GENOMIC CHARACTERIZATION OF ISOLATED *Edwardsiella ictaluri* FROM CAGE-CULTURED *Pangasianodon hypophthalmus* IN PAHANG RIVER

BY

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INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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ABSTRACT

Edwardsiella ictaluri is the etiological agent of Bacillary Necrosis of Pangasius (BNP) in *Pangasianodon hypophthalmus*, causing substantial economic losses in freshwater aquaculture. Infected fish commonly exhibit clinical signs such as erratic swimming behaviour, patchy liver, and congested kidney. Despite its significance, the genomic characteristics, population structure, and epidemiological context of *E. ictaluri* in Malaysia, particularly in major production areas such as Temerloh and Pekan, remain limited. This knowledge gap hinders effective disease surveillance and control strategies in local aquaculture systems. Therefore, this study aimed to investigate the genomic characteristics, antimicrobial resistance determinants, and population-level genetic variation of *E. ictaluri* isolates from Temerloh and Pekan using whole-genome sequencing (WGS) and polymorphism-based approaches. Eleven *E. ictaluri* isolates were recovered from the tissues of clinically diseased *P. hypophthalmus* cultured in riverine cage systems in these areas. As all isolates originated from the same water body, one representative isolate, PLS31B, obtained from the brain of an infected fish, was selected for whole-genome sequencing (WGS) using Oxford Nanopore long-read technology. The assembled genome was 3.8 Mbp in size, with a high-contiguity N50 of 3,761,778 bp and a GC content of 57.39%. Antimicrobial resistance (AMR) analysis revealed resistance to fluoroquinolones, macrolides, and penams, highlighting potential challenges for disease management in aquaculture. Functional annotation identified 3,620 protein-coding genes and 126 RNA genes. Comparative genomic analysis indicated that PLS31B is closely related to *E. ictaluri* strains from China and Vietnam, suggesting potential regional connectivity or transboundary dissemination associated with the importation of fish seed. A large conserved core genome comprising of 3,246 genes was observed alongside of 51 strain-specific genes linked to those encoding outer membrane protein C precursor (OMP), ATP-binding protein, acid shock protein, and hemolysin secretion protein. Meanwhile, genetic polymorphism among all isolates was further assessed using Random Amplified Polymorphic DNA (RAPD) profiling and Sanger sequencing. Seven 10-mer primers were used for RAPD analysis, while the 23S rRNA gene region was sequenced for comparative analysis. Polymorphism analysis revealed minimal genetic differentiation among the isolates, irrespective of sampling locations or host age groups. This low genetic diversity is likely influenced by a shared aquatic environment and interconnected farming systems. In conclusion, these findings highlight the epidemiological relevance of regional dissemination pathways and guide the development of targeted control strategies, including early detection tools, preventive measures, and effective treatment approaches for BNP in *P. hypophthalmus* aquaculture.

ملخص البحث

تعد *Edwardsiella ictaluri* العامل المسبب لمرض Bacillary Necrosis of Pangasius (BNP) في *Pangasianodon hypophthalmus*، مما يؤدي إلى خسائر اقتصادية كبيرة في الاستزراع المائي للمياه العذبة. وتظهر الأسماك المصابة عادة علامات سريرية مثل السباحة غير المنتظمة، وتبقع الكبد، واحتقان الكلية. وعلى الرغم من هذه الأهمية، فإن الخصائص الجينومية، والبنية السكانية، والسياق الوبائي لـ *E. ictaluri* في ماليزيا، ولا سيما في مناطق الإنتاج الرئيسة مثل تميرلوه وبيكان، لا تزال محدودة. وتعيق هذه الفجوة المعرفية وضع استراتيجيات فعالة لرصد المرض ومكافحته في أنظمة الاستزراع المائي المحلية. لذلك، قد هدفت هذه الدراسة إلى استقصاء الخصائص الجينومية، ومحددات مقاومة مضادات الميكروبات، والتباين الوراثي على مستوى الجماعة لعزلات *E. ictaluri* من تميرلوه وبيكان، باستخدام تسلسل الجينوم الكامل (WGS) والمقاربات القائمة على تعدد الأشكال. وقد استخلصت إحدى عشرة عزلة من *E. ictaluri* من أنسجة *P. hypophthalmus* المصابة سريريا والمستزرعة في أنظمة الأقفاص النهرية في هذه المناطق. ونظرا إلى أن جميع العزلات نشأت من المسطح المائي نفسه، فقد اختيرت عزلة ممثلة واحدة، هي PLS31B مأخوذة من دماغ سمكة مصابة؛ لإجراء تسلسل الجينوم الكامل (WGS) باستخدام تقنية أكسفورد نانوبور للقراءات الطويلة. وقد بلغ حجم الجينوم المجمع Mbp 3.8، مع قيمة N50 عالية الاستمرارية بلغت bp 3,761,778، ومحتوى GC بنسبة 57.39%. وكشف تحليل مقاومة مضادات الميكروبات (AMR) عن وجود مقاومة للفلوروكينولونات، والماكروليدات، والبنامات، مما يسلط الضوء على تحديات محتملة في إدارة المرض في الاستزراع المائي. كما حدد التوصيف الوظيفي 3,620 جينا مرمزا للبروتين و126 جينا من جينات الحمض النووي الريبوزي (RNA). وأشار التحليل الجينومي المقارن إلى أن PLS31B ترتبط ارتباطا وثيقا بسلالات *E. ictaluri* من الصين وفيتنام، مما يشير إلى احتمال وجود ترابط إقليمي أو انتشار عابر للحدود مرتبط باستيراد زريعة الأسماك. ولوحظ وجود جينوم أساسي محفوظ كبير يضم 3,246 جينا، إلى جانب 51 جينا خاصا بالسلالة، ترتبط بجينات تشفر طليعة البروتين C للغشاء الخارجي (OMP)، وبروتين الارتباط بـ ATP، وبروتين الصدمة الحمضية، وبروتين إفراز الهيموليسين. وفي

الوقت نفسه، جرى تقييم تعدد الأشكال الوراثية بين جميع العزلات بمزيد من التفصيل باستخدام التنميط القائم على الحمض النووي متعدد الأشكال المضخم عشوائيا (RAPD) وتسلسل سانغر. وقد استخدمت سبعة بادئات عشارية النوكليوتيدات لتحليل RAPD، في حين جرى تسلسل منطقة جين S rRNA23 للتحليل المقارن. وكشف تحليل تعدد الأشكال عن تمايز وراثي ضئيل بين العزلات، بغض النظر عن مواقع أخذ العينات أو الفئات العمرية للعائل. ويحتمل أن هذا الانخفاض في التنوع الوراثي قد تأثر ببيئة مائية مشتركة وبأنظمة تربية مترابطة. وفي الختام، تبرز هذه النتائج الأهمية الوبائية لمسارات الانتشار الإقليمي، وتسهم في توجيه تطوير استراتيجيات مكافحة موجهة، بما في ذلك أدوات الكشف المبكر، والتدابير الوقائية، والمقاربات العلاجية الفعالة لمرض BNP في استزراع *P. hypophthalmus*.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my parents for laying the foundation of what I turned out to be in life.

