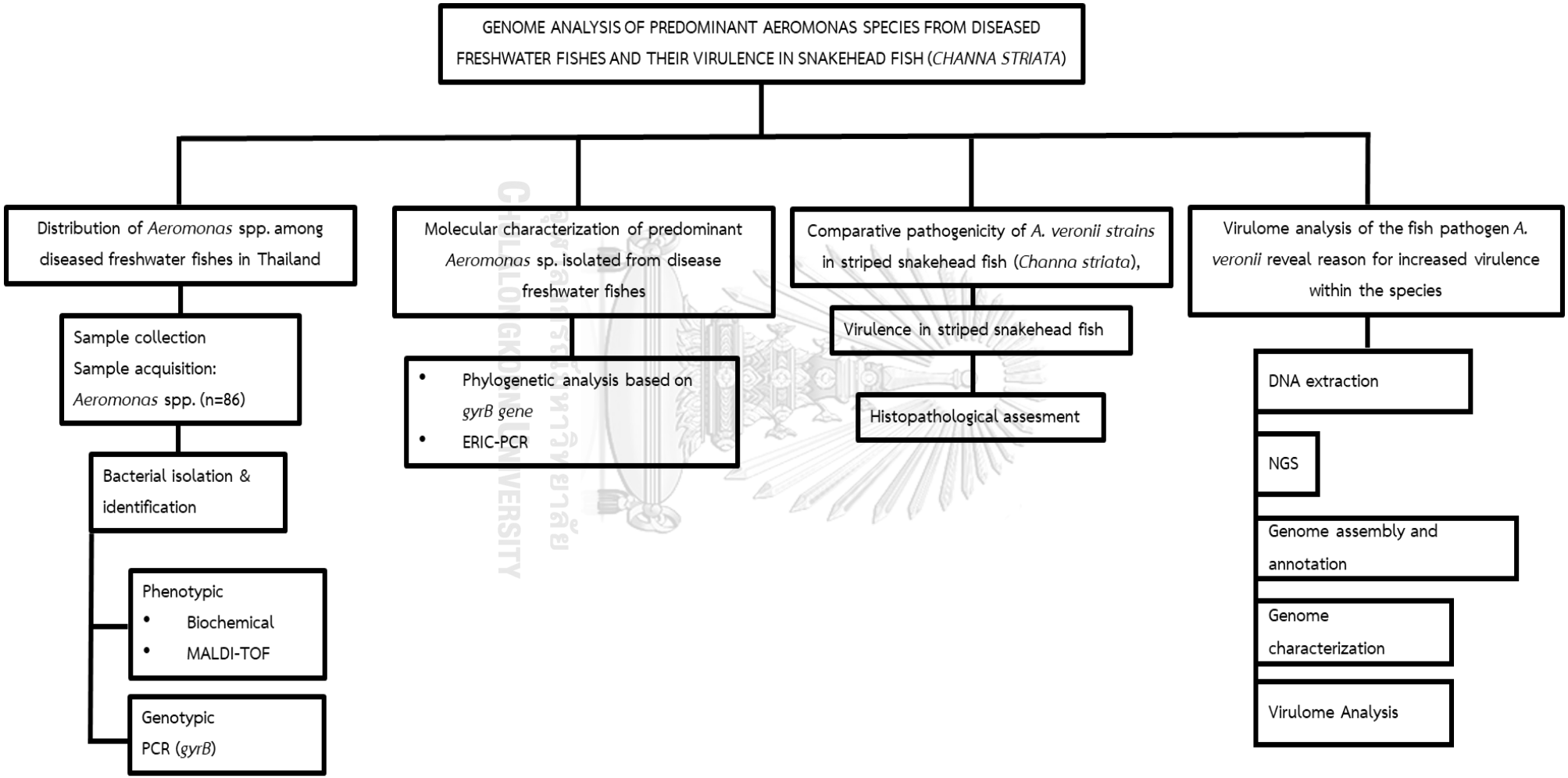

Figure 2 Research design of this study

3.1 Distribution of *Aeromonas* spp. among diseased freshwater fishes in Thailand

3.1.1 Sample collection

The fish handling in this study was performed following the methods approved by the Institutional Animal Care and Use Committee, Kasetsart University (ACKU64/F1S/008). All *Aeromonas* isolates were obtained from 171 diseased freshwater fish species (moribund or recently died) that exhibited at least one clinical sign of septicemia (skin ulcer, hemorrhages in skin and fins, fin rot, exophthalmia, abdominal distension, and loss of scales). In addition, irregular swimming, lethargy, and spinning movements were seen in infected fish and moribund fish. The diseased fishes were from nine provinces around Thailand (Figure 3). The isolates were obtained from red tilapia (*n*=101), striped snakehead fish (*n*=44), Asian sea bass (*n*=11), walking catfish (*n*=5), and Nile tilapia (*n*=10). The cultured fish were sampled in 2014 and between 2018-2020 periods (Table 1).

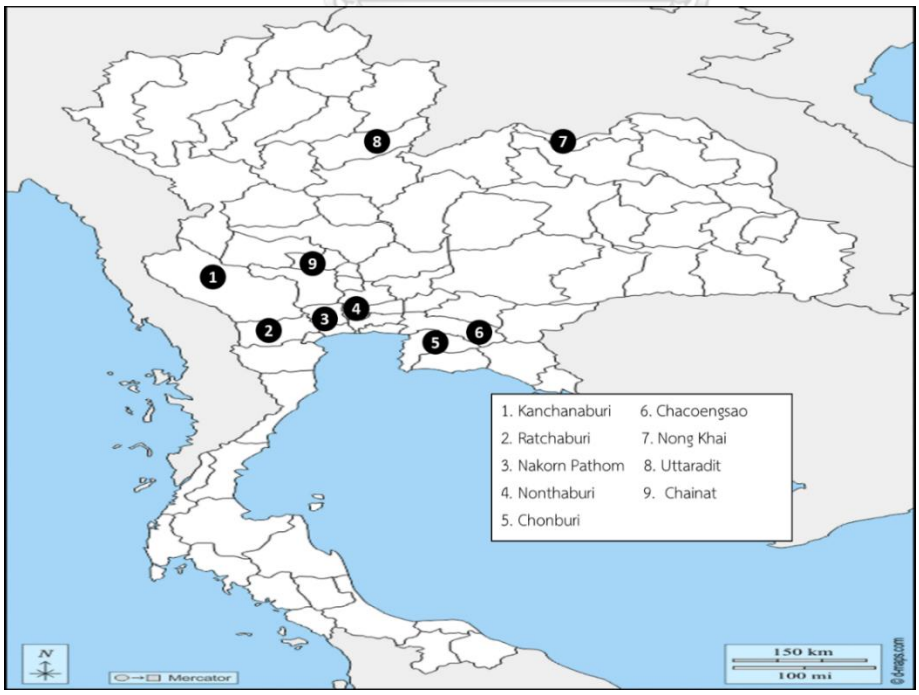

Figure 3 The map of geographic locations was sampled

Table 1 Details of fish sampling

Date of collection	Provinces	Type	Fish species	n*	Tissue sample					Reference
					Liver	Kidney	Spleen	Brain	Internal organs (Fry fish)	
Aug-2014	Nong Kai	Floating cage (pond)	Nile tilapia (*Oreochromis niloticus*)	10	-	7	-	-	-	(Dong et al., 2015)
Feb-2018	Uttaradit	Floating cage (pond)	Red tilapia (*Oreochromis* sp.)	18	-	1	1	-	-	This Study
Feb-2018	Chainat	Floating cage (pond)	Red tilapia (*Oreochromis* sp.)	7	-	6	-	-	-	This Study
Jul-2018	Chacoengsao	Floating cage (pond)	Asian sea bass (*Lates calcarifer*)	8	3	-	2	2	-	This Study
Jul-2018	Nonthaburi	Floating cage (pond)	Walking catfish (*Clarius batrachus*)	5	2	1	1	3	-	This Study
Mar-2019	Ratchaburi	Floating cage (river)	Red tilapia (*Oreochromis* sp.)	31	7	4	-	2	-	This Study
Oct-2019	Kanchanaburi	Floating cage (river)	Red tilapia (*Oreochromis* sp.)	12	2	1	4	-	-	This Study
Dec-2019			Red tilapia (*Oreochromis* sp.)	17	1	4	8	-	-	This Study

Jun-2020			Red tilapia (*Oreochromis* sp.)	16	-	5	-	-	-	This Study
Jul-2019			Striped snakehead fish (*Channa striata*)	5	2	-	2	-	-	This Study
Aug-2019	Nakorn Pathom	Cement tank	Striped snakehead fish (*Channa striata*)	5	-	-	-	1	-	This Study
Aug-2019			Striped snakehead fish (*Channa striata*)	18	-	-	-	-	1	This Study
Jul-2020			Striped snakehead fish (*Channa striata*)	16	-	-	-	5	6	This Study
Feb-2020	Chonburi	Floating cage (pond)	Asian sea bass (*Lates calcarifer*)	3	-	2	-	-	-	This Study
	Total Farms	13		171	17	31	18	13	7	This study

*Different numbers of samples were obtained per fish and per farm due to a limited number of moribund fish available at the sampling time

(-); no collecting sample

3.1.2 Bacterial isolation

In brief, moribund fishes were immersed in clove oil (1 g/L) for euthanasia at the sampling site for approximately 10 min (Underwood et al., 2020). Internal organs (liver, spleen, kidney, and brain) were aseptically swabbed from individual fish by placing sterile loops into the target tissue and streaked onto tryptic soy agar (TSA) (Difco™, USA) supplemented with 5% sheep blood for determining dominant colony, then incubated at 28 °C for 24 hrs. After growing the dominant colony with white and hemolysis, the colony was picked and sub-culture on Rimler Shotts (RS) selective agar medium (HIMEDIA, Mumbai, India). Yellow colonies from RS medium were picked and streaked on TSA for pure colony isolation, then incubated at 28 °C for 24 hrs. Subsequently, single colonies of each isolate were then identified for colony morphology, Gram staining, oxidase, and catalase test. Presumptive *Aeromonas* spp. were determined based on Gram-negative bacteria and positive for oxidase and catalase (Abbott et al., 2003). For further investigation, single colonies of each isolate were prepared in tryptic soy broth (TSB) containing 20% glycerol and kept at 80 °C until used.

3.1.3 Bacterial identification by MALDI-TOF MS

Initially, the presumptive bacteria were recovered from glycerol stocks and then inoculated on Tryptic soy Agar (TSA) (Difco™, USA) for 24 hours at 28 °C. A single colony was taken from each isolate, placed onto metal steel, and then directly extracted using an acetonitrile/formic acid method, as described previously. Approximately 1 µl of each extract was spotted on the MALDI plate target, dry on air, then overlaid with an HCCA (a cyano-4 hydroxycinnamic acid) matrix solution and loaded into BIOTYPER 2.0 (Bruker Daltonics, Bremen Germany).

Samples were performed using Bruker Daltonics UltrafleXtreme MALDI-TOF MS/TOF equipment and the FlexControl software v. 3.0 (Brucker Daltonics, Bremen,

Germany) to acquire mass spectra within a mass range from 2 to 20 kDa. The Biotyper Real-Time Classification software v3.1 (Brucker Daltonics) was used for microbial identification by comparing the spectra with the manufacturer's corresponding database (MALDI Biotyper database, 5989 entries, Bruker Daltonics) (Patel, 2015). This application shows the result with the score, starting from 0 to 3, indicating the similarity between the sample and spectra database, then revealed in score with two matching results as first and second identification with a different color. Different colors mean the accuracy of identifying the genus or species level of this tool (**Table 2**).

Table 2 Score range indicating the reliability of the identification at genus or species level

Score	Description
2.300-3.000	**Highly probable species identification**
2.000-2.299	**Secure genus and probable species identification**
1.700- 1.999	**Probable genus identification**
0.000-1.699	**Not reliable identification**

3.1.4 DNA extraction and purification

Genomic DNA was extracted using the boiling method with modification (Yáñez et al., 2003). Briefly, a loop full of the bacterial colony was suspended in 200 µl ultrapure water by rapid centrifugation at 11,000 x g for 5 min and boiled at 100 °C for 15 min. Then, the cell pellet was directly cooled down to -20 °C for 10 min and subsequently centrifuged at 11,000 x g for 5 min. The supernatant was transferred to sterile tube and stored at -20 °C before being used.

3.1.5 PCR amplification housekeeping gene

The suspected *Aeromonas* DNA was amplified with *gyrB* genes. The primers used in this study are listed in **Table 3**. The PCR condition of *gyrB* was set to 30 cycles of amplification as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2 min preceded by an initial denaturation step at 94 °C for 2 min and followed by terminal extension at 72 °C For 5 min. The PCR products were stained using red safe and then analyzed by electrophoresis on 1% agarose gel in 1x TBE buffer. Gels were captured and examined under UV illumination. The DNA was cleaned up from agarose gel using Nucleospin® Gel and PCR clean-up (MACHEREY-NAGEL, USA), then subjected to the Sanger sequencing.

Table 3 Primers used in this study

Primers	Sequence (5'-3')	Size	Reference
gyrB3F	TCCGGCGGTCTGCACGGCGT	1,100 bp	(Yáñez et al.,
gyrB14R	TTGTCCGGGTTGTACTCGTC		2003)

3.1.6 Phylogenetic data analysis

The quality of the forward and reverse from *gyrB* sequence were checked and assembled using BioEdit® version 7.1.1. The sequence data were submitted and analyzed by The BLAST sequence search tool (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was conducted using ClustalW in MEGA X (Kumar et al., 2018). Genetic distances and clustering will determine using Kimura two-parameter model. The phylogenetic tree will generate using the neighbor-joining with 1000 replicates of the bootstrap test.

3.1.7 Biochemical identification

All identified *Aeromonas* spp. were then performed additional Biochemical tests by microbial biochemical identification tubes referring to Cowan and Steel (Phillips, 1993). The test includes indole, motility, esculin hydrolysis and observed their phenotypic characteristics.

3.1.8 Antimicrobial susceptibility test

All *Aeromonas* isolates were examined to evaluate antimicrobial susceptibility test by agar disc diffusion method according to the Clinical Laboratory Standards Institute guideline VET04 (CLSI, 2014). *Escherichia coli* ATCC 25922 was used for quality control. Seven antimicrobial drugs are routinely used for prevention & treatment, including amoxicillin (AMC, 10 µg), ampicillin (AMP, 10 µg), oxytetracycline (OT, 30 µg), oxolinic acid (OA, 2 µg), enrofloxacin (ENR, 2 µg), florfenicol (FFC, 30 µg), and sulfamethoxazole-trimethoprim (SXT, 30 µg) (Oxoid, Hampshire, UK). In brief, All *Aeromonas* isolates were grown in TSB at 28 °C for 24 h with constant shaking at 160 rpm. Bacterial concentration was then adjusted to 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL) with sterilized saline water before being placed onto Mueller-Hinton agar (MHA) (Difco, Michigan, USA) and spread evenly using a sterile spreader rod. Subsequently, seven antimicrobial disks were set into Mueller-Hinton agar (MHA) (Difco, Michigan, USA) and incubated at 28 °C for 24h. After incubating period, the result was interpreted as inhibition zone (clear zone) based on susceptible (S), intermediate (I), and resistance (R).

3.2 Molecular characterization of predominant *Aeromonas* spp. isolated from diseased freshwater fish

3.2.1 Molecular characterization using *gyrB* gene

Molecular characterization was performed according to the previous part of this study.

3.2.2 Molecular typing using ERIC-PCR

Several predominant *Aeromonas* strains were performed using ERIC-PCR. The primers used are; ERIC 1R (5'- ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'- AAG TAA GTG ACT GGG GTG AGC G-3') (Versalovic et al., 1991). According to the previous study, ERIC-PCR was conducted with modification (Szczuka and Kaznowsky, 2004). PCR conditions are shown in **Table 4.**

Table 4 PCR condition of ERIC- PCR

ERIC PCR Conditions			
Stage	**Temperature**	**Duration**	**Cycle**
Pre-denatured	95°	7 min	
Denaturation	95°	30 s	
Annealing	52°	1 min	30 cycles
Extension	72°	8 min	
Final extension	72°	16 min	

Electrophoresis and visualization. A 10 µl of each PCR product was analyzed by gel electrophoresis in 1.5% agarose gel in TBE buffer, then run the product at 56 volts for 2 hours. Gene Ruler 100-bp DNA Ladder Plus (MBI Fermentas) was used as a molecular size standard. The gel was stained with red safe and visualized using Pop-Bio Imaging. Computer analyses were carried out by Bionumeric version 8.0 software. The similarity between fingerprints was calculated by the Dice coefficient. Cluster

analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) and 5% tolerance for band matching.

3.3 Comparative pathogenicity of *Aeromonas veronii* strains in striped snakehead fish (*Channa striata*)

3.3.1 Virulence in striped snakehead fish

The virulence of the isolated bacteria was evaluated by challenge test using healthy striped snakehead fish. Five representative *A. veronii* strains (NK01, CBSB03, CNRT12, NBCF28, and NPSF 15) were selected from different fishes considering the resistant profile and specific lesion. Initially, bacterial strains will be recovered from glycerol stocks. Bacteria were streaked directly onto TSA plates and incubated at 30 °C for 18 h. . A single pure colony of each isolate was cultured in 5 mL of BHI broth overnight at 28 °C, shaking for 24 h. Bacterial cells were harvested, washed twice with 0.85% of normal saline, and suspended in sterile PBS. The bacterial suspension was adjusted to OD600 = 0.8, and doses of each strain were determined by standard plate count (Dong et al., 2017). Infection doses were determined as 1.9 x 10^6 CFU mL^{-1} for *A. veronii* NK01, 2.3 x 10^6 CFU mL^{-1} for *A. veronii* CBSB03, 1.5 x 10^6 CFU mL^{-1} for *A. veronii* CNRT12, 8,7x 10^5 CFU mL^{-1} for NPSF15, and 7.3 x 10^5 for *A. veronii* NBCF28.