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In the name of Allah, the most Gracious, the Most Merciful

I bear witness that there is no God but Allah alone, and I bear witness that Muhammad (peace be upon him) is His slave and Messenger.

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization of the United Nations
ºC	Degree Celcius
µl	Microlitre
mg	Miligram
mm	Mililitre
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
TSA	Trytic Soy Agar
BHI	Brain Heart Infusion
BNP	Bacillary Necrosis of Pangasius
ESC	Enteric Septicemia of Catfish
NGS	Next-generation sequencing
WGS	Whole Genome Sequencing
PacBio	Pacific Biosciences
SMRT	Single Molecule Real-Time
ONT	Oxford Nanopore Technologies
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
PFGE	Pulsed-field gel electrophoresis
COG	Clusters of Orthologous Genes
CDS	Protein-coding sequences
CRP	cAMP receptor protein
AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
CARD	Comprehensive Antibiotic Resistance Database
SNPs	Single nucleotide polymorphisms
GBS	Genotyping by sequencing

WSSV	White spot syndrome virus
CNVs	Copy number variations
indels	Insertions or deletions
MLST	Multilocus sequence typing
BUSCO	Benchmarking Universal Single-Copy Orthologs
NCBI	National Center for Biotechnology Information

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Aquaculture is one of the biggest contributions to a country's food security due to its fast-growing sectors. In 2022, global aquaculture production has surpassed capture fisheries, producing 94.4 million tons (FAO, 2024). *Pangasianodon hypophthalmus* or commonly known as Patin Hitam is a catfish species native to the rivers in the Southeast Asia. It is farmed extensively in the Mekong Delta, Vietnam, marking a significant aquaculture development both in Vietnam and worldwide (Nguyen, 2009). The sector's explosive growth can be attributed primarily to advancements in hatchery technology and the commercialization of techniques for the artificial propagation of the species (Nguyen et al., 2013). *P. hypophthalmus* is in high demand worldwide, especially within the fillet industry due to its firm and white flesh (Guimarães et al., 2016). Its fillets have been exported to more than 138 countries, generating an estimated value of about 1.6 billion USD (Tan et al., 2023). The increasing popularity of this species has also driven extensive cultivation across various regions. Specifically, in Malaysia, this catfish species has become a significant part of the aquaculture sector. It is predominantly cultured in the state of Pahang, where it is farmed in a variety of settings, including cages suspended in rivers and man-made ponds on land (Padilah et al., 2023).

By 2050, the global population is expected to increase from 7.8 billion to nearly 9 billion people, intensifying pressure on food production and distribution (Gul et al., 2024). In 2024, around 700 million people with approximately one in eleven globally continue to face hunger, highlighting persistent challenges to food security (FAO et al., 2025). In

response to these challenges, sustainable aquaculture has become an essential component of global food security strategies (Mansour, 2025). The fisheries and aquaculture sectors contribute significantly by supplying essential nutrition, employment and economic benefits to millions worldwide. Despite these benefits, disease outbreaks remain a significant challenge in aquaculture productivity and are expected to persist in the future (Cain, 2022). Farmed fish are particularly susceptible to bacterial pathogens, largely due to inadequate farm regulations and poor management practices (Mzula et al., 2021). These pathogens can lead to serious infections and disease outbreaks, resulting in high fish mortality and substantial economic losses for the farmers. *Edwardsiella ictaluri*, a Gram-negative bacterium, is the causative agent of Enteric Septicemia of Catfish (ESC) (Esmail et al., 2015) and Bacillary Necrosis of Pangasius (BNP) (Crumlish et al., 2002). This pathogen is capable of causing considerable damage even in well-managed aquaculture system.

Characterization of *E. ictaluri* is essential for effective prevention and management of disease outbreaks in aquaculture. A comprehensive understanding of its genetic polymorphism provides insight into strain diversity, adaptability and potential pathogenicity (Saini et al., 2024). Genetic polymorphisms are inheritable DNA sequence variations that can influence gene function and disease susceptibility. In this study, WGS was employed to generate a comprehensive genomic profile of *E. ictaluri*. This high-throughput approach provides a detailed overview of the organism's genetic makeup, including potential virulence factors (Bagger et al., 2024). As a powerful modern tool, WGS generates extensive genomic data that greatly enhances diagnostic capabilities and produces an unprecedented amount of information that tremendously facilitates the diagnostic process (Brlek et al., 2024). In parallel, RAPD and Sanger sequencing were utilized to evaluate the genetic diversity among the *E. ictaluri* isolates obtained. RAPD is a PCR-based analysis that serves as a rapid and cost-effective technique for identifying polymorphic loci, even in the absence of prior genomic information (Amiteye, 2021). The resulting sequences were further used to construct phylogenetic trees, allowing comparison among the *E. ictaluri* isolates and providing a clearer insight into their genetic relationships.

1.2 PROBLEM STATEMENTS

In Malaysia, from the year 2000 to 2011, the industry of Pangasius spp. experienced an impressive surge in production. Starting with a modest 1,625.21 tonnes at the beginning of the millennium, it saw a tenfold increase by the end of the period. The number reached a substantial figure of 10,891.51 tonnes, thus proving its potential for large-scale production. In 2023, Malaysia's freshwater aquaculture production reached 113,070.5 tons, contributing significantly to the country's output of 506,867.5 tons. The freshwater sector is dominated by the cultivation of Pangasius sp, driven largely by strong domestic demand (Haimid and Abu-Dardak, 2024). However, despite these positive figures, a significant challenge looms over the industry where mortality rate of the catfish was reported up to 30% per culture cycle especially in Pahang River due to a complex mix of bacterial and viral infections of *E. ictaluri*, Aeromonas spp., herpes virus and some ecto- and endoparasites infestations (Mahmud et al., 2019). Among them, *E. ictaluri* outbreak is the most serious issue that requires immediate attention as it may lead to mass mortality in the farm.