Healthy fingerling striped snakehead fish (n= 160, mean weight 4.2 ± 1.1 g) The snakehead fish used in the experiment came from a commercial snakehead fish hatchery in Nakorn Pathom Province. Five groups of fish were formed (5 groups x 3 bacterial dilution x 15 fish/tank) of bacterial strains and one control group (n=10). Then fish were anaesthetized using clove oil at concentration of 60 mg/L. Five groups were i.p. (0.1 ml/fish) injected with five isolates, and the control group was i.p. injected with 0.1 ml of PBS sterile. After injection, the fish were returned to and cultured in 150-litre water tanks with constant gentle aeration, and they were fed

with snakehead commercial feed three times a day (3% of body weight). Uneaten food was removed from the tank following feeding. All fish were initially acclimatized for 14 days in fiber plastic tank capacity of 1,000 l in a wet lab (Liu and Li, 2012).

Clinical signs and mortality were recorded daily for 14 days. Representatives of freshly dead or dying fish from each treatment were subjected to bacterial isolation and histopathological study. The lethal doses (LD_{50}) were determined according to the study described previously (Reed and Muench, 1938).

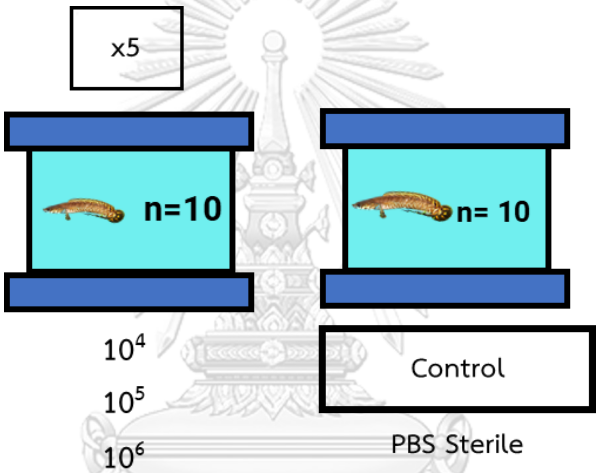

Figure 4 Experimental designs of virulence assay

3.3.2 Histopathological assessment

All moribund fish (groups with high concentration) were immediately sampled before. Initially, the fish were checked for lesions and hemorrhages before histopathological examination. The kidney, liver, and spleen samples were collected (n=5 samples/group) and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were embedded in paraffin wax and processed by standard paraffin wax techniques. Sectioned at 4 µm, then stained with hematoxylin-eosin (HE) were prepared according to a standard protocol.

3.4 Comparative genome analysis of the fish pathogen *Aeromonas veronii* reveals reasons for increased virulence within the species

3.4.1 *A. veronii* genomes used in this study

A total of 12 *A. veronii* genomes were analyzed in this study, including five genomes sequenced in this study and seven publicly available (Table 5). The five *A. veronii* strains sequenced in this study were isolated from five different freshwater fish species samples in Thailand during routine diagnostic procedures.

3.4.2 Whole-genome sequencing

Briefly, genomic DNA of representatives of *A. veronii* was selected and extracted using Nucleospin® DNA purification kit with RNase A treatment (MACHEREY-NAGEL, USA). DNA concentration was then checked by Qubit™ Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA, USA) before being stored at -20°C. Qualified samples were addressed to Next-generation sequencing. The libraries were performed using Nextera XT (Illumina, San Diego, CA, USA), and sequencing was performed using Illumina HiSeq instrument (San Diego, CA, USA).

3.4.3 Genome assembly and annotation

Sequencing product from WGS was examined for their quality using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and cut the low-quality nucleotides and WGS-adapters out by Trimmomatic (ver. 0.35) (Bolger et al., 2014). Then, *de novo* assembly was carried out using SPAdes ver. 3.12.0 through galaxy web server (https://usegalaxy.org/) (Bankevich et al., 2012). Assembled contigs were scaffolded using SSPACE ver 2.0 software, and GapFiller (ver 2.1) was used for gap filling (unknown nucleotide) within the scaffolds (Boetzer et al., 2011; Nadalin et al., 2012). Finally, the QUAST program examined the quality of the scaffold (Gurevich et al., 2013). Assembled genomes were deposited to the NCBI whole genome shotgun (WGS) submission portal. Subsequently, genome annotation was performed

using Rapid Annotations Subsystems Technology (RAST) and PROKKA (Overbeek et al., 2014; Seemann, 2014).

3.4.4 Phylogenomic study

Phylogenetic analysis of the newly assembled genomes and other reference strains was performed. The sequence type (ST) of the *A. veronii* genomes was determined by web base service MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST-2.0) using Aeromonas schemes. The MLST scheme for *Aeromonas* spp. including the *gltA*, *groL*, *gyrB*, *metG*, *ppsA*, and *redA* genes.

The DNA sequence for each locus was exported from the PubMLST: Sequence Query output page. CLUSTALW in MEGAX was used for multilocus sequence analysis (MLSA) and multiple sequence alignment of each locus (Kumar et al., 2018). All aligned sequences were then concatenated into a contig, and the maximum likelihood tree was constructed and visualized using MEGA X under the T29+G+I (Kumar et al., 2018)

Further investigations of the phylogenetic relationship between five *A. veronii* strains in this study and the reference strain from different host and geographical origins were conducted to compare single nucleotide polymorphisms (SNPs) sequences. A total of 12 strains *A. veronii*, including strains in this study, were used in this phylogenomic analysis. Full-length genome and whole-genome shotgun (WGS) sequences of the bacteria, obtained from GenBank database, were submitted to CSI Phylogeny ver. 1.4 web tool (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) to generate the concatenated SNP sequences within the core genome region of *A. veronii* strains (Kaas et al., 2014). The CSI Phylogeny server performed multiple sequence alignment, and the obtained alignment file was then used to construct a maximum-likelihood tree, as described in the MLSA analysis. In this case, *A. veronii* B565 was selected as a reference genome in the CSI Phylogeny automated pipeline.

3.4.5 Pangenome analysis

Core- and pan-genome analyses were performed using Roary (Page et al., 2015). Briefly, gff3 files from RAST were submitted to ROARY. Subsequently, The pangenome result was visualized using Phandango (Hadfield et al., 2018).

3.4.6 Virulence properties

The existence of putative virulence factors of the five *A. veronii* strains (NK01, CNRT12, CBSB03, NBCF28, and NPSF15) was analyzed by virulence factor database (http://www.mgc.ac.cn/cgibin/VFs).

3.4.7 Prophages

The presence of prophages in *A. veronii* genomes was analyzed using PHAge Search Tool Enhanced Release (PHASTER) web server (Zhou et al., 2011; Arndt et al., 2016). Contig sequences of all genomes were concatenated to serve as an input file prior to submission to the PHASTER server. Computed results were arranged into three categories: score more than 90 was considered an intact phage element; a score between 70 and 90 was deemed questionable, and a score less than 70 was considered an incomplete phage region.

3.4.8 CRISPRs

CRISPRs distribution in *A. veronii* genomes was analyzed by submitting the genome to the CRISPRCas Finder web service (https://crisprcas.i2bc.parissaclay.fr/CrisprCasFinder/Index) (Couvin et al., 2018)

3.6 Data Analysis

Data in this study were presented using descriptive analysis and bioinformatics tool analysis.

Table 5 List of *A. veronii* genomes used in this study

Strains	Country	Host	Stage	Year	Accession no.	References
CNRT12	Thailand	Hybrid Red tilapia,	Contig	2018	GCA_012029535.1	(Sakulworakan et al., 2021)
NK01	Thailand	Nile tilapia,	Contig	2015	GCA_012029545.1	(Sakulworakan et al., 2021)
CBSB03	Thailand	Asian sea bass	Contig	2020	JALJOI000000000	This study
NBCF28	Thailand	Walking catfish	Contig	2018	JALJOG000000000	This study
NPSF15	Thailand	Striped snakehead fish	Contig	2020	JALJOH000000000	This study
TH0426	China	Yellowhead catfish	Complete genome	2016	NZ_CP012504.1	(Kang et al., 2016)
X12	China	Whucang bream	Complete genome	2017	NZ_CP024933	N/A
X11	China	Whucang bream	Complete	2017	NZ_CP024930	N/A
Ae52	Sri lanka	Goldfish	Contig	2016	BDGY00000000.1	(Jagoda et al., 2014)
CB51	China	Grass carp	Complete genome	2016	CP015448	N/A
MS1837	USA	Catfish	Complete genome	2018	NZ_CP033604.1	(Abdelhamed et al., 2019)
ML09-123	USA	Catfish	Contig	2018	PPUW01000001	(Hasan et al., 2019)

N/A: Not available

CHAPTER IV. RESULT

4.1 Distribution and antimicrobial resistance pattern of *Aeromonas* spp. among diseased freshwater fishes in Thailand

4.1.1 Sign of diseased fish

Generally diseased fish in this study showed abnormal swimming, lethargy, sluggish movement, and reduced fish intake. Clinical signs of infected fish with motile *Aeromonas* septicemia are presented in Figure 5. Most infected fish displayed varying typical clinical signs of motile *Aeromonas* septicemia, such as hemorrhage of skin, fin bases, around the mouth, exophthalmia in Asian sea bass, whereas deep skin ulceration in walking catfish, white nodules in striped snakehead fish, detached scales with hemorrhagic operculum in Nile tilapia, and hemorrhagic in enlarged liver of red tilapia were the most typical clinical signs.

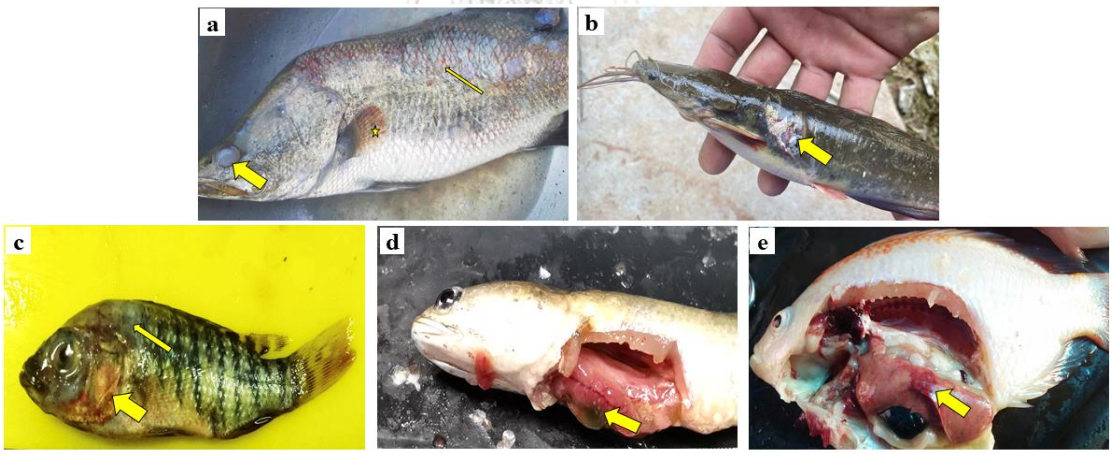

Figure 5 Clinical sign and gross lesions investigated in necropsied fishes suspected with *Aeromonas* spp. infection. The clinical sign exhibited on a) Asian sea bass with exophthalmia (thick arrow), hemorrhages in the skin (thin arrow), and fin bases (star); b) Walking catfish with deep skin ulceration, c) Nile tilapia with hemorrhage on operculum (thick arrow) and loss of scales (thin arrow); and gross lesion in d) Striped snakehead fish with white nodule and hemorrhage in the liver; e) Red tilapia with enlarged hemorrhagic liver

4.1.2 Isolation and identification

Of the bacterial isolates selected from cultures grown on TSA and *Aeromonas* selective media, 86 isolates were presumptive *Aeromonas* by confirmed Gram-negative and oxidase and catalase positive. All of the presumptive *Aeromonas* were then submitted to MALDI-TOF MS identification. Results of MALDI-TOF MS identification are shown in appendix. Sequencing the PCR product targeting *gyrB* gene (1,100 bp) confirmed all 86 *Aeromonas* isolates into six species in which *A. veronii* (72.1%) was the predominant species, followed by *A. jandaei* (11.6%), *A. schubertii* (9.3%), *A. diversa* (3.5%), *A. hydrophila* (2.3%), and *A. caviae* (1.2%) (Figure 6). Comparison results between MALDI-TOF MS and *gyrB* gene sequencing as gold standard correctly confirmed all 86 *Aeromonas* isolates (100%) at the genus level, while at the species level showed only 76 isolates (88.4%) were correctly confirmed (Table 6). The percentage of correct identification at the species level of MALDI-TOF MS, including *A. veronii* (96.8%), *A. schubertii* (100%), *A. jandaei* (60%), and *A. hydrophila* (100%). Further identification was evaluated through nucleotide BLAST showed results of nucleotide similarity for *A. veronii* (94.03–99.81%), *A. hydrophila* (95.04–97.56%), *A. schubertii* (92.45%-97.87%), *A. jandaei* (95.70–99.15%), *A. diversa* (92.97%-97.81%), and *A. caviae* (95.98%).

Distribution and diversity of *Aeromonas* spp. based on location are presented in Figure 7. Overall, *A. veronii* showed the most abundant species and is widely distributed in all provinces and fish hosts. *A. caviae* isolated from Asian sea bass was the lowest *Aeromonas* species found. Asian sea bass at Chachoengsao province had the highest *Aeromonas* spp. diversity compared to other provinces with *A. veronii*, *A. schubertii*, *A. diversa*, and *A. caviae.*

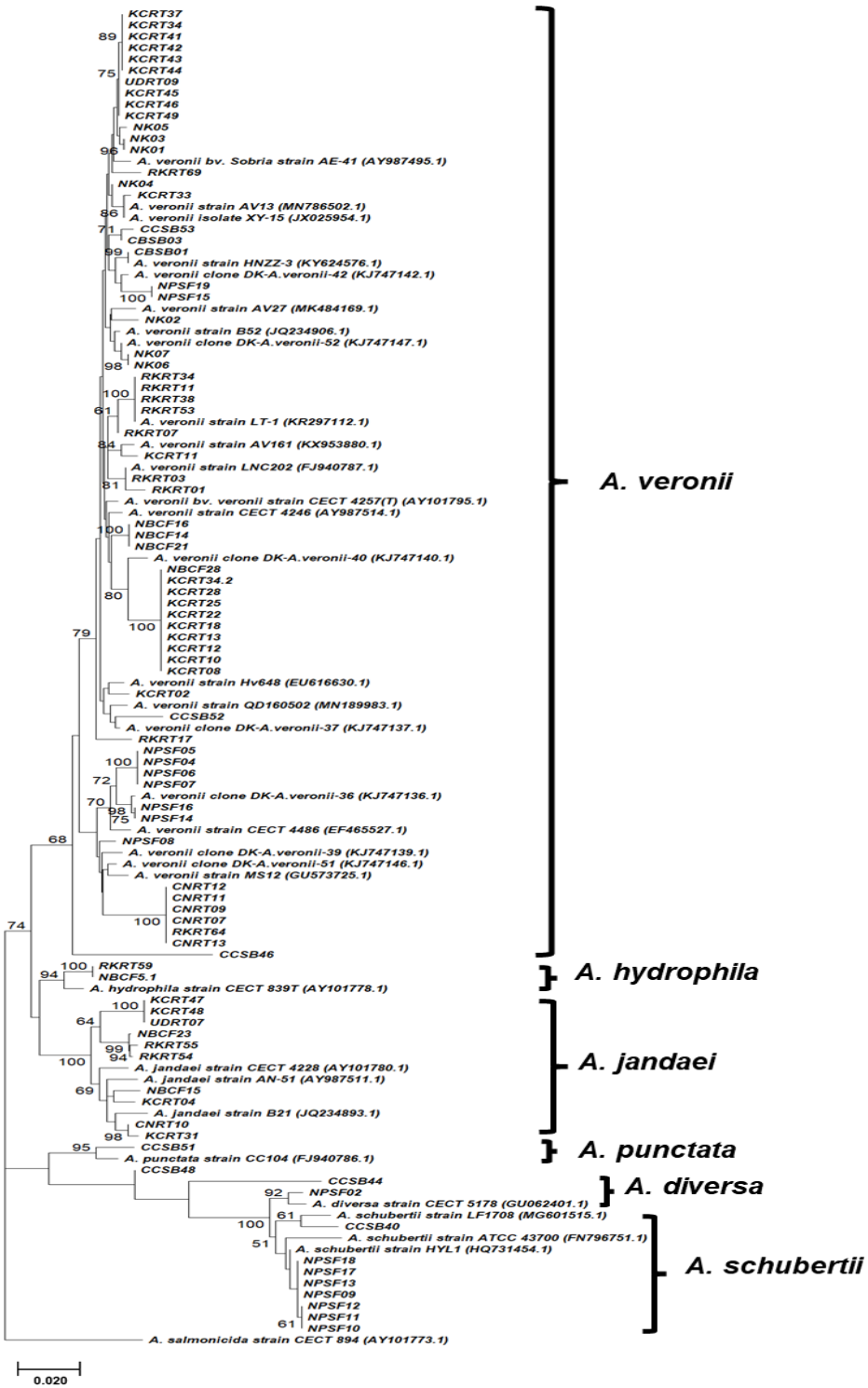

Figure 6 Neighbor-joining tree of 86 *Aeromonas* strains and reference based on sequence of *gyrB* gene with bootstrap replication of 1000. The scale bar indicated 2% sequence divergence

Table 6 Comparison result of MALDI-TOF MS with *gyrB* gene sequencing

Identification methods		**% agreement**
***gyrB* sequencing**	**MALDI TOF**	
A. veronii (*n*=62)	*A. veronii* (*n*=46)	96.8
	A. ichtiosmia[a] (*n*=14)	
	A. hydrophila (*n*=1)	
	A. jandaei (*n*=1)	
A. jandaei (*n*=10)	*A. jandaei* (*n*=6)	60
	A. veronii (*n*=2)	
	A. ichtiosmia (*n*=1)	
	A. hydrophila (*n*=1)	
A. schubertii (*n*=8)	*A. schubertii* (*n*=8)	100
A. diversa (*n*=3)	*A. hydrophila* (*n*=1)	0
	A. veronii (*n*=1)	
	A. schubertii (*n*=1)	
A. hydrophila (*n*=2)	*A. hydrophila* (*n*=2)	100
A. caviae[b] (*n*=1)	*A. schubertii* (*n*=1)	0
	Total	**88.4**

[a]*A.ichtiosmia* is synonim of *A.veronii* (Collins et al., 1993).