However, in Malaysia, *E. ictaluri* has not been widely studied, particularly from a molecular perspective. Publications focusing on its genetics remain limited, and to the best of our knowledge, no whole-genome or genetic polymorphism studies have been reported. This lack of genomic information restricts our understanding of pathogen diversity, virulence variation, and potential transmission patterns within local aquaculture settings. Given these gaps, there is an urgent need for comprehensive genomic investigations of *E. ictaluri* strains circulating in Malaysia. The findings from such studies will not only support better disease management strategies but also contribute valuable knowledge for maintaining the long-term growth, productivity, and sustainability of the Pangasius aquaculture industry.

1.3 RESEARCH OBJECTIVES

1. To purify and sequence the whole genome of *Edwardsiella ictaluri* isolated from cage-cultured *Pangasianodon hypophthalmus* in Pahang River.
2. To study the gene polymorphisms of *Edwardsiella ictaluri* isolated from cage-cultured *Pangasianodon hypophthalmus* in Pahang River.

1.4 RESEARCH QUESTIONS

1. What is the complete genomic sequence of *Edwardsiella ictaluri* isolated from cage-cultured *Pangasianodon hypophthalmus* in the Pahang River?
2. What gene polymorphisms are present among *Edwardsiella ictaluri* isolates obtained from cage-cultured *Pangasianodon hypophthalmus* in Pahang River?

1.5 RESEARCH HYPOTHESIS

1. The *Edwardsiella ictaluri* isolates from the Pahang River possess a distinct whole-genome profile that differs from strains reported in other regions.
2. Low genetic diversity observed among *Edwardsiella ictaluri* isolates obtained from cage-cultured *Pangasianodon hypophthalmus* in the Pahang River.

1.6 SIGNIFICANT OF STUDY

This study aims to be the first comprehensive genomic investigation of *E. ictaluri* isolated from cage-cultured *P. hypophthalmus* in Malaysia, particularly in the Pahang River where high mortality events have been reported. Despite the economic importance of the Pangasius aquaculture industry, genomic research on *E. ictaluri* in Malaysia remains very limited, with no published whole-genome sequences or gene polymorphism studies available to date. By purifying and sequencing the whole genome of local isolates and characterizing their genetic polymorphisms, this research will provide crucial baseline data on strain diversity, potential virulence determinants, and molecular signatures associated with pathogenicity. The findings will not only enhance scientific understanding of *E. ictaluri* at the genomic level but also contribute valuable insights for improving disease diagnostics, monitoring, and management practices in aquaculture.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Pangasianodon hypophthalmus* (Patin Hitam)

P. hypophthalmus, locally known as Patin Hitam in Malaysia, belongs to the family Pangasiidae. This species is extensively farmed in several Asian countries including Vietnam, Malaysia, Indonesia, Laos, Cambodia, and China (Phan et al., 2009, Shamsuzzaman et al., 2020). Due to its high adaptive capacity, *P. hypophthalmus* is currently one of the most important species cultured in both tropical and subtropical environments, within and beyond its natural distribution range (Nugroho et al., 2019). In 2022, the global production of *P. hypophthalmus* reached approximately 3.1 million tons, with Vietnam as the leading producer, followed by India and Bangladesh (FAO, 2024). The species has gained considerable international attention and is widely exported due to its high acceptability, attributed to its mild taste and good flesh quality and white meat (Sarnes et al., 2020). Its affordability has positioned *P. hypophthalmus* as a potential substitute for more expensive white fish species, such as cod (Guimarães et al., 2016).

Beyond Asia, *P. hypophthalmus* has also entered markets in South America, particularly Brazil, where production reached 3,734 tons, and the species is notably utilized in the manufacture of fish-based sausages (Costa et al., 2025). Additionally, this species exhibits high fecundity, rapid growth and ease of culture, reaching market size of approximately 1.2 – 1.3 kg within six months of cultivation (Gurung et al, 2016). These biological and production advantages make *P. hypophthalmus* a significant contributor to the aquaculture industries in many countries (Singh and Lakra, 2012; Nugroho et al., 2019).

2.1.1 Biological Features and Production

P. hypophthalmus can be morphologically identified based on its elongated, laterally flattened, scaleless body and a broad mouth equipped with small, sharp teeth (Griffiths & Trinh, 2020; Chakraborty et al., 2021). The dorsal surface is grey with a slight dark bluish hue, while the lateral sides are silver-blue with a faint yellowish tint near the pelvic fins. The dorsal and caudal fins appear blackish-grey, with a reddish coloration observed at the distal end of the caudal fin (Figure 2.1) (FAO, 2020). Under culture conditions, optimal growth occurs at water temperatures ranging from 28 – 32 °C and pH values between 6.5 - 7.5 (Islam et al., 2019). Mature individuals may attain a maximum length of up to 130 cm and a body weight of approximately 44 kg (FAO, 2020).

Figure 2.1: *Pangasianodon hypophthalmus*. Weight = 265 g; Total length = 28 cm

In captivity, female *P. hypophthalmus* generally reach sexual maturity after a minimum of three years, at a body weight exceeding 3 kg. Meanwhile, males often mature during their second year. A mature female weighing approximately 10 kg is capable of producing over one million eggs. In contrast, wild populations of *P. hypophthalmus* displays distinct migratory behaviour, with adults migrating upstream during the monsoon

season to spawn, followed by downstream drift of larvae and fingerlings to nursery and growth areas (Poulsen et al., 2004). During the dry season, this species gathers and shelters in deeper refuge habitats. Their natural spawning grounds typically consist of river rapids and sandbanks scattered with deep rocky channels and pools (FAO, 2020).

Historically, *P. hypophthalmus* was cultured in small-scale pond systems using wild-caught seeds for several decades (Phan et al., 2009; Ouma & Barasa, 2022). In Vietnam, early culture practices involved rearing the species in every household backyard ponds, primarily for domestic consumption and as a source of supplementary income (De Silva and Phuong, 2011; Nash et al., 2021). The transition to intensive commercial farming using cages, pens and ponds was facilitated by the successful development of artificial mass seed production in the early 2000s (Tri et al., 2021). This technological advancement marked a turning point for the industry, enabling rapid expansion and large-scale production, generating employment for hundreds of thousands of people in Vietnam. Attributed to its rapid growth performance and strong market demand, *P. hypophthalmus* has been widely referred to as the "Princess of Vietnamese aquaculture" (Phuong and Oanh, 2009; Balami et al., 2022). The success of hatchery technology and its widespread adoption by farming communities remain key drivers of industry growth.

2.1.2 Bacterial Infections in *Pangasianodon hypophthalmus*

The expansion of *P. hypophthalmus* culture has been accompanied by an increased incidence of infectious diseases, particularly bacterial infections, which pose a major threat to productivity and farm sustainability. These infections are often associated with intensive stocking densities, suboptimal water quality, environmental stress, and inadequate biosecurity measures, all of which facilitate the proliferation and transmission of pathogenic bacteria. Among the bacterial diseases reported in *P. hypophthalmus*, infections

caused by *E. ictaluri*, *Aeromonas hydrophila*, and *Flavobacterium columnare* are of particular concern due to their high morbidity and mortality rates (Table 2.1).

Table 2.1: Common Pathogenic Bacteria in *Pangasianodon hypophthalmus*

Species	Diseases	Management Practices	References
Edwardsiella ictaluri	• Bacillary necrosis of pangasius (BNP) in striped catfish • Enteric septicaemia of catfish (ESC) in channel catfish • Red-head disease in yellow catfish • Edwardsiellosis in tilapia	• Restricted feeding, administration of medicated feed and water chemical treatment • Antibiotics treatments including Romet ® and Aquaflor ® (USA), enrofloxacin and florfenicol (Vietnam), and doxycycline (DC) (China) • Vaccines – Alpha Ject Panga 1 and Alpha Ject Panga 2 (Vietnam) and live attenuated *E. ictaluri* bacterium lacking the *evp*B gene and AQUAVAC-ESC® (USA)	Machimbirike et al., (2022)
Aeromonas hydrophila	• Motile aeromonas septicemia (MAS)	• Effective water management in dry season • Vaccines – Recombinant live-vectored vaccine, Freund's adjuvant (Malaysia) and recombinant protein vaccine ISA 763 adjuvant (China)	Mzula et al., (2019)
Flavobacterium columnare	• Freshwater columnaris disease	• Water treatment by UV light • Vaccine – AQUAVAC-COL™	Tran et al., (2025)

2.2 *Edwardsiella ictaluri*

E. ictaluri is a Gram-negative, non-zoonotic bacterium that is primarily associated with diseases affecting catfish species. Although it poses no direct risk to human health, *E. ictaluri* represents a major threat to the aquaculture industry, particularly in catfish farming systems (Kumar et al., 2019; Phuoc et al., 2020). The bacterium belongs to the family Enterobacteriaceae and was first established based on 37 isolates found in diseased and seemingly healthy humans and animals from the United States, Brazil, Ecuador, Israel and Japan (Ewing et al., 1965; Wang et al., 2020). It was first reported in 1979 as the causative agent of disease in *Ictalurus punctatus* in the United States and was subsequently identified in Asian fish species, including *P. hypophthalmus* and *Pelteobagrus fulvidraco* (Li et al., 2017; Machimbirike et al., 2022). Over time, its host range has expanded substantially, with reports documenting infections in up to 44 fish species, highlighting its global significance to aquaculture (Machimbirike et al., 2022).

2.2.1 General Characteristics

E. ictaluri is a pleomorphic, rod-shaped bacterium that forms whitish, pinpoint colonies on Tryptic Soy Agar (TSA). Colonies typically measure 1-2 mm in diameter after incubation (Dong et al., 2015). When incubated at 28 ºC for 48 hours, colonies appear off-white to translucent, with irregular edges and surfaces, and reported colony sizes range from 0.03 to 0.14 mm (Crumlish et al., 2002). Notably, the size of this bacterium, specifically its cell dimensions, is not fixed and may vary depending on the host environment (Crumlish et al., 2002). It has been described as a small, straight rod measuring approximately 1µm x 2-3 µm (Plumb, 1993), while Vietnamese isolates have been reported to exhibit greater heterogeneity in both length and width (Figure 2.2) (Dung, 2002). The bacterium possesses peritrichous flagella all over its body, which contribute to its motility. However, at optimal

growth temperatures, this bacterium exhibits only weak motility (Hawke et al., 1981; Adepoju et al., 2021). *E. ictaluri* grows best in nutrient-rich media, with optimal culture temperatures ranging from 25 - 30 ºC (Machimbirike et al., 2022).

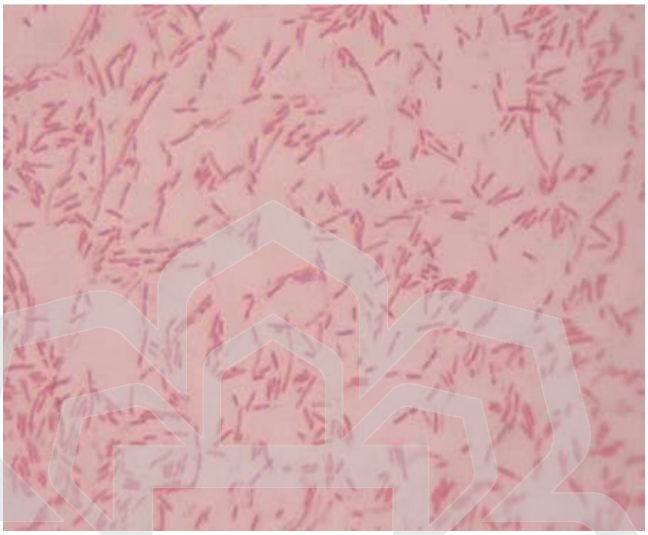


Figure 2.2: Vietnamese isolates of *Edwardsiella ictaluri* (Gram stain x 1,000) (https://backoffice.biblio.ugent.be/download/5951321/5951323).

2.2.2 Bacillary Necrosis of Pangasius (BNP)

E. ictaluri is the etiological agent of Bacillary Necrosis of Pangasius (BNP), one of the most frequently reported bacterial disease affecting cultured catfish species (Crumlish et al., 2002). In the Mekong Delta, BNP represents the most significant health challenge for nursing farms, with a reported prevalence of approximately 75% per production cycle (Hoa et al., 2021). Infected *P. hypophthalmus* exhibit behavioural abnormalities, including erratic and spiral swimming, along with pathological changes such as congested kidneys, enlarged spleens and whitish nodules on the liver and spleen (Wagner et al., 2002; Khang

et al., 2022). Diagnosis of the infected fish range from behavioural shifts, such as lethargy and spiral swimming, to severe physical lesions including small red and white skin ulcers, petechial haemorrhages around the mouth and fins, pale and swollen gills, exophthalmia (bulging eyes), and abdominal distension caused by ascitic fluid accumulation (Nordin et al., 2024). In the severe cases of *E. ictaluri* infection, characteristics lesions such as 'hole in the head', haemorrhagic opercula (gill cover) and haemorrhages of the lower mandible may be observed. (Liu et al., 2010; Zeng et al., 2021).