[b]*A. caviae* is synonim of *A. caviae*(Holmes and Farmer III, 2020)

4.1.3 Biochemical identification of *Aeromonas* isolates

All detected Aeromonas isolates were subjected to four additional biochemical assays (Table 7) to evaluate their phenotypic characteristics, showing 80% of the *Aeromonas* isolates being motile. Furthermore, all *Aeromonas* isolates (100%) tested positive for catalase, while 35% of isolates tested positive for indole, and 17% of isolates tested positive for esculin hydrolysis.

Table 7 Biochemical result of *Aeromonas* spp. isolated from diseased freshwater fish

Aeromonas species	Number of isolates	Percentage of positive isolates					
		Gram stain (-)	Esculin hydrolysis	Motility	Indole production	Oxidase	Catalase
A. veronii	62	100	16	79	44	100	100
A. jandaei	10	100	10	100	10	100	100
A. schubertii	8	100	0	50	0	100	100
A. diversa	3	100	33	100	33	100	100
A. hydrophila	2	100	100	100	0	100	100
A. caviae	1	100	100	100	100	100	100
Total	86	100	17	80	35	100	100

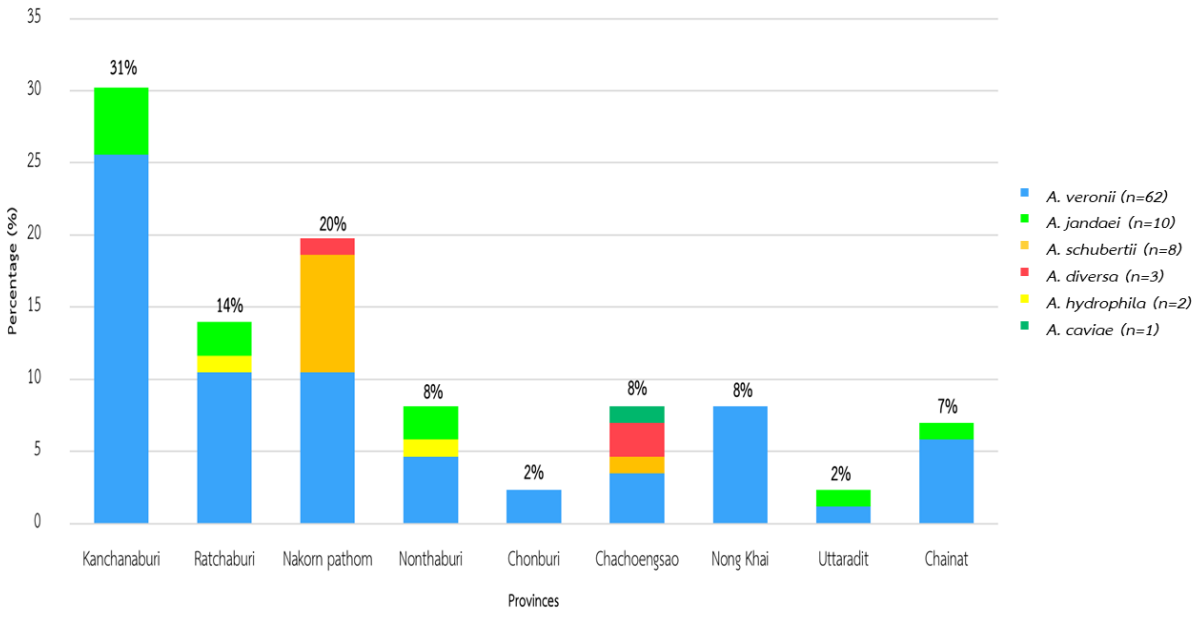

Figure 7 Distribution and diversity of *Aeromonas* spp. based on different locations as identified using *gyrB* gene.

4.1.4 Antimicrobial resistance of *Aeromonas* isolates

Evaluation of antimicrobial susceptibility to seven common antimicrobial agents was conducted on 86 *Aeromonas* isolates confirmed in this study (Table 8). *Aeromonas* isolated from diseased freshwater fishes exhibited resistance against AMC (99%), AMP (98%), OA (81.4%), and OT (77%). In contrast, ENR (21%) and SXT (24%) had the lowest resistant isolates. All *Aeromonas* spp. have at least 50% of their isolates confer resistance for AMC and AMP. In addition, *A. veronii* is the most resistant species to AMC and AMP, with 98% and 97%, respectively. Two species were resistant to ENR, including *A. veronii* (24%) and *A. jandaei* (40%). None of *Aeromonas* isolates was resistant to FFC. *A. diversa* and *A. caviae* were susceptible to OA and SXT. Most of *Aeromonas* spp. were resistant to OT except *A. caviae*. None of *A. hydrophila* isolate was founded to be resistant to SXT.

Table 8 Antimicrobial susceptibility test of *Aeromonas* by disc diffusion interpreted as susceptible (S), intermediate (I), and resistant (R)

Aeromonas spp.	No of Isolates	Percentage of isolates (%)																				
		AMC			AMP			ENR			FFC			OA			OT			SXT		
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
A. veronii	62	2	0	98	3	0	97	63	13	24	95	5	0	11	0	89	21	0	79	76	6	18
A. jandaei	10	0	0	100	0	0	100	60	0	40	100	0	0	30	0	70	20	0	80	60	10	30
A. schubertii	8	0	0	100	0	0	100	100	0	0	37.5	62.5	0	0	0	100	12.5	0.	87.5	12.5	0	87.5
A. diversa	3	0	0	100	0	0	100	100	0	0	100	0	0	67	33	0	67	0	33	67	33	0
A. hydrophila	2	0	0	100	0	0	100	100	0	0	100	0	0	50	0	50	0	50	50	100	0	0
A. punctata	1	0	0	100	0	0	100	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Total	86	1	0	99	2.3	0	98	71	8	21	91	9	0	16.3	2.3	81.4	22	1	77	69	7	24

Abbreviations: AMC, amoxicillin; AMP, ampicillin; ENR, enrofloxacin; FFC, florfenicol; OA, oxolinic acid; OT, oxytetracycline; SXT, trimethoprim-sulfamethoxazole;

4.2 Molecular characterization of predominant *Aeromonas* spp. isolated from diseased freshwater fish

4.2.1 Phylogenetic analysis based on *gyrB* gene

Phylogenetic tree based on construction *gyrB* sequence (1100 bp) revealed that 62 isolates were accurately identified as *A. veronii*. Blast alignments showed nucleotide similarities compared to the references, ranging from 94.03–99.81% (Figure 8).

4.2.2 ERIC-PCR

The enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) patterns allowed differentiating the 48 of 62 *A. veronii* isolates are shown in Figure 9. All 48 *A. veronii* isolates under analysis produced 1 to 11 amplification bands ranging from 100 to 3000 bp. The strains within each cluster appeared to have identical fingerprints and were considered to belong to the same clone. The dendrogram obtained from the ERIC-PCR analysis revealed six clusters with 95% similarity level in the current study. Four clusters came from *A. veronii,* while *A. jandaei* (KCRT04 and NBCF15), and *A. schubertii* (NPSF09 and NPSF10) are one cluster each. Isolates within these clusters were considered genetically related. A total of 14 isolates of *A. veronii* were untypeable, and 15 isolates only have one band by using this method. Most identical *A. veronii* ERIC-PCR patterns were obtained from freshwater fishes, indicating that genetically related isolates could infect other fish species.

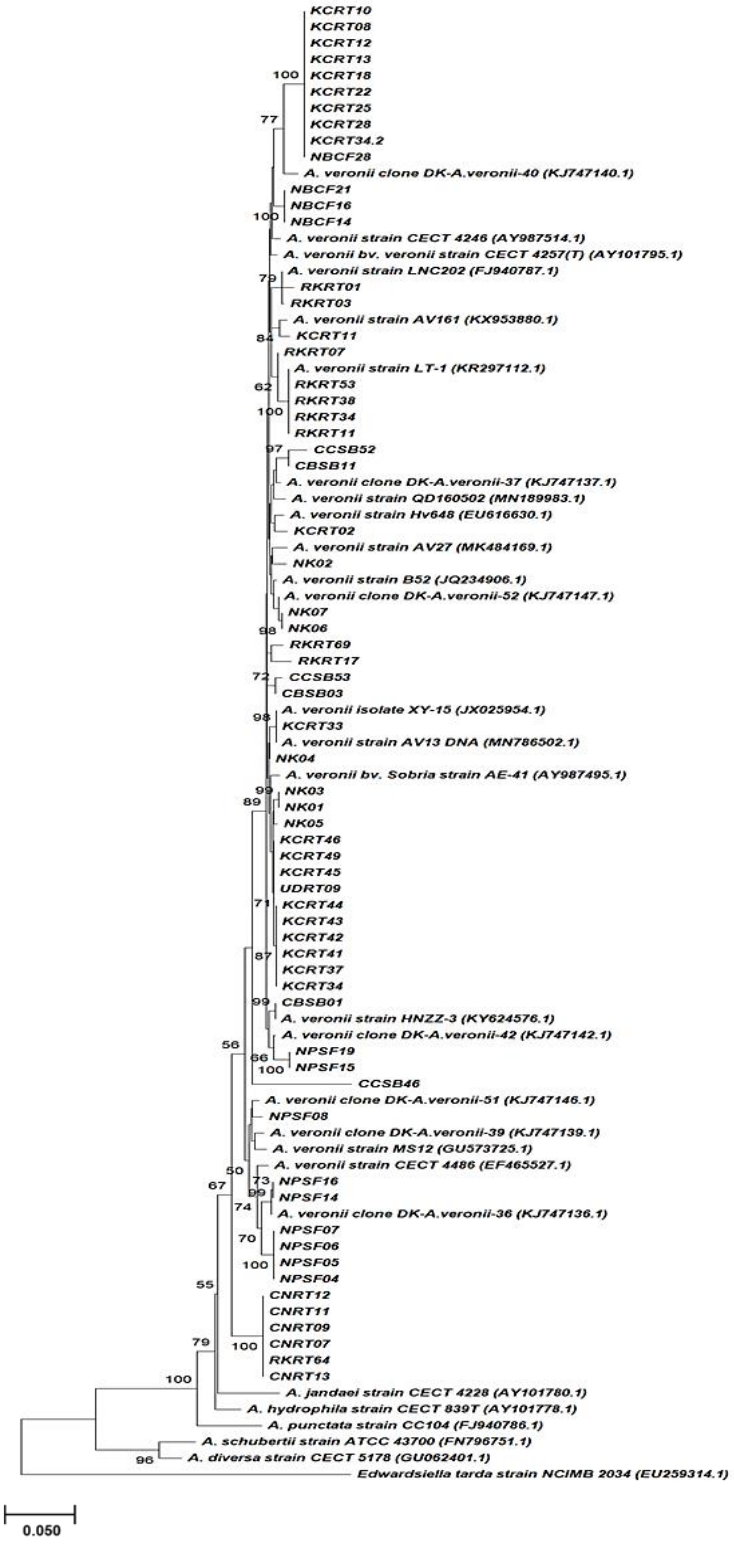

Figure 8 Unrooted Phylogenetic tree of 62 *A. veronii* and reference strains based on the *gyrB* gene sequences using the neighbor-joining method with bootstrap replication of 1000

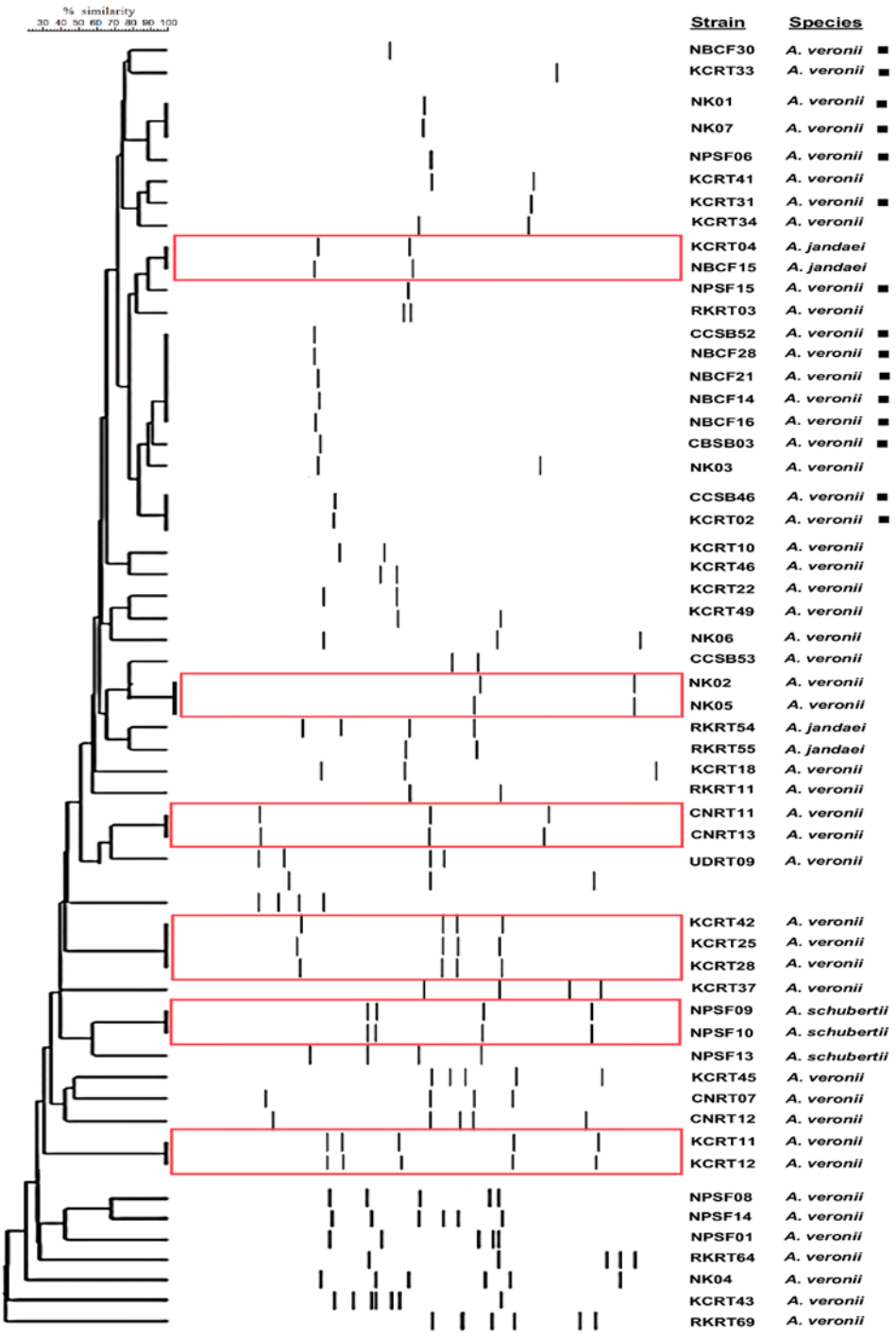

Figure 9 Dendrogram showing ERIC fingerprints of the 48 *A. veronii* isolates using dice similarity coefficient and UPGMA cluster method with tolerance of 5%. Red rectangles indicate similar ERIC-PCR patterns, and black boxes showed isolates with one band

4.3 Comparative genome analysis of the fish pathogen *Aeromonas veronii* reveals reasons for increased virulence within the species

4.3.1 Clinical signs and virulence

The animal handling and experimental challenge following the method approved by the Institutional Animal Care and Use Committee, Kasetsart University (ACKU64/F1S/008). In general, experimental infected striped snakehead fish displayed clinical signs such as abnormal breathing, lethargy, reduced food consumption, and erratic swimming. The gross lesion included loss of scales (Figure 10A) and skin ulceration with hemorrhage (Figure 10B). Post mortem displayed enlarged spleen and liver with hemorrhages and congestion (Figure 10C). Enlarged kidney is also shown in infected fish (Figure 10D). Overall, fish infected by *A. veronii* NBCF28 showed more severe gross lesions than other strains. All control fish exhibited neither clinical signs nor gross lesions.

The cumulative mortality rate and LD_{50} of *A. veronii* strains from different hosts are shown in Table 9. Mortality within 14 days of fish challenged with 10^3 CFU/mL of bacteria is 30%, between 10% and 40% with 10^4 CFU/mL, between 20% and 80% with 10^5 CFU/mL, and between 50% and 60% with 10^6. *A. veronii* strain NBCF28 isolated from walking catfish had the highest cumulative mortality (80%). The lowest LD_{50} of 1.2 x 10^5 CFU/mL was for *A. veronii* strain NBCF28, while the highest LD_{50} of 5.3 x 10^5 CFU/mL was calculated for *A. veronii* strain CBSB03.