BNP affects fish at all production stages, however, fingerlings and juveniles are particularly susceptible, resulting in a severe impact on economic losses due to high mortality, reduced production, and increased treatment costs (Faruk & Anka, 2017; Hoa et al., 2021). Susceptible host sizes are commonly reported to range between 200-250 g, or approximately ¾ to 2 inches in length (Mawardi et al., 2018). Disease outbreaks are often associated with stress-inducing factors including environmental degradation, poor management practices, overstocking, temperature fluctuations and inadequate biosecurity measures (Faruk & Anka, 2017; Machimbirike et al., 2022). The highest occurrence of this disease was reported in June and July, which corresponds with the onset of the wet season and increased rainfall (Phan et al., 2009; Pokhrel & Oanh, 2021). Similarly, in the Pahang River, the highest prevalence of *E. ictaluri* infection has been observed around June, with the most susceptible size of *P. hypophthalmus* ranging from 1 to 50 g in body weight (Nordin et al., 2024).

The transmission and pathogenesis of *E. ictaluri* involve rapid systemic dissemination and multiple routes of entry. The bacterium persists in the environment through fecal shedding by infected fish and the decomposition of infected carcasses, resulting in high bacterial loads in the water body (Mawardi et al., 2018). Infection may occur through the olfactory system, where *E. ictaluri* colonizes the olfactory epithelium and migrates along the olfactory nerve to the brain, contributing to meningoencephalitis and "hole-in-the-head" lesion (Mawardi et al., 2018; Nordin et al., 2024). Ingestion of contaminated water or feed also represents a major route of infection, as the pathogen can rapidly cross the intestinal epithelium and disseminate via the bloodstream to internal organs such as the kidney and spleen, leading to septicemia (Abdelhamad et al., 2018). In

intensive aquaculture systems, horizontal transmission is further enhanced by the ability of *E. ictaluri* to persist in pond sediments and on submerged surfaces, allowing continued exposure even after disease outbreaks (Hoa et al., 2021; Uma, 2025).

2.3 Biotechnology Approaches in Bacterial DNA Characterization

While clinical observation and histopathology allow a presumptive diagnosis, effective disease control in aquaculture relies on accurate molecular characterisation of bacterial strains (Karacalar & Kemerlioğlu, 2025). Molecular approaches enable the differentiation of closely related strains, identification of virulence-associated genes, and assessment of genetic diversity that cannot be resolved through conventional diagnostic methods alone (De Carvalho et al., 2022). Conventional methods include polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), microsatellite, and Sanger sequencing (Gohil et al., 2019). Collectively, these tools have been widely applied in aquaculture systems to characterize bacterial DNA, support outbreak tracing, and develop disease management strategies.

Despite the usefulness of these approaches, their resolution remains limited when addressing complex genomic traits such as virulence regulation, antimicrobial resistance, and evolutionary relationships (Swain et al., 2022). In Malaysia, genomic data on *E. ictaluri* remain scarce, and there is a lack of comprehensive studies examining strain diversity, population structure, and transmission dynamics within aquaculture systems. This gap in molecular epidemiology restricts the understanding of how *E. ictaluri* strains circulate, adapt, and spread among farms, thereby constraining the development of effective control measures (Machimbirike et al., 2022). Therefore, the emergence of next-generation sequencing (NGS) technologies has revolutionized bacterial characterization by enabling whole genome sequencing (WGS), which provides comprehensive insights into genome

structure, gene content, and strain-level variation (Klinkenberg et al., 2017; Cassidy et al., 2020). For *E. ictaluri*, particularly in Malaysia, gaps remain in understanding genomic diversity and evolutionary relationships among isolates circulating in aquaculture systems, justifying the application of WGS in this study. To complement genome-wide analysis and further discriminate closely related strains, polymorphism-based methods, including RAPD profiling and 23S rRNA Sanger sequencing, were additionally employed to assess genetic variability and phylogenetic relationships among *E. ictaluri* isolates.

2.3.1 Next Generation Sequencing (NGS)

Next-generation sequencing (NGS) technology has instigated a revolutionary shift in the field of genomic research by providing the capability to generate massively parallel, high throughput DNA sequencing data (Park & Kim, 2016; Espitia-Navarro et al., 2020). This advancement has not only transformed the landscape of genomic studies but also paved the way for a new era of in-depth research. Introduced between 2004 and 2006, NGS marked a major breakthrough in molecular biology by dramatically increasing sequencing speed while reducing cost, compared with first-generation Sanger sequencing (Grada and Weinbrecht, 2013; Hu et al., 2021). Unlike traditional Sanger capillary electrophoresis, NGS platforms are capable of generating large-scale genomic datasets at substantially lower cost, making population-level and comparative genomic studies feasible (Heger, 2011). This technological shift has ushered in a new era of genomics research across diverse fields, including human genomics, microbial genomics and aquaculture health management.

NGS technologies offer several key advantages over first-generation sequencing methods. These include the ability to process millions of sequencing reactions simultaneously and capacity to perform comprehensive whole-genome analysis with superior sensitivity and specificity (Satam et al., 2023). Among the various applications of

NGS, whole genome sequencing (WGS) has become one of the most widely used approaches, particularly following its initial development in human genomics and subsequent adoption in microbial research (Balloux et al., 2018). Advances in sequencing platforms have significantly enhanced the accuracy and analytical power of bacterial genomics. Short-read technologies such as Illumina's HiSeq and MiSeq, provide high-precision data, while long-read platforms including Pacific Biosciences' (PacBio) Single Molecule Real-Time (SMRT) and Oxford Nanopore Technologies' (ONT) MinION offer greater genome completeness (De Maio et al., 2019). In order to overcome individual platform limitations, researchers increasingly employ hybrid sequencing approaches that combine the strengths of both technologies.

2.3.2 Whole Genome Sequencing (WGS)

Whole genome sequencing (WGS) is a comprehensive genomic approach that enables the analysis of an organism's entire genetic content (Ng and Kirkness, 2010; Liu et al., 2024). It provides complete genomic coverage and the most comprehensive representation of nucleotide-level genetic variation (Van El et al., 2013; Elbehiry & Abalkhail, 2025). This technique generates enormous amounts of raw sequencing data, which require complex bioinformatic pipelines for quality control, assembly, annotation and downstream analysis. Two primary strategies are commonly employed in WGS data analysis which are de novo assembly and reference-based assembly (Park and Kim, 2016; Barbadikar et al., 2024). De novo assembly involves overlapping short sequencing reads to construct contiguous sequences without relying on an existing reference genome (Liao et al., 2019). This method is particularly valuable for novel organisms, highly divergent strains or when reference genomes are unavailable or incomplete. In contrast, reference-based assembly aligns sequencing reads to a previously established reference genome sequence, enabling faster and more computationally efficient genome reconstruction (Ng and Kirkness, 2010; Scheunert et al., 2020).