Table 9 The number of mortalities of striped snakehead fish infected with six strains of *A. veronii*

Species	Host	Doses	Number of Deaths at Specific Time (Day Post Infection)														Number of samples	Cumulative mortality (%)	LD_{50}
		CFU/ml	1	2	3	4	5	6	7	8	9	10	11	12	13	14			
A. veronii NK01	Nile tilapia	1.9×10^4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	10	10%	4.1×10^5
		1.9×10^5	0	0	0	0	0	0	0	0	1	0	0	1	0	0	10	20%	
		1.9×10^6	0	0	2	0	0	1	0	1	0	0	1	1	0	0	10	60%	
A. veronii CBSB03	Asian sea bass	2.3×10^4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	10	10%	5.3×10^5
		2.3×10^5	0	0	0	0	0	0	0	0	0	1	0	1	0	0	10	20%	
		2.3×10^6	1	0	0	0	0	1	0	1	1	1	0	0	0	0	10	50%	
A. veronii CNRT12	Red tilapia	1.5×10^4	0	0	0	0	0	0	1	0	0	0	0	1	0	0	10	20%	2.1×10^5
		1.5×10^5	0	0	0	0	0	0	0	1	1	0	0	1	0	1	10	40%	
		1.5×10^6	0	0	1	0	0	1	2	0	0	0	1	1	0	0	10	60%	
A. veronii NPSF15	Striped snakehead fish	8.7×10^3	0	1	0	0	0	0	0	1	0	0	1	0	0	0	10	30%	1.8×10^5
		8.7×10^4	0	0	0	0	0	1	0	0	1	0	0	1	1	0	10	40%	
		8.7×10^5	0	0	0	1	1	0	1	1	0	0	0	2	0	0	10	60%	
A. veronii NBCF28	Walking catfish	7.3×10^3	0	0	0	1	1	0	0	0	0	0	0	1	0	0	10	30%	1.2×10^5
		7.3×10^4	0	0	0	0	0	1	0	0	0	0	1	1	1	0	10	40%	
		7.3×10^5	0	1	1	1	1	0	1	1	1	0	1	0	0	0	10	80%	
Control	PBS sterile	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10		

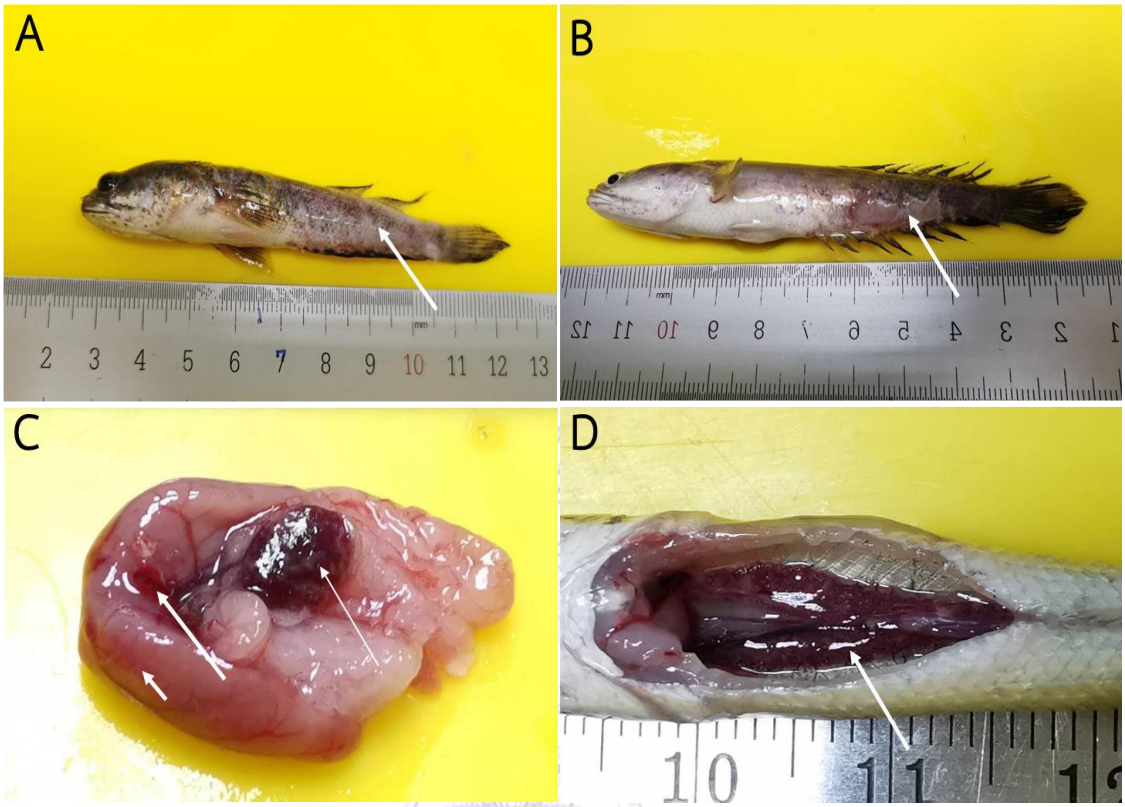

Figure 10 Clinical signs in *C.striata* inoculated with *A. veronii* strains. (A) Arrow indicated the loss of scales, (B) arrow showed skin ulcers with hemorrhage, (C) enlarged spleen (thin arrow), hemorrhage (thick arrow), and congestion (short arrow) in the enlarged liver, (D) enlarged kidney

4.3.2 Histopathology study

The histopathological lesions in experimentally infected fish showed degenerative changes in the parenchymatous organs, including the spleen, liver, and kidney. Macrophage aggregates, also known as melanomacrophage, were abundant in multiple spleen areas (Figure 11A). Liver showing fibrosis (F), vacuolation (V), and hyperemia in the sinusoid liver were dominant (Figure 11B). In kidney showed severe pathology alteration, including inflammatory cell infiltrates (I), tubules dilatation, tubules dilatation (TD), hypermia (H), hypoplasia of tubules (HYO), and hyperplasia of proximal tubules (HYP) (Figure 11C).

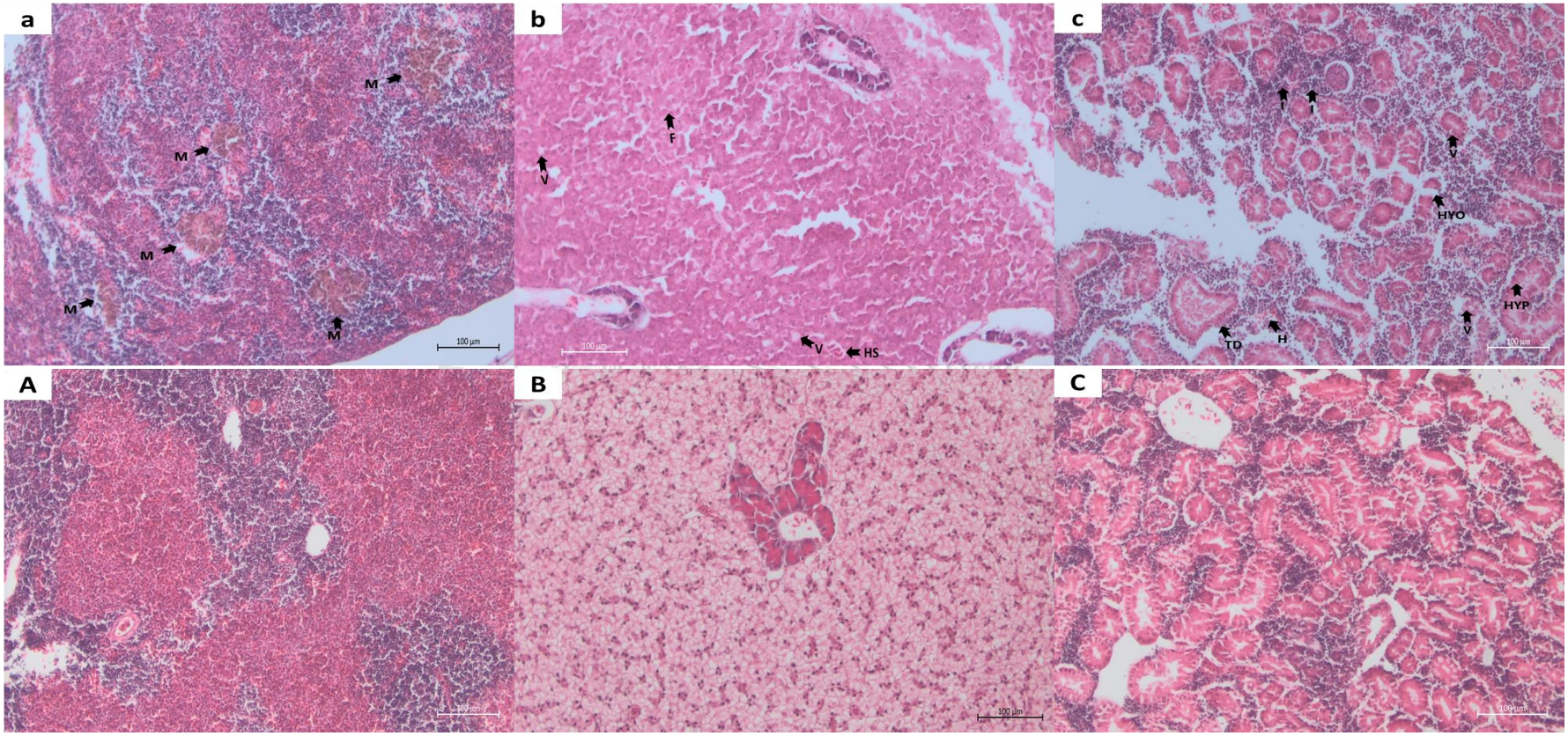

Figure 11 Histopathological changes in striped snakehead fish with *A. veronii* strains **(a)** Spleen showing numerous melanomacrophage (M) in several areas in the blood vessel **(b)** Liver showing, fibrosis (F), vacuolation (V) and hyperemia in sinusoid liver (HS) **(c)**. Kidney showing infiltration of inflammatory cell (I), tubules dilatation (TD), hypoplasia of tubules (HYO), and hyperplasia of proximal tubules (HYP)**.** Typical morphology of (A) Spleen, (B) liver, and (c) kidney from the control group

4.4 Virulome analysis of predominant *Aeromonas* spp.

4.4.1 Genome characteristic of *A. veronii* isolates

In this study, five strains of *A. veronii,* specifically NK01, CNRT12, CBSB03, NBCF28, and NPSF15 previously isolated from freshwater fishes associated with septicemia diseases, were submitted to Illumina HiSeq instrument. Basic information on *A. veronii* isolates used in the present study is presented in Table 10. In total, the SPAdes de novo assembler can generate 93 to 324 contigs for each bacterial strain. The size of *A. veronii* genome isolates varies significantly, ranging from 4.56 Mb to 4.83 Mb, and GC content ranges from 58.5 to 58.7% contains and 4,097 to 4611 coding sequences (CDSs). RAST annotation revealed that *A. veronii* isolates yielded 4097 to 4611 predicted coding sequences (CDS), in which isolate CNRT12 bears the largest genome size and the highest amount of CDS. Among all isolates, 342-520 subsystems were characterized, and the top 3 subsystem features were counted into amino acid and derivatives, carbohydrate, and protein metabolism association by 31-57% coverage of subsystem. The major genome characteristics and subsystem statistics are also presented in the appendix.

Table 10 Genomic information of five representative *A. veronii* obtained from RAST annotation server

Genome	Size (Mb)	G+C content (%)	N50	L50	Number of contigs (with PEGs)	Number of subsystem	Subsystem coverage	Number of coding sequences	Number of RNAS
NK01	4,56	58.5	171547	9	95	520	57%	4097	133
CNRT12	4,84	58.3	265081	5	324	348	31%	4611	78
CBSB03	4,63	58.6	162758	10	137	342	32%	4379	129
NBCF28	4.63	58.7	199087	8	179	350	32%	4461	131
NPSF15	4,50	58.7	356083	4	93	342	32%	4220	128

4.4.2 Phylogenomic analysis

Among the 12 *A. veronii* genomes used for the genome analysis, ST could be assigned to only one genome, MS1837 (ST254). On the other hand, the other isolates were classified as an unknown ST due to new allele variants (Appendix). In this study, only four MLST loci (*gltA*, *groA*, *metG*, and *recA*), with a total length of 2,100 bp, were included for the phylogenetic tree reconstruction since *gyrB* and *ppsA* were absent in some isolates due to the incompleteness of the genomes. The results MLSA based tree suggested a high genetic diversity among the *A. veronii* isolates. The observed phylogeny was likely unrelated to the geographical origin, host species, and virulence. Only three isolates (MS1837, TH0426, and ML09-123) from the reference genome showed a close genetic relationship due to the same host and caused high mortality in their country of origin. The same problem was also obtained from concatenated SNPs that revealed 12 strains with high genetic variability and independently clustered according to their hosts and geographical sources. There are no Thai isolates closely related and genetically identical due to a high SNPs matrix-bound by the tree topology.

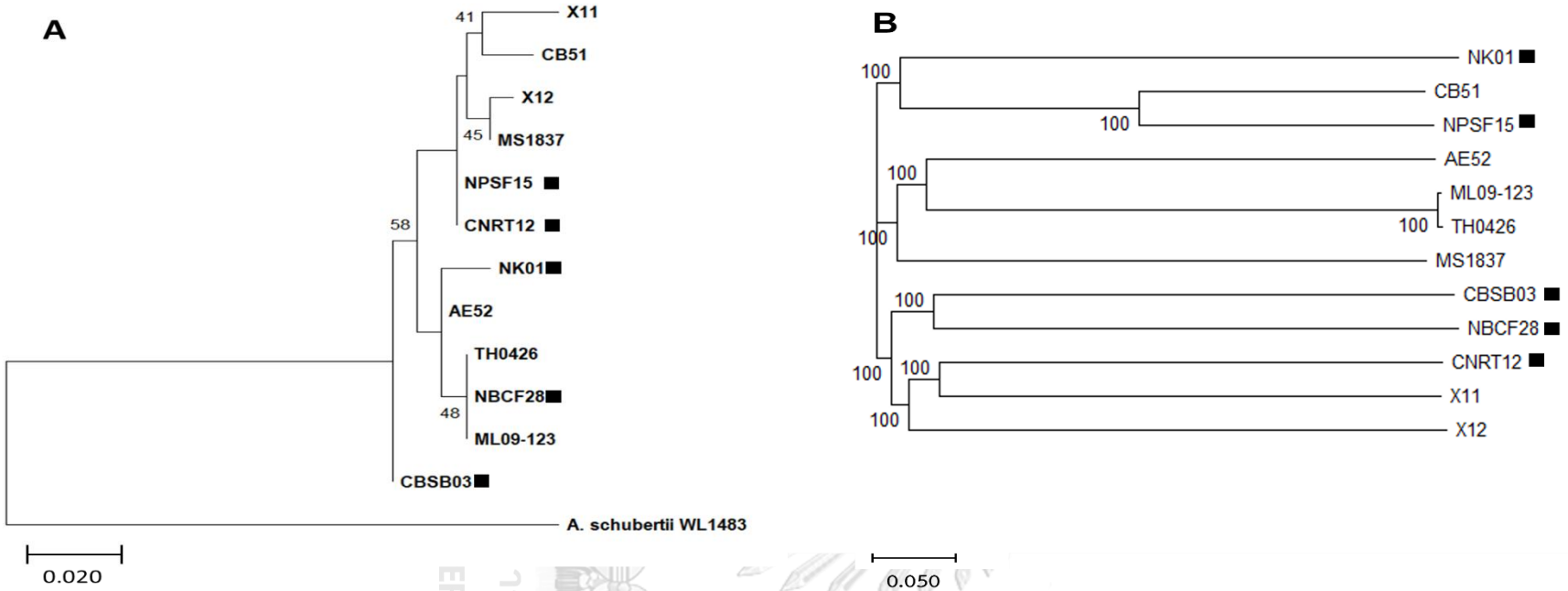

Figure 12 Maximum likelihood tree generated from concatenated MLST loci (A) and genome-wide SNPs (B). MLST loci included *gltA*, *groA*, *metG*, and *recA* with a total length of 2,100 bp. Genome-based phylogenetic tree generated from the concatenated SNPs. The CSIPhylogeny v1.4 web server constructed the tree with total length of 176,411 bp. Numbers at the tree node represent the bootstrap value in percentage (only ≥ 50 value is shown). The newly assembled genomes (Thai isolates) are indicated by the filled rectangle adjacent to the taxa. Scale bar represents the nucleotide substitution per site

4.5 Pangenome analysis

The pangenome analysis was generated by Roary and identified 6823 genes. The core genome, which consists of genes shared by at least 99% of the isolates, included 3150 genes that were ubiquitous in all tested isolates. The accessory genome included 3673 shell genes shared by 15–95% of isolates and 0 cloud genes in less than 15% of the isolates **(Figure 13**).

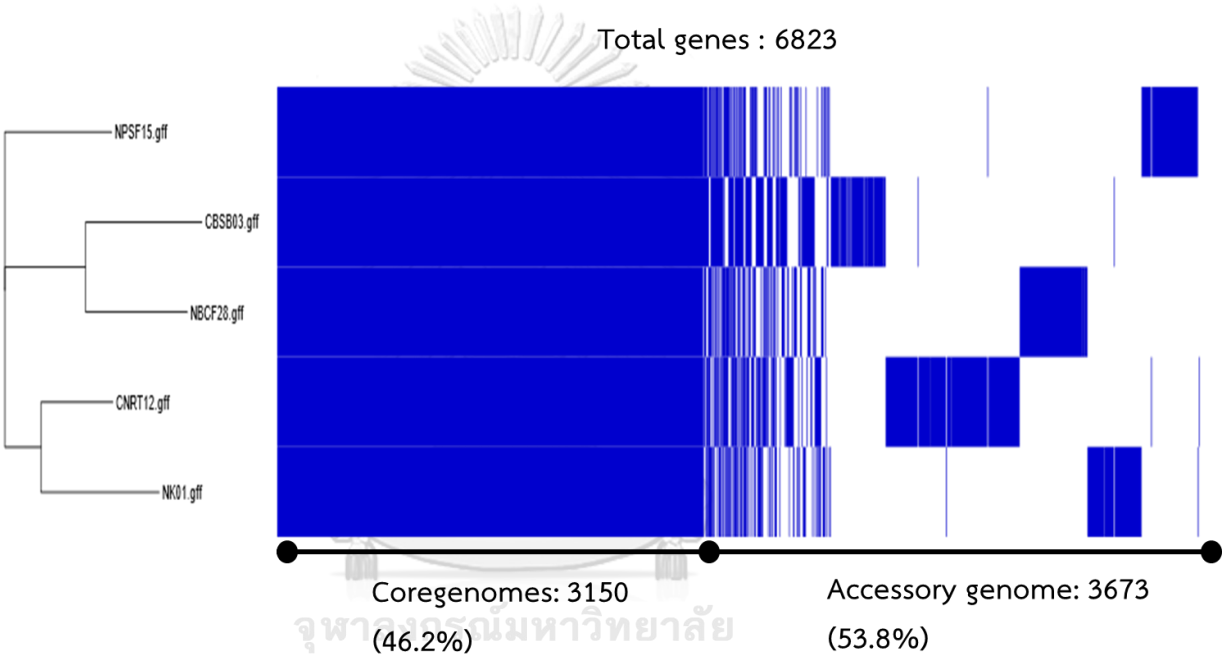

Figure 13 Pangenome analysis of the five *A. veronii* isolates using Roary Genomes were clustered according to the presence/absence of core genes. Blue: the presence of the gene, white: absence of the gene

4.6 Putative virulence properties identification

The complete list of virulence genes investigated in *A. veronii* strains in this study (NK01, CNRT12, CBSB03, NBCF28, and NPSF15) is in the appendix. Simultaneously, we conducted further research on the known virulence factors of *Aeromonas*, including those associated with pili, flagella, toxins, iron ion uptake

system, and secretion system. The results of comparative analysis of the main virulence factors of these five *A. veronii* strains are summarized in Table 11.