In aquaculture research, WGS has provided powerful insights into the genetic makeup of the microbial pathogens (Sudheesh et al., 2012). It enables the identification of outbreak-associated lineages through the integration of epidemiological data with phylogenetic analysis (George et al., 2017; Klinkenberg et al., 2017). Through WGS, *E. ictaluri* strains isolated from different geographical locations or hosts species can be genetically characterized and compared. This approach facilitates the identification of virulence genes, antimicrobial resistance (AMR) profiling and genomic polymorphisms, thereby providing insights into pathogen evolution, transmission dynamics and disease epidemiology (Balloux et al., 2018). Ultimately, such genomic information contributes to the development of effective disease control strategies, supporting sustainable and profitable aquaculture systems (Iqbal et al., 2023).

2.3.3 Long-Read Sequencing Technologies

Long-read sequencing technologies have emerged as powerful tools in genomic research, addressing several limitations associated with short-read sequencing platforms (George et al., 2017). While traditional short-read methods are restricted to 50 - 300 base pairs, long-read platforms are capable of producing reads spanning several kilobases, thereby enabling improved resolution of complex genomic regions (Amarasinghe et al., 2020). The most widely used long-read sequencing platforms include Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) (Tedersoo et al., 2021). A primary advantage of long-read sequencing is the ability to resolve repetitive regions, structural variations and complex rearrangements that typically result in fragmented assemblies when using short-read data alone (Mahmoud et al., 2019). This capability results in more contiguous genome assemblies, thus improving the completeness of bacterial genomes (Koren et al., 2017). Among these technologies, ONT stands out as the only commercially available nanopore-based platforms. It operates by measuring the ionic current fluctuations when single-

stranded nucleic acids pass through biological nanopores (Figure 2.3) (Jain et al., 2016). Each nucleotides confer distinct electrical resistances, the sequence of bases will produce specific patterns (Amarasinghe et al., 2020).

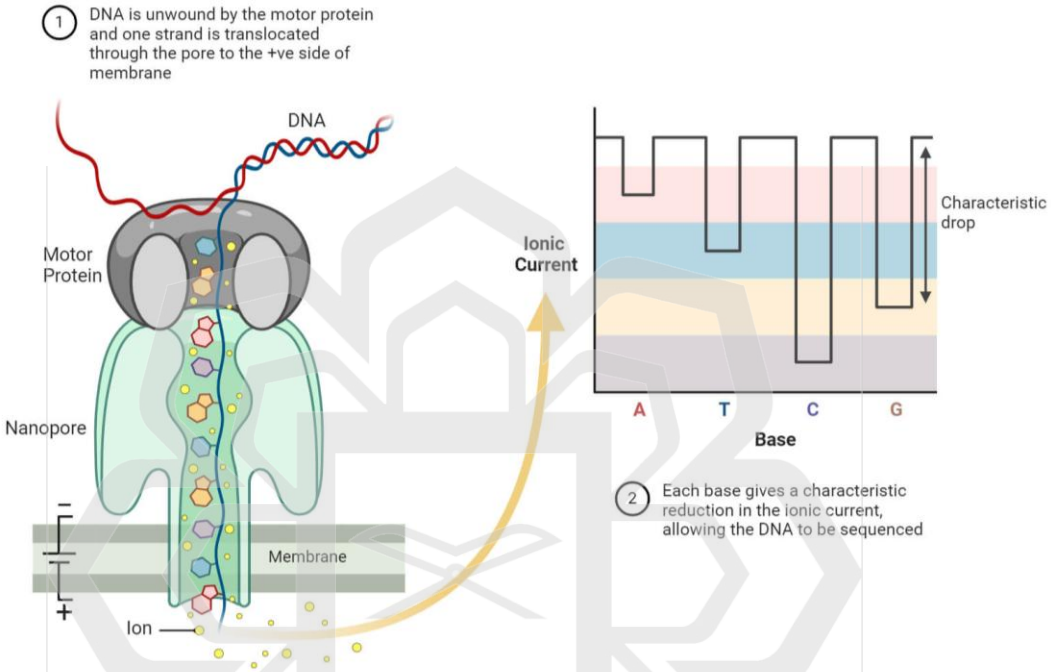


Figure 2.3: Nanopore Sequencing Principle (https://microbenotes.com/oxford-nanopore-sequencing/)

Historically, long-read platforms exhibited higher raw error rates, however, recent technological advancements such as PacBio HiFi reads and ONT chemistry upgrades have substantially improved sequencing accuracy (Wenger et al., 2019). These have substantially narrowed the performance gap with short-read platform, enabling the generation of closed or near-complete bacterial genomes in a single run without extensive computational scaffolding (Sereika et al., 2022). In the context of microbial genomics, this high-contiguity sequencing is critical for the accurate reconstruction of plasmids, genomic islands and mobile genetic elements which often carry virulence factors and antimicrobial

resistance (AMR) genes (Tedersoo et al., 2021). For specific pathogens like *E. ictaluri*, these complete assemblies facilitate accurate identification of horizontal gene transfer events and tracking of outbreak-associated strains. Furthermore, the portability of ONT instruments, such as the MinION and the low-cost Flongle flow cell, allows for rapid, field-based analysis, offering unprecedented flexibility for real-time disease monitoring in aquaculture (Sereika et al., 2022).

2.3.4 Next-generation Sequencing (NGS) Technologies in Aquaculture

Next-generation sequencing (NGS) technologies have transformed genomic research and are widely applied across multiple disciplines including biomedical sciences, agriculture, ecology and forensics. In fisheries and aquaculture, NGS enables genome-wide analyses of genetic diversity, population structure, and evolutionary relationships, supporting disease surveillance and management (Sahu et al., 2024). This approach allows large-scale sequencing of whole genomes or targeted regions, allowing the detection of single nucleotide polymorphisms (SNPs) and other genetic variations linked to important traits (Yang et al., 2025). In addition, the integration of NGS into genomic selection programs has further accelerated selective breeding by identifying genetic markers linked to economically important traits, including disease resistance and growth performance (Swain et al., 2022). Genotyping by sequencing (GBS), targeted sequencing and PCR-based genotyping are some of the examples that can be used for choosing the best individuals that showed the greatest resistance or survival (Saura et al., 2019). Genomic-assisted selection is being adopted in shrimp breeding research programmes for improved disease and stress tolerance (Luo et al., 2022). Research shows that 80% of individuals in genomically-selected families for white spot syndrome virus (WSSV) resistance survived when challenged with the disease (Robinson & Lillehammer, 2020).

The application of NGS in aquaculture pathogens has provided valuable genomic insights. For instance, the complete genome sequence of *E. ictaluri* strain 93-146 from the United States, obtained using high-throughput shotgun sequencing, is 3,812,315 bp in length and comprises 3,783 predicted protein-coding genes, with an average GC content of 57.4% (Williams et al., 2012). Similarly, *E. ictaluri* isolates recovered from diseased *P. hypophthalmus* in the Mekong Delta, Vietnam, have been characterized using using Oxford Nanopore MinION sequencing, yielding genome sizes ranging from 3.55 to 3.75 Mbp and GC contents between 57.0 and 57.7% (Erickson et al., 2024). These findings indicate a relatively conserved genome size and GC content among *E. ictaluri* strains across different geographical regions. In the Malaysian context, the importation of fish seed from Vietnam suggests a potential epidemiological link, whereby, local *E. ictaluri* strains may share genomic similarities with Vietnamese isolates (Nguyen et al., 2024). However, the extent of this relationship remains unclear due to the limited availability of genomic data from Malaysian isolates, highlighting the need for further comparative genomic studies.

2.4 Genetic Polymorphisms Study

Genetic polymorphisms are heritable variations in DNA sequence within a species' genome that contribute to genetic diversity, phenotypic differences and variation in disease susceptibility (Aga et al., 2022). Formally, it is defined as the inheritance of a trait controlled by a single genetic locus where the least common allele occurs at a frequency of 1% or greater within a population (Ismail and Essawi, 2012; Hailemariam & Yadeta, 2020). Genetic polymorphisms include single nucleotide polymorphisms (SNPs), copy number variations (CNVs), insertions or deletions (indels) and structural variants (Sameer et al., 2021). These variations arise through mutational processes such as nucleotide substitutions and DNA rearrangements. A distinction is commonly made between the terms *mutation* and *polymorphism*. Rare and often irreversible DNA sequence changes, whether occurring spontaneously or induced by external factors are referred to as *mutations*, whereas,

common, stable and heritable sequence variations are classified as *polymorphisms* (Karki et al., 2015; Sameer et al., 2021).

The distribution of genetic polymorphisms is not uniform across the genome. Polymorphisms can occur within coding regions of genes as well as in non-coding regions. Non-coding regions often exhibit a higher density of polymorphisms than protein-coding sequences due to lower selective pressure (Mu et al., 2011; Durward-Akhurst et al., 2021). In contrast, polymorphisms occurring within coding regions may result in amino acid substitutions that alter protein structure or function, potentially influencing phenotypic traits, disease susceptibility, and responses to environmental stressors (Hailemariam & Yadeta, 2020). SNPs represent the simplest and most common form of genetic variation, accounting for approximately 90% of all DNA polymorphisms in the human genome. SNPs are predominantly found in non-coding regions and serve as excellent genetic markers disease associated studies, evolutionary analyses and population genetics (Lin et al., 2022; MedlinePlus, 2022). In bacterial populations, the detection of genetic polymorphisms relies on various molecular techniques. Among these, Random Amplified Polymorphic DNA (RAPD-PCR) is commonly employed for rapid genomic fingerprinting and for assessing genetic diversity among closely related isolates (Babu et al., 2020).

2.4.1 Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR)

Random Amplified Polymorphic DNA polymerase chain reaction (RAPD-PCR) is a PCR-based fingerprinting technique that amplifies random genomic loci using short, arbitrary primers to produce multi-band patterns on agarose gels (Lee & Chang, 1994). The collective pattern of amplified fragments constitutes a unique genetic "fingerprint" for each isolate (Williams et al., 1990). Polymorphisms detected by RAPD-PCR arise primarily from sequence variations within primer binding sites or base changes in the amplified

regions (Babu et al., 2020). Since RAPD-PCR does not require prior knowledge of DNA sequence information, it has been widely applied as a rapid and cost-effective approach for screening genetic diversity among microbial isolates or populations (William et al., 1990; Fadel et al., 2022). Compared with sequencing-based molecular techniques, which offer higher discriminatory power at the intra-genus or species level, RAPD-PCR is less expensive and less labor-extensive, making it particularly suitable for preliminary genetic analyses or laboratories with limited resources (Robinson and Harris 1999; Zhang et al., 2024).

The standard RAPD-PCR workflow involves genomic DNA extraction, amplification using multiple random primers, and scoring presence or absence of bands to generate a binary data matrix for similarity, clustering or phylogenetic analyses (Welsh & McClelland, 1990; Fadel et al., 2022). The key advantages of RAPD-PCR include its speed, simplicity and minimal DNA input requirements, which make it attractive for preliminary diversity assessments of genetic diversity and population structure (Robinson and Harris, 1999). In fish health research, RAPD-PCR has been successfully used for strain differentiation, population structure analysis, and preliminary epidemiological investigations of bacterial pathogens (Nur-Nazifah et al., 2011; Sudheesh et al., 2012). In studies involving fish pathogens, RAPD-PCR enables rapid detection of genetic heterogeneity among isolates collected from different farms, host species, or geographical regions (Nur-Nazifah et al., 2011). This approach is particularly useful for identifying potential outbreak clusters or transmission patterns prior to more detailed sequencing-based analyses. Given the significant economic impact of *E. ictaluri* infections in catfish aquaculture, RAPD-PCR provides a practical first-line tool to assess genetic variation among local isolates.

2.4.2 Sanger Sequencing

Sanger sequencing is a first-generation DNA sequencing method that generates highly accurate nucleotide reads for specific PCR-amplified regions and remains the gold standard for targeted sequence validation (Sanger et al., 1977; Cheng et al., 2023). In microbial and pathogen studies, this technique is commonly used to sequence conserved genetic markers such as 16S rRNA, housekeeping genes, or selected virulence-associated loci to confirm species identity, detect single nucleotide polymorphisms (SNPs), and generate alignable sequences for phylogenetic analysis (Janda & Abbott, 2007; Woo et al., 2008; Drevinek et al., 2023). Compared with fingerprinting techniques such as RAPD-PCR, Sanger sequencing provides unambiguous base-level sequence information that allows robust evolutionary inference and accurate detections of point mutations or indels (Clarridge, 2004). The resulting sequence data are highly reproducible and suitable for comparative analyses across studies and databases, making Sanger sequencing particularly valuable for validating genetic variation detected by rapid screening methods.

In comparison to NGS platforms, its application is generally restricted to a limited number of genes or representative isolates rather than large-scale genome-wide analysis. Therefore, in many molecular epidemiological studies, Sanger sequencing is applied strategically to validate representative RAPD-PCR clusters or to resolve phylogenetic relationships among closely related isolates identified through preliminary fingerprinting (Power, 1996; Robinson & Harris, 1999). Overall, RAPD-PCR and Sanger sequencing are often used in a complementary manner, where RAPD-PCR serves as a rapid and cost-effective screening tool to detect broad genetic diversity among multiple isolates, while Sanger sequencing provides precise nucleotide-level confirmation for selected strains and loci of interest. This combined approach enables efficient characterization of genetic polymorphisms while maintaining high analytical accuracy, particularly in studies involving bacterial pathogens of aquaculture importance such as *E. ictaluri*.