Comparative analysis of the pili genes among five *A. veronii* strains indicated significant differences in their pili types. All evaluated genomes had Tap type IV and MSHA type IV pili clusters, and their essential characteristics were consistent. In contrast, only the CBSB03 strain had Flp type IV pili and typed I fimbriae. Flagella analysis revealed that all five strains possessed polar and lateral flagella and distinct clusters of flagella genes. Among them, the number of polar and lateral flagella-related genes was, respectively, 61 and 36 in the NK01, 60 and 36 in the CNRT12 strain, 60 and 36 in the CBSB03 strain, 60 and 36 in the NBCF28 strain, and 60 and 36 in the NPSF15 strain, which was considered to be very similar. Aerolysin is encoded by the *aerA* gene and is one of the important factors determining the virulence of *A. veronii*. Our genome sequencing and analysis results revealed that only NK01 strain did not consist of the *aerA* gene. After comparative analysis of T2SS, T3SS, and T6SS systems of five *A. veronii* strains, the results indicated that all strains had T2SS and T3SS system gene clusters containing 14 and 45 genes, respectively, thus, basically the same. In contrast, only CNRT12 and CBSB03 strains with T6SS system gene clusters were observed. Surprisingly, both the Rtxa toxin and ion uptake system gene did not found in all strains.

4.7 Genome element of *A. veronii* strains

4.7.1 CRISPRs

CRISPR-Cas Finder only detected two sequences with confirmed CRISPR in *A. veronii* strain NK01 genome. These sequences contained 4 and 71 spacers, respectively, despite only one sequence having a CAS-TypeIE gene sequence (Table 12).

4.7.2 Prophage

The distribution of prophage in *A. veronii* genomes in this study is shown in Table 13. Strain NBCF28 has the highest number of prophages, including two intact and two incomplete prophages, while strain CNRT12 only carries one intact prophage. Strain CBSB03 and NPSF15 have one incomplete prophage, respectively. Two incomplete prophages were found in NK01 strain. The general G+C content for phage elements identified within *A. veronii* genomes vary from 36.36–59.05%. The schematic representation of this phage found in each *A. veronii* strain is exhibited in appendix.

Table 11 Comparative analysis of main virulence factor of five *A. veronii* strains

Types	Virulence factor	Strains				
		NK01	CNRT12	CBSB03	NBCF28	NPSF15
Pili	Tap type IV pili	+	+	+	+	+
	MSHA type IV pili	+	+	+	+	+
	Flp type IV pili	-	-	+	+	+
	Type I fimbrae	+	+	+	-	-
Flagella	Polar flagella	+	+	+	+	+
	Lateral flagella	+	+	+	+	+
Secretion system	T2SS	+	+	+	+	+
	T3SS	+	+	+	+	+
	T6SS	-	+	+	-	-
Toxin	Aerolysin	-	+	+	+	+
	RtxA (repeat toxin A)	-	-	-	-	-
Iron ion intake system	Related genes of iron ion uptake system	-	-	-	-	-

+, carry; -, not carry;

Table 12 General features of CRISPR-Cas loci in five *A. veronii* genomes

Strain	CRISPR region and their features					Cas element availability	
	Confirmed	Questionable	Direct repeat Length	Number of spacers	Crispr length	Cas-TypeI	Cas-TypeIII
NK01	2	8	23-47	24-71	106-4383	Cas-TypeI	-
CNRT12	-	7	24-37	102-207	81-124	-	-
CBSB03	-	2	35-43	81-207	106-148	-	-
NBCF28	-	5	23-52	1	97-161	-	-
NPSF15	-	6	81-147	1	81-147	-	-

Table 13 Prophages distribution in *A. veronii* genomes

Strains	Region	Region	Completeness	Score	Total protein	GC(%)
NK01	1	9.8 Kb	incomplete	20	11	36.36
	2	7.5 Kb	incomplete	10	10	47.43
CNRT12	1	25.6 Kb	intact	150	35	58.41
CBSB03	1	28.4 Kb	incomplete	60	29	56.98
NBCF28	1	35.9 Kb	intact	150	44	58.20
	2	41.8 Kb	intact	110	40	56.58
	3	33.9 Kb	incomplete	30	21	54.94
	4	26 Kb	incomplete	30	32	59.05
NPSF15	1	6.4 Kb	incomplete	30	6	58.70

CHAPTER V. DISCUSSION

5.1 Distribution of *Aeromonas* spp. isolated from diseased freshwater fishes in Thailand

The major findings of this study revealed that five different fish species in Thailand were infected with various *Aeromonas* spp. Numerous studies about the distribution of *Aeromonas* spp. have been frequently associated with MAS in fish farming worldwide (Azzam-Sayuti et al., 2021; Hassan et al., 2017; Nhinh et al., 2021). Our study with 86 *Aeromonas* isolates exposes that *A. veronii* (72.1%) was the predominant among all six confirmed species. Conversely, previous studies reported that *A. hydrophila* is the most common species associated with MAS in cultured freshwater fish (Austin, 2011; El-Bahar et al., 2019; Nielsen et al., 2001). Research by Ran et al. (2018) supports these findings that revealed *A. veronii* was stronger than *A. hydrophila* due to its virulence factor, which could cause septicemia in fish. These findings indicated that *A. veronii* is dominant to *A. hydrophila* because of its virulence factor. Nevertheless, to better understand the increased distribution of *A. veronii* isolated from cultured freshwater fish, further monitoring and understanding the pathogenicity of *A. veronii* is necessary.

In our study, MALDI-TOF MS should be considered a rapid identification method of *Aeromonas*, especially in aquaculture. Although it correctly confirmed all isolates at the genus level, several *Aeromonas* isolates were unsuccessful in identifying species. These inaccurate identification aligned with previous studies on the same species (Benagli et al., 2012; Shin et al., 2015; Perez-Sancho et al., 2018). Inaccurate identification of this device may be due to the database of this device not being updated. Thus, updating the database and selecting the reference of isolates should be done regularly (Piamsomboon et al., 2020).

Lately, the accuracy of biochemical tests for *Aeromonas* identification at the species level has been proven inappropriate due to inconsistent strain results (Janda and Abbott, 2010). Beaz-Hidalgo et al. (2010) revealed that 119 *Aeromonas* strains mainly isolated from diseased fish were identified phenotypically and then compared using molecular techniques (16S rRNA-RFLP and *rpoD* sequences). The results showed that only 35.5% were correctly identified phenotypically at the species level (Beaz-Hidalgo et al., 2010). From our findings, biochemical identification was successfully confirmed at the genus level, while at the species level, misidentifications often occurred due to inconsistent results. We found inconsistencies in esculin hydrolysis, indole production, and motility. For instance, some studies have reported 100% positive indole isolates for *A. hydrophila, A. veronii, A. jandaei, A. diversa,* and *A. caviae* (Gilardi, 1967; Carnahan et al., 1991; Abbott et al., 2003; Miñana-Galbis et al., 2010); even though, in the present study, only *A. caviae*was showed to be 100% indole-positive whereas *A. hydrophila* (0%), *A. jandaei* (20%), *A. veronii* (44%), *A. schubertii* (0%), and *A. diversa* (33%). *A. caviae* might not be used as a reference because the number of isolates was only one.

The result of motility test shows 100% positive on *A. hydrophila, A. jandaei, A. diversa, and A. caviae.* In contrast, it was observed that *A. veronii* (79%) and *A. schubertii* (50%) were not universally motile-positive. Even though many studies reported *Aeromonas* to be generally motile, few studies have been notified otherwise, such as *A. salmonicida* isolates reported as non-motile (McIntosh and Austin, 1991; Tomas, 2012). Furthermore, 20% *A. jandaei* and 33% *A. diversa* isolate showed ability to hydrolyze esculin, contradicting the results of Carnaham et al. (1991) and Abbot et al. (2003), that found that both *A. jandaei* and *A. diversa* could not hydrolyze esculin. From our study, 16% of *A. veronii* showed ability to hydrolyze esculin. According to Carnahan et al. (1991), based on ability to hydrolyze esculin

might be speculated that *A. veronii* refers to *A. veronii* bv. *veronii*. Despite the inconsistency of biochemical test results presented above, all of our *Aeromonas* were oxidase-catalase positive, supported by previous studies (Carnahan et al., 1991; Beaz-Hidalgo et al., 2010).

Six different species were successfully clustered based on phylogenetic analysis based on the *gyrB* gene, including *A. veronii*, *A. hydrophila*, *A. schubertii*, *A. jandaei*, *A. diversa*, and *A. caviae*. However, from our study, *A. scubertii* and *A. diversa* were in the same cluster; it was appropriate to the previous study (Fernández-Bravo and Figueras, 2020). *A. veronii* (72.1%) was the most abundant isolates among *Aeromonas* species. *A. veronii* is an important pathogen, has a wide range of hosts, and can cause symptoms including wound infection, pneumonia, and gastroenteritis in humans (Janda & Abbott, 2010). Recently, *A. veronii* has received global attention for its ability to infect a wide variety of freshwater fish such as Asian sea bass (Mursalim et al., 2020), Nile tilapia (Dong et al., 2015), red tilapia (Amal et al., 2018), channel catfish (Hoai et al., 2019), and snakehead fish (Zheng et al., 2012). The main manifestations of diseased fish are deep ulcers on the body surface and hemorrhages on internal and external (Dong et al., 2017; Raj et al., 2019; Wang et al., 2020). In some cases, exophthalmia has also been observed (Raj et al., 2019). This species reported consists of two biovars, *A. veronii* bv. *veronii* and *sobria*, with the latter being concerned due to its ability to cause epizootic ulcerative syndrome (EUS) (Janda & Abbott, 2010; Rahman et al., 2002; Silver et al., 2011). *A. jandaei* was first isolated from clinical specimens (blood, wounds, stool) and a prawn (Carnahan et al., 1991). According to recent research, *A. jandaei* contributed to causing high mortality of cultured Nile tilapia in Brazil (Assane et al., 2021). In Thailand, *A. schubertii* has been isolated from shrimp and Asian sea bass (Mursalim et al., 2020; Sangpo et al., 2020). In the present study, all *A. schubertii* isolates were mainly isolated from striped snakehead fish (87.5%) and Asian sea bass (12.5%). These results are

supported by several previous studies where isolates of *A. schubertii* become the primary pathogen causing white spot nodules in internal organs of the family Channidae and have since spread rapidly throughout China. According to their report, the pathogen infects the main snakehead species, *C. maculata* and *C. argus*, as well as the hybrid cross of *C. maculata* females and *C. argus* males, causing high mortality (45%) and becomes one of the most severe diseases in the snakehead farming industry (Chen et al., 2012; Liu & Wang, 2012; Zhang et al., 2019). This is the first time *A. diversa* and *A. caviae* have been recovered from cultured freshwater fish since they were first described in human sources (Miñana-Galbis et al., 2010). Although, a previous study reported *A. diversa* had been found in ornamental fishes (Jagoda et al., 2014).

The comparison between MALDI-TOF MS and *gyrB* gene sequencing revealed that *gyrB* gene sequencing was more accurate at confirming *Aeromonas* at the species level than MALDI-TOF MS. However, MALDI-TOF MS is used for rapid examination or screening to carry out treatment to reduce losses in aquaculture immediately.

Antimicrobial susceptibility tests contributed to valuable insight into the resistance capabilities of these *Aeromonas* species. This study observed seven antimicrobials agents allowed and registered for prevention and treatment in Thai aquaculture (Sumpradit et al., 2021). Our findings revealed that more than 90% of *Aeromonas* strains showed resistance to β-lactam antibiotics such as AMC and AMP. These results also agree with the previous studies reported by many researchers (Azzam-Sayuti et al., 2021; Sakulworakan et al., 2021). Many *Aeromonas* strains isolated from diseased freshwater fish in the studied ponds exhibited high antibiotic resistance against AMC and AMP, indicating that chromosomal encode β-lactam resistance genes frequently produced in *Aeromonas* isolates microbes in the studied aquatic environment (Saavedra et al., 2004; Soto-Rodriguez et al., 2018). OA, a

quinolone antibiotic group, was resistant primarily due to mutations in the quinolone resistance determining regions (QRDRs) (Kim et al., 2011). According to Jia et al. (2017), the presence of the qnrS2 gene indicates the presence of the plasmid-mediated quinolone resistance protein, which was discovered in *Salmonella enterica* and is involved in horizontal gene transfer. However, few publications reported that *A. veronii* encoded the *qnrS2* gene on a plasmid since the first report in 2008 (Sánchez-Céspedes et al., 2008; Alcock et al., 2020; Sakulworakan et al., 2021). However, no isolate was resistant to FFC, and ENR was the antibiotic with the least resistant *Aeromonas* isolates (22%). ENR was reported effectively prevent and treat various systemic bacterial fish infections such as those of *Aeromonas* spp., *Vibrio* spp., and *Plexiomonas shigelloides* (Maluping et al., 2005). ENR, with high potential efficacy and safety for treating diseases in animals, has received growing attention (Fan et al., 2017). A previous study also supported these findings, showing that FFC effectively works against MAS, and no resistant strain was founded (Godoy et al., 2008; Mursalim et al., 2020). Therefore, evaluating antimicrobial susceptibility is crucial for regularly monitoring the severity of antimicrobial resistance to treat diseased fish in aquaculture and avoiding risks to human health. In addition, to reduce antibiotic resistance, alternative environmentally-friendly treatments such as probiotics, herbal therapies, phages, and vaccinations need to be developed immediately. This will improve food quality and minimize negative impacts on human health and the environment.

Since the number of fish samples collected is not equal on each farm with lengthy collection duration, the results cannot be confirmed as a prevalence study. Furthermore, we could not find a significant difference in comparing species distribution.

In conclusion, our findings revealed that *A. veronii* is the predominant species in freshwater fishes of Thailand, raising concerns about public health. Most of the

A. veronii isolates tested positive for antibiotic resistance when tested with the seven antibiotics used in this study. These findings should raise awareness among fish farmers and aquaculture workers about the spreadability of *A. veronii* in aquaculture systems and the prudent use of antibiotics in fish farms. As a result, more monitoring and research should be conducted to develop effective treatments, prevention, and vaccines to reduce antibiotic use in aquaculture worldwide.

5.2 Molecular typing of predominant *Aeromonas veronii* isolated from diseased freshwater fish

Previous studies mention that *A. veronii* is the predominant species in Thai cultured freshwater fish. The number of fish infections caused by *A. veronii* is increasing continuously and becoming a prominent concern in aquaculture. The *gyrB* genes have been used successfully as accurate, decisive molecular chronometers to identify *A. veronii* (Yáñez et al., 2003; Chen et al., 2019; Hoai et al., 2019). The genetic diversity expressed by the *gyrB* gene exhibited isolates from different fish species in the same cluster. It indicates that *A. veronii,* which has genetic closeness, can infect other fish species.

In our study, ERIC fingerprints suggested that the *A. veronii* were highly diverse across freshwater fishes in Thailand. According to our result, ERIC PCR fingerprinting formed four clusters of 9 isolates at the 95 % similarity level. In contrast, other *A. veronii* isolates showed different fingerprint bands, suggesting that they were not clonally associated. These findings supported the findings of Jagoda et al. (2014), who discovered increased intraspecific diversity in *A. veronii* using this method. This variety of genetic diversity was observed among environmental and clinical isolates of *Aeromonas* from various sources that were typed using the technique mentioned above (Szczuka and Kaznowsky, 2004). In 2015, the ability of ERIC-PCR to display the clonality of isolates, where 30 isolates out of 122 *Aeromonas* isolated from Malaysian lakes successfully formed 10 clusters (Khor et al., 2015). ERIC-PCR confirmed its discriminatory power in this work and is rapid and easy to perform for routine epidemiological investigations. This kind of high genetic diversity has been observed among clinical and environmental isolates of aeromonads from different sources typed by the above technique (Szczuka and Kaznowsky, 2004).

In the present study, 14 isolates of *A. veronii* produced an absence of bands, and 15 isolates only produced one band pattern in ERIC-PCR despite multiple

repetitions of amplification. This problem is similar to Cheok et al. (2020). The lack of amplification may be due to the atypical ERIC sequences and thus to the lack of binding sites of the currently used primers (Cheok et al., 2020). Therefore, the major limitation of this study is several isolates showing erroneous due to being untypeable and appearing one band only. This serious problem should be considered to selecting other typing methods for the genus *Aeromonas*.

In conclusion, the present study highlights that ERIC-PCR revealed the diversity of several *A. veronii* associated with MAS in culture freshwater fish. However, ERIC PCR is not recommended for characterizing *Aeromonas* due to its limitation.

5.3 Comparative of pathogenicity *Aeromonas veronii* strains in striped snakehead fish (*Channa striata*)

We evaluated the virulence level of *A. veronii* isoalated from naturally infected fishes to challenge striped snakehead fish by intraperitoneal injection and described experimental infection. Our findings revealed that all *A. veronii* strains are susceptible to cause high mortality (≥ 50%) in striped snakehead fish, which might potentially cause significant harm in terms of public health and safety. Generally, skin ulcers, intra-abdominal hemorrhage, and other clinical symptoms are caused by *Aeromonas* infection in fish. Interestingly, different *Aeromonas* infections cause a wide range of symptoms (Chen et al., 2019). In catfish, for example, clinical signs of bacterial septicemia produced by *A. veronii* included pale gills, slight abdominal distension, and swollen and inflamed vents (Mohammed and Peatman, 2018). When *A. veronii* infects Nile tilapia, loss of scale appears, hemorrhages all over the body surface, and bilateral exophthalmia (Hassan et al., 2017; Raj et al., 2019).

During the post-infection period of the current study, fingerling striped snakehead fish displayed several general clinical signs such as abnormal swimming, lethargy, and aggregated on the water surface. Others appeared to be sluggish and lost in their escape reflex. Skin ulceration and loss of scales were discovered on the fish's body surface. This result is in agreement with the findings of (Zheng et al., 2012) in snakehead fish (*Ophiocephalus argus*), (Wassif, 2018) in Nile tilapia (*Oreochromis niloticus),* and (Hoai et al., 2019) in channel catfish (*Ictalurus punctatus*). The most prevalent gross lesion seen in recently moribund dead and sick fish was enlargement and internal organ hemorrhages (spleen, liver, and kidney). The fishes' confinement in aquaria during the experimental infection with growing bacterial load on the host fish may have played a key role in developing these symptoms (Mazumder et al., 2021).

Our histopathological examination revealed that *A. veronii* infection could cause alteration in internal organs of striped snakehead fish, also typical following *A.*

veronii infection. In the liver, alterations were manifested as fibrosis, vacuolation and hyperemia in the blood vessel and sinusoid liver (Dong et al., 2017; Hassan et al., 2017; Hal and El-Barbary, 2020). The abundance of melanomacrophage (MMC) was prominent in multiple spleen areas (Azzam-Sayuti et al., 2021). Necrosis of the kidney was described by infiltration of inflammatory cells, interstitial hemorrhages, dilatation of tubules, atrophy of tubules, and hypertrophy of proximal tubules were also reported in previous studies (Azzam-Sayuti et al., 2021; Mazumder et al., 2021). Alternatively, hemocyte aggregation and congestion in all selected internal organs indicated that the liver, spleen, and kidney are the target organs for *Aeromonas* systemic infection (Abdelhamed et al., 2017; Chen et al., 2018). Histopathological abnormalities in the liver, spleen, and kidney also pointed to acute to subacute cellular damage. These lesions might result from the bacterial products and their adverse effect on the tissue. The cause of mortality in *Channa striata* was determined by these signs.

The current findings of this study demonstrated that *A. veronii* is an important bacterial pathogen that is highly infectious in striped snakehead fish and causes massive mortality. The pathogenicity test showed that the smaller the LD50 and the stronger the pathogenicity. Hence, we could confirm that *A. veronii* strain NBCF 28 (1.2 x10^5 CFU/ml) obtained from walking catfish was the most virulent strain based on LD_{50}. On the other hand, *A. veronii* strain CBSB03 (5.3 x 10^5) from Asian sea bass is the least virulent. As a result, infected striped snakehead fish are susceptible to all *A. veronii* strains from various fish species. More research is needed, particularly on the genomes of *A. veronii*, which influenced its virulence characteristics.

5.4 Virulome analysis of the fish pathogen *Aeromonas veronii* reveals reasons for increased virulence within the species

The number of cases related to *A. veronii* outbreaks is growing gradually, particularly in the aquaculture sector (Hasan et al., 2019). However, there is still a lack of relevant reports about the pathogenicity of *A. veronii*, especially concerning to genomic approach of virulent factors. This confines when it comes to deep study on the pathogenicity of *A. veronii*. The current study sequenced and assembled the complete genomes of five *A. veronii* strains, including NK01, CNRT12, CBSB03, NBCF28, and NPSF15. The complete genome sequence information allows us to better understanding the separation between virulent, attenuated, and avirulent strains and serve as the foundation for investigating the causes of the increased virulence of *A. veronii* strain NBCF28 during experimental challenge.

The frequency of 'intraspecies' SNP between bacterial isolates could vary due to organisms' genetic heterogeneity. For example, the geographically diverse isolates between *A.veronii* strains ML09-123 (USA) and TH0426 (China) were highly similar and both caused ulcerative syndrome disease in yellow catfish, carrying only 720 SNPs (Hasan et al., 2019; Yang et al., 2021). On the contrary, our findings compared with 12 isolates of distinct biotypes of *A. veronii*, most isolates collected from freshwater fishes, revealed 53,682 SNPs present among the core genome totaling 2.1 Mb in size. Hence, the large number of SNPs identified between *A. veronii* genomes suggested that the species has a relatively high degree of genetic heterogeneity.

The MLSA scheme for the epidemiological study of *A. veronii* was recently proposed based on DNA sequence variation of six housekeeping genes. (Martino et al., 2011). Further analysis of these five *A. veronii* strains revealed distantly associated global sequence types, indicating that they are not related to the STs in the *Aeromonas* MLST database.

Pangenome analysis using Roary revealed a relatively low proportion of core-genome genes (46.2%) compared to pan-genome genes, indicating a substantial amount of gene acquisition in *A. veronii* clades and strains. However, the core genome size (3150 genes) relative to the average number of protein-coding genes for *A. veronii* (4357 genes) is 63.8%. Thus, the species has a relatively large set of core functions with highly variable sections of the genome that potentially enable adaptations in different environmental.

The ability of pathogenic *A. veronii* to adhere to and colonize the host surface is critical for successful infection. This pathogenic mechanism involves major virulence factors, such as adherence, effector delivery system, motility, and exotoxin. Comparative genome analysis of the NK01, CNRT12, CBSB03, NBCF28, and NPSF15 strains showed differences in the main virulence factor between these five strains (Table 10). Gene cluster-related Flp type IV was found in the genome of CBSB03, NBCF28, and NPSF15 strains, while neither the NK01 nor CNRT12 strain had this type of pili. Flp type IV pili, the gene encoding biosynthetic proteins of peritrichous pili, has contributed to biofilm formation and autoagglutination (Boyd et al., 2008). According to Boyd et al. (2008), the *flp* gene had minimal or no contribution to the virulence of the strains while compared with *tap* gene, which was absent in the salmon experiment challenged with *A. salmonicida* (Boyd et al., 2008). The gene cluster related to type I fimbrae was found in the genome of NK01, CNRT12, and CBSB03. Type I fimbrae are structurally characterized as short-rigid and are frequently reported in environmental samples; generally, these structures are unrelated to the pathogenicity of the bacteria and are present in *A. hydrophila* the majority of the time (Lowry et al., 2014; Liu, 2015). Flp type 4 pili and type 1 fimbria may assist in their continued existence and multiplication, a first step in the developing infection. (Boyd et al., 2008; Dacanay et al., 2010; Yang et al., 2021).

Gram-negative bacteria have developed various secretion systems to deliver small molecules, proteins, and DNA into the extracellular space or target cells. Secretion systems play critical roles in bacterial metabolism and pathogenesis, including host invasion, immune evasion, tissue damage, as well as bacterial competition. (Green and Mecsas, 2016). This study has found three secretion systems among all *A. veronii* isolates, i.e., T2SS, T3SS, and T6SS. Those secretion systems are generally found in the genus *Aeromonas*. However, our findings found that T2SS and T3SS were found in all genome strains, while T6SS was found only in CNRT12 and CBSB03 genomes. T2SS and T3SS secretion systems have been reported in *A. veronii* (Silver et al., 2007). Our findings revealed that five *A. veronii* strains possess both T2SS and T3SS systems. T2SS is contributed to secretion of many virulences, including aerolysis, proteases, hemolysin, and Dnase (Burr et al., 2001). In this way, T2SS supports *A. veronii* in colonizing the gut of leeches; in particular, T2SS transports hemolysin to the leech gut in early colonization. T2SS is used by many strains of *A. veronii* and is maintained as one of the secretory systems (Maltz and Graf, 2011). The T3SS , well known as injectisome, is evolutionarily related to the flagellum that serves as an injection needle that can bridge the environmental gap between the bacterium and the host cell and transport the substances to the host (Frey and Origgi, 2016). In this study, all the *A. veronii* genomes possess the mandatory genes for flagella. A previous study reported that T3SS-delivered effector molecules and toxins of *A. salmonicida* impair the host cytoskeleton, thus damaging cell physiology and phagocytosis and related to immunosuppression of the host (Origgi et al., 2017). However, T3SS is closely related to pathogenicity and affects the virulence of pathogenic bacteria in many secretion systems of Gram-negative bacteria (Tampakaki et al., 2010).

T6SS or Vas (Virulence associated secretion) is a multiprotein weapon that kills eukaryotic predators or prokaryotic competitors by delivering toxic effectors.

Bioinformatic analysis highly conserved T6SS gene cluster presence in 25% Gram-negative bacteria (Ho et al., 2014). T6SS genes are typically found on chromosomes, but they can also be found in mobile genetic elements and plasmids, where they can be transferred and increase genetic diversity. This secretion system has phage mechanism as structural components that are able to inject protein effectors directly into the cytosol of the target cell. It was reportedly responsible for producing the hcp (hemolysin-coregulated protein) for causing apoptosis and VgrG (valine-glicyne repeat G) gene family for preventing phagocytosis, with eukaryotic and bacterial cells as the main destinations (Suarez et al., 2010; Sha et al., 2013). Nevertheless, this study's presence of T6SS in *A. veronii* might be speculated based on geographical location and host species. For instance, Bruto et al. (2017) detected T6SSs in *V. crassostreae* isolates from Brest, near the Atlantic coast of north-west France, but not in isolates from Sylt in northern German. By host, for instance, *B. uniformis* isolated from the human gut microbiota. Whereas *B. uniformis* isolates with T6SS type GA1 were isolated from one individual (CL06), isolates from another individual (CL11) did not carry the T6SS type GA1 (García-Bayona et al., 2021). More research is required to comprehend the predominance of the T6SS among bacteria fully.

Aerolysin is the gene that is essential for hemolytic, cytotoxic, and enterotoxin activities (Degiacomi et al., 2013). Aerolysin that induces **β** hemolysin (encoded by the gene *aerA*) is commonly isolated from fish, one of the essential components in *A. veronii* pathogenicity (Yang et al., 2021). On the contrary, in this study, only strain NK01 did not encode Aerolysin, which is important for changing blood cells' permeability. Instead, the *hyl*A gene plays the same role as Aerolysin in producing hemolysin gene that can affect lysis blood cells (González-Serrano et al., 2002; Oleiwi et al., 2014).

CRISPR-Cas systems are composed of two primary components: the CRISPR array and the CRISPR-associated genes (Cas), separated by spacers (Grissa et al.,

2007). They establish defense mechanisms against viral infection and invasion of foreign DNA (lysogenic bacteriophages, plasmids, or transposons) (Horvath and Barrangou, 2010). Our findings found at least two intact CRISPR systems in *A. veronii* strain NK01 and encode CAS elements in addition to CRISPR areas. While the other only showed questionable CRISPR. It might be speculated that the number of CRISPR enhances the immune system of bacteria to inhibit exogenic infection.

The bacteriophage inserted a portion of the genome called prophage. Most aquatic bacteria (about 70%) are infected with prophages (Chen et al., 2006). Of particular interest, bacteriophages can mediate horizontal gene transfer, including genes encoding virulence factors and antibiotic resistance (Colomer-Lluch et al., 2011). These abilities may contribute to bacteria providing specific mechanisms for bacterial attachment, invasion, and survival, particularly in harsh environments (Canchaya et al., 2003). Increasing phage numbers may be the reason for concern since they can enhance the pathogenicity of a bacterial strain or transform an avirulent strain into virulent (Canchaya et al., 2004). The *A. veronii* strain NBCF28 has more phage regions (four regions) than the other strains in this study. Each region contains proteins contributing to virulence factors, including Integrase, tail shaft, hypothetical protein, transposase, terminase, and tRNA. This finding was supported by a previous study that mentioned two virulence Strains of MS-17-88 and TH0426 had the maximum number of identified prophage elements (six elements per strain) (Tekedar et al., 2020; Yang et al., 2021). This finding indicated that the number of putative prophages might be increased the virulence of *A. veronii* strain NBCF28 previously.

To summarize, the genomic comparisons among five *A. veronii* strains of distinct origins in Thailand revealed a significantly diverse genetic relationship and the variations in genome size, number of genes, distribution, STs, SNPs, CRISPR spacer

profiles, number of prophages, and the putative virulence factors were equally distributed among the sequenced strains.

CHAPTER VI. GENERAL DISCUSSION AND CONCLUSION

This study found the distribution and diversity of *Aeromonas* spp. based on a phylogenetic construction of the *gyrB* gene and MALDI-TOF MS revealed that *A. veronii* is the predominant freshwater fish species in Thailand, including its high resistance to several antibiotics. Furthermore, the utility of MALDI-TOF MS in aquaculture can be a solution for rapid and low-cost identification in routine diagnosis. Therefore, treatment and management improvement could be immediately prepared. The role of *A. veronii* as the predominant pathogen replacing *A. hydrophila* during disease outbreaks should be reinvestigated. Molecular characterization using *gyrB* sequencing and ERIC-PCR revealed that *A. veronii* strains from different fish species form clusters, indicating that different strains can infect other freshwater fishes. ERIC-PCR discovered the intraspecies diversity found in the *A. veronii* isolated in this study. Due to several isolates could not be typing with this method and several isolates only having one band, the use of other typing methods can be considered. According to the challenge test, all *A. veronii* strains are susceptible to striped snakehead fish with mortality ≥50%. This finding also indicated that *A. veronii* is an important species that is responsible for disease prevalence in cultured freshwater fish in Thailand. Genome comparison strains revealed multiple virulence factors which affect *A. veronii* strains in this study, such as fimbriae, flagella, toxins, iron ion uptake systems, type II, type III and type VI secretion systems, CRISPR, and prophages. These particular virulence factors may be the fundamental reasons for the increased virulence.

Due to the high potential for pathogenicity of *A. veronii* to fish, investigating the high prevalence of *A. veronii* in this study suggests that fish farmers should be aware of this pathogen because of its significant impact on Thai freshwater aquaculture. Further research on the combination between *gyrB* gene and MALDI-

TOF MS to develop the MALDI spectra database should update and improve the library with high log scores in routine diagnosis. This study provides genomic information related to the pathogenesis and spread of *A. veronii* in several fish species, which will greatly assist in vaccine research. Besides that, more surveillance and research to develop effective treatments, prevention, and vaccines. Therefore, careful use of antimicrobials, intensive hygiene management, and monitoring should be promoted as key strategies to address these challenges.

REFERENCES

REFERENCES

Abbott SL, Cheung WK and Janda JM 2003. The Genus *Aeromonas*: Biochemical Characteristics, Atypical Reactions, and Phenotypic Identification Schemes. J Clin Microbiol. 41: 2348-2357.

Abdelhamed H, Ibrahim I, Baumgartner W, Lawrence ML and Karsi A 2017. Characterization of Histopathological and Ultrastructural Changes in Channel Catfish Experimentally Infected with Virulent Aeromonas hydrophila. 8.

Abdelhamed H, Lawrence ML and Waldbieser G 2019. Complete genome sequence data of multidrug-resistant *Aeromonas veronii* strain MS-18-37. Data Brief. 23: 103689.

Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen A-LV, Cheng AA, Liu S, Min SY, Miroshnichenko A, Tran H-K, Werfalli RE, Nasir JA, Oloni M, Speicher DJ, Florescu A, Singh B, Faltyn M, Hernandez-Koutoucheva A, Sharma AN, Bordeleau E, Pawlowski AC, Zubyk HL, Dooley D, Griffiths E, Maguire F, Winsor GL, Beiko RG, Brinkman FSL, Hsiao WWL, Domselaar GV and McArthur AG 2020. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 48(D1): D517-D525.

Aliyu-Paiko M, Hashim R and Shu-Chien AC 2010. Influence of dietary lipid/protein ratio on survival, growth, body indices and digestive lipase activity in Snakehead (*Channa striatus*, Bloch 1793) fry reared in re-circulating water system. Aquac Nutr 16: 466-474.

Ambak MA, Bolong AA, Ismail P and Tam BM 2006. Genetic variation of Snakehead fish (*Channa striata*) populations using Random Amplified Polymorphic DNA. . Biotechnology. 1: 104-110.

Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y and Wishart DS 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44(W1): W16-21.

Azzam-Sayuti M, Ina-Salwany MY, Zamri-Saad M, Annas S, Yusof MT, Monir MS, Mohamad A, Muhamad-Sofie MHN, Lee JY, Chin YK, Amir-Danial Z, Asyiqin A, Lukman B, Liles MR and Amal MNA 2021. Comparative Pathogenicity of *Aeromonas* spp. in Cultured Red Hybrid Tilapia (*Oreochromis niloticus* × *O. mossambicus*). 10(11): 1192.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA and Pevzner PA 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19(5): 455-477.

Barton ES, Andrew FG and Frederick MA 2018. Overview of Next Generation Sequencing Technologies. Curr Protoc Mol Biol. 122: e59.

Beaz-Hidalgo R, Alperi A, Bujan N, Romalde JL and Figueras M 2010. Comparison of phenotypical and genetic identification of *Aeromonas* strains isolated from diseased fish. Systematic and Applied Microbiology. 33: 149-153.

Beaz-Hidalgo R and Figueras M 2013. *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease. J Fish Dis. 36: 371-388.

Beaz-Hidalgo R, Latif-Eugenin F, Hossain MJ, Berg K, Niemi RM, Rapala J and Figueras MJ 2015. *Aeromonas aquatica* sp. nov., *Aeromonas finlandiensis* sp. nov. and *Aeromonas lacus* sp. nov. isolated from Finnish waters associated with cyanobacterial blooms. Syst Appl Microbiol. 38: 161-168.

Behjati S and Tarpey PS 2013. What is next generation sequencing? Archives of disease in childhood. Education and practice edition. 98(6): 236-238.

Benagli C, Demarta A, Caminada A, Ziegler D, Petrini O and Tonolla M 2012. A rapid MALDI-TOF MS identification database at genospecies level for clinical and environmental *Aeromonas* strains. PLoS One. 7(10): e48441.

Boetzer M, Henkel CV, Jansen HJ, Butler D and Pirovano W 2011. Scaffolding preassembled contigs using SSPACE. Bioinform Biol Insights. 27(4): 578-579.

Bolger AM, Lohse M and Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. J. Bioinform (Oxford, England). 30(15): 2114-2120.

Boonyaratpalin M, McCoy EW and Chittapalapong T 1985. Snakehead culture and its socio-economics in Thailand. In: National Inland Fisheries Institute, Bangkok, Thailand.

Boyd JM, Dacanay A, Knickle LC, Touhami A, Brown LL, Jericho MH, Johnson SC and Reith M 2008. Contribution of type IV pili to the virulence of Aeromonas salmonicida subsp. salmonicida in Atlantic salmon (Salmo salar L.). Infect Immun. 76(4): 1445-1455.

Bruto M, James A, Petton B, Labreuche Y, Chenivesse S, Alunno-Bruscia M, Polz MF and Le Roux F 2017. Vibrio crassostreae, a benign oyster colonizer turned into a pathogen after plasmid acquisition. The ISME Journal. 11(4): 1043-1052.

Burr SE, Diep DB and Buckley JT 2001. Type II secretion by *Aeromonas salmonicida*: evidence for two periplasmic pools of proaerolysin. J Bacteriol. 183(20): 5956-5963.

Canchaya C, Fournous G and Brüssow H 2004. The impact of prophages on bacterial chromosomes. Mol Microbiol. 53(1): 9-18.

Canchaya C, Proux C, Fournous G, Bruttin A and Brüssow H 2003. Prophage genomics. Microbiol Mol Biol Rev. 67(2): 238-276, table of contents.

Carnahan AM, Behram S and Joseph SW 1991. Aerokey II: A Flexible Key for Identifying Clinical *Aeromonas* Species. Journal of Clinical Microbiology,. 29 2843-2849.

Carnahan AM and Joseph SW 2005. Aeromonas. In: Brenner, Krieg, Staley, Garrity (Eds.), Bergey's Manual of Systematic Bacteriology. Vol 2. Second edition ed. In: Williams and Wilkins, New York.

Chandra S and Banerjee TK 2004. Histopathological analysis of the respiratory organs of *Channa striata* subjected to air exposure. Veterinarski Arhiv. 74(1): 37-52.

Chen F, Sun J, Han Z, Yang X, Xian J-a, Lv A, Hu X and Shi H 2019. Isolation, Identification and Characteristics of *Aeromonas veronii* From Diseased Crucian Carp (Carassius auratus gibelio). Front. Microbiol. 10:2742.

Chen F, Wang K, Stewart J and Belas R 2006. Induction of multiple prophages from a marine bacterium: a genomic approach. Appl Environ Microbiol. 72(7): 4995-5001.

Chen N, Jiang J, Gao X, Li X, Zhang Y, Liu X, Yang H, Bing X and Zhang X 2018. Histopathological analysis and the immune related gene expression profiles of mandarin fish (*Siniperca chuatsi*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol. 83: 410-415.

Chen YF, Liang RS, Zhuo XL, Wu XT and Zou JX 2012. Isolation and characterization of *Aeromonas schubertii* from diseased snakehead, *Channa maculata* (Lacepede). J Fish Dis. 35: 421-430.

Cheok YY, Puah SM, Chua KH and Tan J 2020. Isolation and molecular identification of *Aeromonas* species from the tank water of ornamental fishes. Acta Vet Hung. 68(2): 130-139.

CLSI 2014. Performance standards for antimicrobial testing of Bacteria isolated from aquatic animal. Vol 34. In, CLSI document VET03/Vet04-S2.

Colomer-Lluch M, Imamovic L, Jofre J and Muniesa M 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. Antimicrob Agents Chemother. 55(10): 4908-4911.

Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Néron B, Rocha EPC, Vergnaud G, Gautheret D and Pourcel C 2018. CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. Nucleic Acids Res. 46(W1): W246-w251.

Dacanay A, Boyd JM, Fast MD, Knickle LC and Reith ME 2010. *Aeromonas salmonicida* Type I pilus system contributes to host colonization but not invasion. Dis Aquat Organ. 88(3): 199-206.

Degiacomi MT, Iacovache I, Pernot L, Chami M, Kudryashev M, Stahlberg H, van der Goot FG and Dal Peraro M 2013. Molecular assembly of the aerolysin pore reveals a swirling membrane-insertion mechanism. Nat Chem Biol. 9(10): 623-629.

Deurenberg RH, Bathoorn E, Chlebowicz MA, Couto N, Ferdous M, Garcia-Cobos S, Kooistra-Smid AMD, Raangs EC, Rosema S, Veloo ACM, Zhou K, Friedrich AW and Rossen JWA 2017. Reprint of "Application of next generation sequencing in clinical microbiology and infection prevention". J Biotechnol. 250: 2-10.

DOF 2020. Anual export values of Aquatic animal product in Thailand Fisheries Statistic. Vol.2021.

Dong HT, Techatanakitarnan C, Jindakittikul P, Thaiprayoon A, Taengphu S, Charoensapsri W, Khunrae P, Rattanarojpong T and Senapin S 2017. Aeromonas jandaei and Aeromonas veronii caused disease and mortality in Nile tilapia, Oreochromis niloticus (L.). J Fish Dis. 40(10): 1395-1403.

Erdem B, Kariptaş E, Çil E and Işik K 2011. Biochemical identifcation and numerical taxonomy of *Aeromonas* spp. isolated from food samples in Turkey. Turkish Journal of Biology. 35: 463-472.

FAO 2016. The State of World Fisheries and Aquaculture 2016. 978-92-5-109185-2.

FAO 2020. The State of World Fisheries and Aquaculture 2020 - Meeting the sustainable development goals. Rome

Fernández-Bravo A 2019. Epidemiology and pathogenic characterization of species of the genus *Aeromonas*. In, Universidad Rovira i Virgili : Tarragona, Spain.

Fernández-Bravo A and Figueras JM 2020. An Update on the Genus *Aeromonas*: Taxonomy, Epidemiology, and Pathogenicity. *Microorganisms*. 8: 1-39.

Figueras MJ, Suarez-Franquet A, Chacon MR, Soler L, Navarro M, Alejandre C, Grasa B, Martínez-Murcia AJ and Guarro J 2005. First record of the rare species *Aeromonas culicicola* from a drinking water supply. *Appl. Environ. Microbiol*. 71: 538-541.

García-Bayona L, Coyne MJ and Comstock LE 2021. Mobile Type VI secretion system loci of the gut Bacteroidales display extensive intra-ecosystem transfer, multi-species spread and geographical clustering. PLoS Genet. 17(4): e1009541.

Gilardi GL 1967. Morphological and biochemical characteristics of Aeromonas punctata (hydrophila, liquefaciens) isolated from human sources. Appl Microbiol. 15(2): 417-421.

Gonçalves Pessoa RB, de Oliveira WF, Marques DSC, Dos Santos Correia MT, de Carvalho E and Coelho L 2019. The genus *Aeromonas*: A general approach. Microb Pathog. 130: 81-94.

González-Serrano CJ, Santos JA, García-López ML and Otero A 2002. Virulence markers in *Aeromonas hydrophila* and *Aeromonas veronii* biovar *sobria* isolates from freshwater fish and from a diarrhoea case. 93(3): 414-419.

Green ER and Mecsas J 2016. Bacterial Secretion Systems: An Overview. Microbiol Spectr. 4(1).

Gurevich A, Saveliev V, Vyahhi N and Tesler G 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics and Biology Insights. 29(8): 1072-1075.

Hadfield J, Croucher NJ, Goater RJ, Abudahab K, Aanensen DM and Harris SR 2018. Phandango: an interactive viewer for bacterial population genomics. Bioinformatics. 34(2): 292-293.

Hal AM and El-Barbary MI 2020. Gene expression and histopathological changes of Nile tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila* and *Pseudomonas fluorescens*. Aquaculture. 526: 735392.

Hasan CT, Salih K, Jochen B, Andy DP, Matt JG, Hossam A, Attila K and Mark LL 2019. Comparative genomics of Aeromonas veronii: Identification of a pathotype impacting aquaculture globally. PLoS One. 14(8).

Hassan MA, Noureldin EA, Mahmoud MA and Fita NA 2017. Molecular identification and epizootiology of *Aeromonas veronii* infection among farmed *Oreochromis niloticus* in Eastern Province, KSA. The Egyptian Journal of Aquatic Research. 43(2): 161-167.

Ho BT, Dong TG and Mekalanos JJ 2014. A view to a kill: the bacterial type VI secretion system. Cell Host Microbe. 15(1): 9-21.

Hoai TD, Trang TT, Tuyen NV, Giang NTh and Kim VV 2019. *Aeromonas veronii* caused disease and mortality in channel catfish in Vietnam. Aquaculture. 513: 734425.

Hoel S, Vadstein O and Jakobsen AN 2017. Species Distribution and Prevalence of Putative Virulence Factors in Mesophilic *Aeromonas* spp. Isolated from Fresh Retail Sushi. Front Microbiol. 8: 931.

Holmes B and Farmer III JJ 2020. Correction of the type strain of *Aeromonas punctata* (Zimmermann 1890) Snieszko 1957 and of *A. caviae*subsp. *punctata* from ATCC 15468T to NCMB 74T (=NCIMB 74T= ATCC 23309T). 70(3): 2155-2157.

Hulton CSJ, Higgins CF and Sharp PM 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. Molecular Microbiology. 5: 825-834.

Jagoda SS, Wijewardana TG, Arulkanthan A, Igarashi Y, Tan E, Kinoshita S, Watabe S and Asakawa S 2014. Characterization and antimicrobial susceptibility of motile aeromonads isolated from freshwater ornamental fish showing signs of septicaemia. Dis Aquat Organ. 109(2): 127-137.

Janda JM and Abbott SL 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev. 23(1): 35-73.

Kaas RS, Leekitcharoenphon P, Aarestrup FM and Lund O 2014. Solving the Problem of Comparing Whole Bacterial Genomes across Different Sequencing Platforms. PLoS One. 9(8): e104984.

Kang Y, Pan X, Xu Y, Siddiqui SA, Wang C, Shan X and Qian A 2016. Complete genome sequence of the fish pathogen *Aeromonas veronii* TH0426 with potential application in biosynthesis of pullulanase and chitinase. J Biotechnol. 227: 81-82.

Khor WC, Suat Moi Puah SM, Jin Ai Mary Anne Tan., Puthucheary SD and Chua KH 2015. Phenotypic and Genetic Diversity of Aeromonas Species Isolated from Freshwater lakes in Malaysia. PLoS One. 10 (12).

Kozinska A 2007. Dominant pathogenic species of mesophilic aeromonads isolated from diseased and healthy fish cultured in Poland. J Fish Dis. 30(5): 293-301.

Kumar S, Stecher G, Li M, Knyaz C and Tamura K 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol. 35(6): 1547-1549.

Lamy B, Laurent F, Verdier I, Decousser J-W, Lecaillon E, Marchandin H, Roger F, Tigaud S, De Montclos H and Kodjo A 2010. Accuracy of 6 commercial systems for identifying clinical *Aeromonas* isolates. Diagn. Microbiol. Infect. Dis. 67: 9–14.

Latif-Eugenin FL 2015. *Aeromonas*, un microorganismo ambiental de importancia en salud humana y animal. Universidad Rovira i Virgili, Tarragona, Spain.

Li Y and Cai S-H 2011. Identification and Pathogenicity of Aeromonas sobria on Tail-rot Disease in Juvenile Tilapia Oreochromis niloticus. Curr Microbiol. 62(2): 623-627.

Liu D 2015. Chapter 61 - Aeromonas. In: Molecular Medical Microbiology (Second Edition). Yi-Wei Tang, Max Sussman, Dongyou Liu, Ian Poxton, and Joseph Schwartzman (eds). Boston: Academic Press. 1099-1110.

Liu JY and Li AH 2012. First case of *Aeromonas schubertii* infection in the freshwater cultured snakehead fish, *Ophiocephalus argus* (Cantor), in China. J Fish Dis. 35(5): 335-342.

Loman NJ, Constantinidou C, Chan JZ, Halachev M, Sergeant M, Penn CW, Robinson ER and Pallen MJ 2012. High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity. Nat Rev Microbiol. 10(9): 599-606.

Lowry R, Balboa S, Parker JL and Shaw JG 2014. *Aeromonas* Flagella and Colonisation Mechanisms. Adv Microb Physiol. 65: 203-246.

Maltz M and Graf J 2011. The type II secretion system is essential for erythrocyte lysis and gut colonization by the leech digestive tract symbiont Aeromonas veronii. Appl Environ Microbiol. 77(2): 597-603.

Martínez-Murcia A, Beaz-Hidalgo R, Navarro A, Carvalho MJ, Aravena-Román M, Correia A, Figueras MJ and Saavedra MJ 2016. *Aeromonas lusitana* sp. nov., isolated from untreated water and vegetables. *Curr. Microbiol*. 72: 795-803.

Martínez-Murcia A and Lamy B 2015. Molecular diagnostics by genetic methods in *Aeromonas*. In: Academic Press: Norfolk, UK.

Martinez-Murcia AJ, Soler L, Saavedra MJ, Chacon MR, Guarro J, Stackebrandt E and Figueras MJ 2005. Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. Int Microbiol. 8: 259–269.

Martino ME, Fasolato L, Montemurro F, Novelli E and Cardazzo B 2014. *Aeromonas* spp: ubiquitous or specialized bugs? Environ Microbiol. 16(4): 1005-1018.

Martino ME, Fasolato L, Montemurro F, Rosteghin M, Manfrin A, Patarnello T, Novelli E and Cardazzo B 2011. Determination of microbial diversity of Aeromonas strains on the basis of multilocus sequence typing, phenotype, and presence of putative virulence genes. Appl. Environ. Microbiol. 77: 4986–5000.

Mazumder A, Choudhury H, Dey A and Sarma DJSR 2021. Isolation and characterization of two virulent Aeromonads associated with haemorrhagic septicaemia and tail-rot disease in farmed climbing perch Anabas testudineus. 11.

McIntosh D and Austin B 1991. Atypical characteristics of the salmonid pathogen *Aeromonas salmonicida*. 137(6): 1341-1343.

Miñana-Galbis D, Farfán M, Gaspar Lorén J and Carmen Fusté M 2010. Proposal to assign *Aeromonas diversa* sp. nov. as a novel species designation for *Aeromonas* group 501. Syst Appl Microbiol. 33(1): 15-19.

Mohammed HH and Peatman E 2018. Winter kill in intensively stocked channel catfish (Ictalurus punctatus): Coinfection with *Aeromonas veronii*, *Streptococcus parauberis* and *Shewanella putrefaciens*. 41(9): 1339-1347.

Mollah MFA, Mamun MSA, Sarowar MN and Roy A 2009. Effects of stocking density on the growth and breeding performance of broodfish and larval growth and survival of shol, *Channa striatus* (Bloch). Journal of the Bangladesh Agricultural. 7(2): 427-432.

Mursalim MF, Budiyansah H, Raharjo HM, Chokomangmeepisarn P, Sakulworakan R, Yindee J, Chanchaithong P and Rodkhum C 2020. Characterization and Antimicrobial Susceptibility of *Aeromonas* spp. Isolated from diseased Asian seabass (*Lates calcarifer). Thai J Vet Med*. 50: 10-13.

Nadalin F, Vezzi F and Policriti A 2012. GapFiller: A de novo assembly approach to fill the gap within paired reads. BMC Bioinformatics. 13.

Navarro A and Martinez-Murcia A 2018. Phylogenetic analyses of the genus *Aeromonas* based on housekeeping gene sequencing and its influence on systematics. J Appl Microbiol. 125: 622-631.

Nhung PH, Hata H, Ohkusu K, Noda M, Shah MM, Goto K and Ezaki T 2007. Use of the novel phylogenetic marker dnaJ and DNA–DNA hybridization to clarify interrelationships within the genus *Aeromonas*. Int J Syst Evol Microbiol. 57: 1232–1237.

Nordmann P and Poirel L 2002. Emerging carbapenemases in Gram-negative aerobes. Clin Microbiol Infect. 8: 323-331.

Oleiwi SR, Musleh RM, Sabah MA and Abdullah FT 2014. Detection of hemolysins (hlyA and aerA) genes in *Aeromonas hydrophila* isolated from raw and drinking water in Baghdad city. Int. J. of Adv. Res. 2: 468-474.

Origgi FC, Benedicenti O, Segner H, Sattler U, Wahli T and Frey J 2017. *Aeromonas salmonicida* type III secretion system-effectors-mediated immune suppression in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 60: 334-345.

Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F and Stevens R 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 42(Database issue): D206-214.

Pablos M, Huys G, Cnockaert M, Rodriguez-Calleja JM, Otero A, Santos JA and Garcia-Lopez ML 2011. Identification and epidemiological relationships of *Aeromonas* isolates from patients with diarrhea, drinking water and foods. Int J Food Microbiol. 147(3): 203-210.

Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, Fookes M, Falush D, Keane JA and Parkhill J 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics. 31(22): 3691-3693.

Park TS, Oh SH, Lee EY, Lee TK, Park KH, Figueras MJ and Chang CL 2003. Misidentification of *Aeromonas veronii* biovar *sobria* as *Vibrio alginolyticus* by the Vitek system. Lett Appl Microbiol. 37(4): 349-353.

Patel R 2015. MALDI-TOF MS for the diagnosis of infectious diseases. Clin Chem. 61(1): 100-111.

Perez-Sancho M, Cerda I, Fernandez-Bravo A, Dominguez L, Figueras MJ, Fernandez-Garayzabal JF and Vela AI 2018. Limited performance of MALDI-TOF MS for

identification of fish *Aeromonas* isolates at species level. J Fish Dis. 41(10): 1485-1493.

Pérez-Sancho M, Cerdá I, Fernández-Bravo A, Domínguez L, Figueras MJ, Fernández-Garayzábal JF and Vela AI 2018. Limited performance of MALDI-TOF MS for identification of fish *Aeromonas* isolates at species level. *J. Fish Dis*. 41: 1485-1493.

Phillips I 1993. Cowan and Steel's Manual for the Identification of Medical Bacteria. J Clin Pathol. 46(10): 975-975.

Piamsomboon P, Jaresitthikunchai J, Hung TQ, Roytrakul S and Wongtavatchai J 2020. Identification of bacterial pathogens in cultured fish with a custom peptide database constructed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). BMC Vet Res. 16(1): 52.

Qin J, Fast AW, DeAnda D and Weidenbach RP 1997. Growth and survival of larval snakehead (*Channa striatus*) fed different diets. Aquaculture. 148(2-3): 105-113.

Rahman M, Colque-Navarro P, Kühn I, Huys G, Swings J and Möllby R 2002. Identification and characterization of pathogenic *Aeromonas veronii* biovar sobria associated with epizootic ulcerative syndrome in fish in Bangladesh. Appl Environ Microbiol. 68(2): 650-655.

Raj NS, Swaminathan TR, Dharmaratnam A, Raja SA, Ramraj D and Lal KK 2019. *Aeromonas veronii* caused bilateral exophthalmia and mass mortality in cultured Nile tilapia, *Oreochromis niloticus* (L.) in India. Aquaculture. 512: 734278.

Reed LJ and Muench H 1938. A simple method of estimating fifty percent endpoints Am J Epidemiol. 27(3): 493-497.

Sakulworakan R, Chokmangmeepisarn P, Dinh-Hung N, Sivaramasamy E, Hirono I, Chuanchuen R, Kayansamruaj P and Rodkhum C 2021. Insight Into Whole Genome of *Aeromonas veronii* Isolated From Freshwater Fish by Resistome Analysis Reveal Extensively Antibiotic Resistant Traits. Front Microbiol. 12(2630).

Sánchez-Céspedes J, Blasco MD, Marti S, Alba V, Alcalde E, Esteve C and Vila J 2008. Plasmid-mediated QnrS2 determinant from a clinical *Aeromonas veronii* isolate. Antimicrob Agents Chemother. 52(8): 2990-2991.

Sechi LA, Deriu A, Falchi MP, Fadda G and Zanetti S 2002. Distribution of virulence genes in *Aeromonas* spp. isolated from Sardinian waters and from patients with diarrhoea. J Appl Microbiol. 92(2): 221-227.

Seemann T 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 30(14): 2068-2069.

Sha J, Rosenzweig JA, Kozlova EV, Wang S, Erova TE, Kirtley ML, van Lier CJ and Chopra AK 2013. Evaluation of the roles played by Hcp and VgrG type 6 secretion system effectors in Aeromonas hydrophila SSU pathogenesis. Microbiology (Reading). 159(Pt 6): 1120-1135.

Shao-wu L, Di W, Hong-bai L and Tong-yan L 2013. Molecular Typing of *Aeromonas hydrophila* Isolated from Common Carp in Northeast China. Journal of Northeast Agricultural University 20: 30-36.

Shin HB, Yoon J, Lee Y, Kim MS and Lee K 2015. Comparison of MALDI-TOF MS, housekeeping gene sequencing, and 16S rRNA gene sequencing for identification of *Aeromonas* clinical isolates. Yonsei Med J. 56(2): 550-555.

Silver AC, Kikuchi Y, Fadl AA, Sha J, Chopra AK and Graf J 2007. Interaction between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations. 104(22): 9481-9486.

Soler L, Figueras MJ, Chacón MR, Guarro J and Martinez-Murcia AJ 2003. Comparison of three molecular methods for typing *Aeromonas popoffii* isolates. *Antonie Leeuwenhoek* 83: 341–349.

Soler L, Yáñez MA, Chacon MR, Aguilera-Arreola MG, Catalan V, Figueras MJ and Martínez-Murcia AJ 2004. Phylogenetic analysis of the genus *Aeromonas* based on two housekeeping genes. Int. J. Syst. Evol. Microbiol. 54: 1511–1519.

Son R, Micky V, Kasing A, Raha AR, Patrick GB and Gulam R 2002. Molecular characterization of *Vibrio cholerae* O1 outbreak strains in Miri, Sarawak (Malaysia). Acta Trop. 83: 169-176.

Soto-Rodriguez SA, Cabanillas-Ramos J, Alcaraz U, Gomez-Gil B and Romalde JL 2013. Identification and virulence of *Aeromonas dhakensis*, *Pseudomonas mosselii* and *Microbacterium paraoxydans* isolated from Nile tilapia, *Oreochromis niloticus*, cultivated in Mexico. J Appl Microbiol. 115(3): 654-662.

Stackebrandt E, Garrity GM, Trüper HG, Whitman WB, Grimont PAD, Nesme X, Frederiksen W, Vauterin L, Kampfer P and Rosselló-Mora R 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. 52: 1043-1047.

Suarez G, Sierra JC, Erova TE, Sha J, Horneman AJ and Chopra AK 2010. A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* that induces host cell toxicity by ADP ribosylation of actin. J Bacteriol. 192(1): 155-168.

Sumpradit N, Wongkongkathep S, Malathum K, Janejai N, Paveenkittiporn W, Yingyong T, Chuxnum T, Vijitleela A, Boonyarit P, Akaleephan C, Manosuthi W, Thienthong V, Srinha J, Wongsrichai S, Laoprasert T, Athipunyakom P, Kriengchaiyaprug N, Intarukdach K, Numsawad S, Somjetanakul N, Punnin S and Kiatying-Angsulee N 2021. Thailand's national strategic plan on antimicrobial resistance: progress and challenges. Bull World Health Organ. 99(9): 661-673.

Szczuka E and Kaznowsky A 2004. Typing of Clinical and Environmental *Aeromonas* sp. Strains by Random Amplified Polymorphic DNA PCR, Repetitive Extragenic Palindromic PCR, and Enterobacterial Repetitive Intergenic Consensus Sequence PCR. J Clin Microbiol. 42: 220-228.

Tampakaki AP, Skandalis N, Gazi AD, Bastaki MN, Sarris PF, Charova SN, Kokkinidis M and Panopoulos NJ 2010. Playing the “Harp”: Evolution of Our Understanding of hrp/hrc Genes. 48(1): 347-370.

Tekedar HC, Arick MA, 2nd, Hsu CY, Thrash A, Blom J, Lawrence ML and Abdelhamed H 2020. Identification of Antimicrobial Resistance Determinants in *Aeromonas veronii* Strain MS-17-88 Recovered From Channel Catfish (*Ictalurus punctatus*). Front Cell Infect Microbiol. 10: 348.

Tekedar HC, Kumru S, Blom J, Perkins AD, Griffin MJ, Abdelhamed H, Karsi A and Lawrence ML 2019. Comparative genomics of *Aeromonas veronii*: Identification of a pathotype impacting aquaculture globally. PLoS One. 14(8): e0221018.

Tomas JM 2012. The Main *Aeromonas* Pathogenic Factors. ISRN Microbiology. 2012: 1-23.

Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre CB and Miller DL 2020. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. In: American Veterinary Medical Association., Schaumburg, IL.

Vavrova A, Balazova T, Sedlacek I, Tvrzova L and Sedo O 2015. Evaluation of the MALDI-TOF MS profiling for identification of newly described *Aeromonas* spp. Folia Microbiol. 60: 375-383.

Versalovic J, Koueth T and and Lupski JR 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acid Research. 19: 6823-6831.

Wassif IM 2018. Biochemical and Molecular Characterization of *Aeromonas* Species Isolated from Fish. Alexandria Journal of Veterinary Sciences. 59(1).

Yamamoto S and Harayama S 1996. Phylogenetic analysis of acinetobacter strains based on the nucleotida sequence of *gyrB* genes and on the amino acid sequences of their products. Int J Syst Bacteriol. 46: 506-511.

Yáñez MA, Catalán V, Apráiz D, Figueras MJ and Martínez-Murcia AJ 2003. Phylogenetic analysis of members of the genus Aeromonas based on *gyrB* gene sequences. Int J Syst Evol Microbiol. 53(Pt 3): 875-883.

Yang B, Chen C, Sun Y, Cao L, Zhang D, Sun W, Zhang L, Wang G, Shan X, Kang Y and Qian A 2021. Comparative genomic analysis of different virulence strains reveals reasons for the increased virulence of *Aeromonas veronii*. J Fish Dis. 44(1): 11-24.

Ye Y, Jiang Y, Fan T, Jiang Q, Cheng Y, Lu J and Lin L 2012. Resistance Characterization, Virulence Factors, and ERIC-PCR Fingerprinting of *Aeromonas veronii* Strains Isolated from Diseased Trionyx sinensis. Foodborne Pathog Dis. 9: 1053.

Yousr AH, Napis S, Rusul GRA, Alitheen NB and Son R 2010. Molecular typing of *Aeromonas* species using RAPD and ERIC-PCR fingerprinting. *International Food Research Journal* 17: 691-698.

Zhang D, Xu D-H and Shoemaker C 2016. Experimental induction of motile *Aeromonas* septicemia in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*. Aquaculture Reports. 3: 18-23.

Zhang DX, Kang YH, Song MF, Shu HP, Guo SN, Jia JP, Tao LT, Zhao ZL, Zhang L, Wang CF, Wang GQ, Qian AD and Shan XF 2019. Identity and virulence properties of *Aeromonas* isolates from healthy Northern snakehead (*Channa argus*) in China. Lett Appl Microbiol. 69(2): 100-109.

Zheng W, Cao H and Yang X 2012. *Aeromonas veronii* infection in the cultured snakehead fish, *Ophiocephalus argus* (Cantor). Afr J Microbiol Res. 6 (44): 7218-7223.

Zhong C, Han M, Yang P, Chen C, Yu H, Wang L and Ning K 2019. Comprehensive Analysis Reveals the Evolution and Pathogenicity of *Aeromonas*, Viewed from Both Single Isolated Species and Microbial Communities. *mSystems* 4 (5): e00252-00219.

Zhou K, Lokate M, Deurenberg RH, Tepper M, Arends JP, Raangs EG, Lo-Ten-Foe J, Grundmann H, Rossen JW and Friedrich AW 2016. Use of whole-genome sequencing to trace, control and characterize the regional expansion of extended-spectrum β-lactamase producing ST15 Klebsiella pneumoniae. Sci Rep. 6: 20840.

Zhou Y, Liang Y, Lynch KH, Dennis JJ and Wishart DS 2011. PHAST: a fast phage search tool. Nucleic Acids Res. 39(Web Server issue): W347-352.

REFERENCES

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APPENDIX

1. Tryptic soy agar with 5% shepp blood (TSA blood agar)

TSA	8 g
Distiled water	200 ml
Sheep whole blood	10 ml

Add components to distilled water and bring volume to 200 ml, as described by manufacturer instruction. Mix thoroughly. Gently heat and bring to boiling. Distribute into the 200 mL bottles. Autoclave for 15 min at 15 psi pressure, 121°C. Cool to 55-60^{0}C in the water bath. Before pouring medium into the petri dishes, one bottle of TSA is carefully mixed with 10-15 mL of sheep whole blood

2. Trypticase soy broth (TSB)

TSB powder	30 g
Distiled water	1000 ml

Source: This medium is available as a pre-mixed powder form BD BactoTM.
Medium preparation: Add components to distilled water and bring volume to 1.000 ml, as manufacturer instruction describes. Mix thoroughly. Gently heat and bring to boiling. Distribute into the tubes. Autoclave for 15 min at 15 psi pressure, 121°C

3. Rimler-Shotts (RS) Medium Base

RS media	45.43 g
Distiled water	990 ml

Suspend 45.43 grams in 990 ml distilled water. Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 45-50°C and aseptically

add rehydrated content of 1 vial of Novobiocin Supplement. Mix well and pour into sterile Petri plates

4. Glycerol preservation

Sterile glycerol 50%	400 ml
Bacterial culture in TSB	600 ml

5. Muller Hinton Agar (MHA)

Muller Hilton Broth	21 g
Distiled water	1000 ml
Agar	15 g

6. TBE electrophoresis buffer (10X)

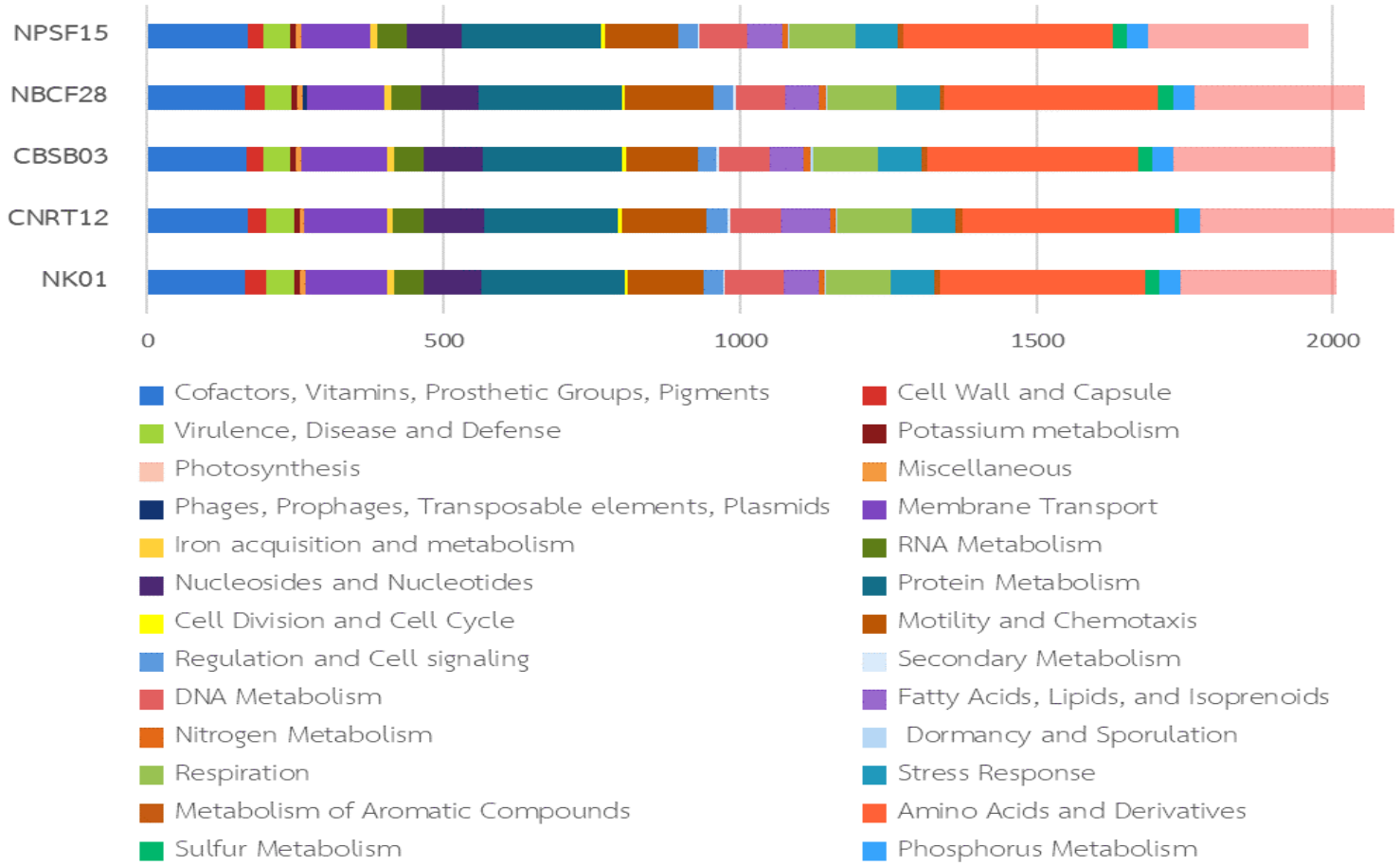
Tris base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 ml
Rnase-free H20	1000 ml

Prepare with RNase-free H2O. Dilute 100 mL to 1 L to make gel running buffer and store for up to 6 months at room temperature.

Supplemented Table 1 Identification result using MALDI-TOF MS for a total of 86 *Aeromonas* isolates

Number of isolates	MALDI-TOF MS			
	1st Identification	Log(score)[a]	2nd Identification	Log(score)[a]
20	*A. veronii*	2.16-2.34	*A. ichthiosmia*	2.06-2.30
14	*A. ichthiosmia*	2.17-2.32	*A. veronii*	2.05-2.25
26	*A. veronii*	2.12-2.33	*A. veronii*	2.06-2.26
1	*A. hydrophila*	2.06	*A. jandaei*	2.01
1	*A. jandaei*	2.09-2.21	*A. veronii*	1.99-2.17
1	*A. hydrophila*	2.30	*A. hydrophila*	2.18
1	*A. hydrophila*	2.06	*A. jandaei*	2.01
3	*A. jandaei*	2.09-2.21	*A. veronii*	1.99-2.17
2	*A. jandaei*	2.14-2.18	*A. hydrophila*	2.11-2.15
1	*A. veronii*	2.29	*A. veronii*	2.17
1	*A. veronii*	2.20	*A. ichtiosmia*	2.18
1	*A. ichtiosmia*	2.21	*A. veronii*	2.20
1	*A. jandaei*	2.16	*A. jandaei*	2.05
1	*A. hydrophila*	2.23	*A. caviae*	2.20
8	*A. schubertii*	2.25-2.66	*A. schubertii*	2.10-2.64
1	*A. hydrophila*	2.23	*A. caviae*	2.20
1	*A. veronii*	2.33	*A. veronii*	2.27
1	*A. schubertii*	2.56	*A. schubertii*	2.54
1	*A. schubertii*	2.45	*A. schubertii*	2.56

a log (score) values according to the results ≤ 1.79 represented no substantial similarity between the spectra and any of the database entries; values 1.8–1.9 represented possibility identification of the genus; log (score) 2.0–2.29 represented confident genus identification and possible identification of the species level, and values 2.3–3.0 represented highly possible identification of the species



Supplemental Figure 1 SEED subsystem category for *A. veronii* genomes isolated from diseased freshwater fishes in Thailand

Supplemental Table 2 MLST allele and sequence type (ST) identified from the genomes of *A. veronii* (n= 12) and *A. schubertii* strain 43700

Strains	ST	*gyrB*	*grol*	*gltA*	*metG*	*ppsA*	*recA*
CNRT12	ND	221		110	81		476
NK01	ND	221					
CBSB03	ND	168		44			
NBCF28	ND		217	143	679		
NPSF15	ND	599		110	612	810	49
TH0426	ND	29	28	17		26	28
ML09-123	ND	29	28	17		26	28
X12	ND	466		56			
X11	ND	479	221	358	227		53
Ae52	ND	232	223	229	46		86
CB51	ND			127			378
MS1837	254	212	216	151	213	84	220
A. schubertii strain 43700	ND	16	15	16	15		

Supplemental Figure 2 Graphical representation of the prophage sequences presented in *A. veronii* genomes strain NK01, CNRT12, CBSB03, NPSF15, and NBCF28 as identified by PHASTER tool