2.4.3 Polymorphism Studies in Aquaculture Industry

Genetic polymorphism studies are foundational to the modern aquaculture industry, improving understanding of genetic variation in cultured species and their associated pathogens. In aquaculture systems, polymorphism analysis is widely applied to assess population structure, genetic diversity, disease susceptibility, and adaptive responses to environmental stressors (Aga et al., 2022). Such information is essential for improving selective breeding programs, supporting sustainable management and effective disease-control framework in the industry (Houston et al., 2020). In host species, genetic polymorphisms serve as molecular markers for identifying Quantitative Trait Loci (QTLs) associated with economically important traits such as growth rate, feed efficiency, stress tolerance, and disease resistance (Nguyen, 2024). Single nucleotide polymorphisms (SNPs), in particular, are frequently applied in marker-assisted selection and genomic selection programs to accelerate genetic improvement in aquaculture species including tilapia, salmon, and catfish (Yáñez et al., 2015; Houston et al., 2020).

Polymorphism analysis is also extensively used in the study of aquatic pathogens, where genetic variation influences virulence, host specificity, and antimicrobial resistance (Islam et al., 2025). In prevalent bacterial pathogens such as *E. ictaluri*, *A. hydrophila*, and *Streptococcus agalactiae*, polymorphism-based techniques including RAPD-PCR, multilocus sequence typing (MLST), and sequencing of conserved genes have been applied to differentiate strains, trace infection sources, and investigate outbreak dynamics (Nur-Nazifah et al., 2011; Bogaerts et al., 2025). These studies provide valuable epidemiological insights that support early detection of disease outbreaks and implementation of targeted biosecurity measures. Furthermore, identifying polymorphism in virulence-associated genes assists in predicting disease severity and evaluating the efficacy of vaccines (Islam et al., 2025; Sahoo et al., 2025).

CHAPTER THREE

MATERIALS AND METHODOLOGY

The third chapter focused on how the research was conducted. The overall research flow is presented in Figure 3.1.

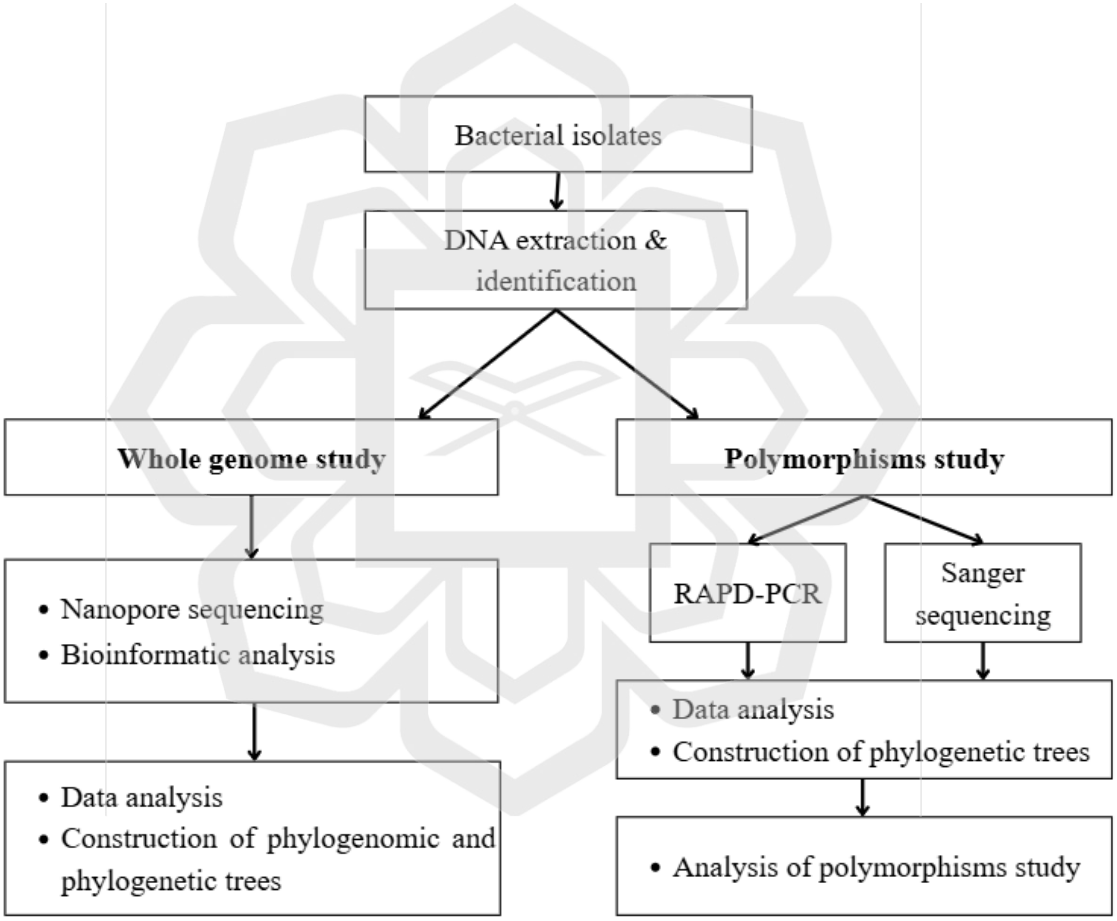


Figure 3.1: Overall methodology

This research was divided into two main approaches to characterize isolates of *Edwardsiella ictaluri* isolated from *Pangasianodon hypophthalmus* cultured in the Pahang River, whole genome study and polymorphisms study. For the whole genome study, genomic DNA of *E. ictaluri* isolate was extracted and sequenced using Oxford Nanopore technology, followed by bioinformatic analysis to generate a phylogenomic tree. Meanwhile, for polymorphisms study, genetic polymorphisms among the isolates were examined using RAPD-PCR and Sanger sequencing. The resulting datasets were subsequently analyzed, and phylogenetic trees were constructed to evaluate genetic diversity and evolutionary relationships among the bacterial isolates.

3.1 BACTERIAL ISOLATES

3.1.1 Background of Isolates

A total of 11 positive isolates of *E. ictaluri* were previously isolated from cage-cultured *P. hypophthalmus* in the Pahang River by Nordin et al., (2024) and stored at Fish Disease Laboratory, Kulliyyah of Science, International Islamic University Malaysia (IIUM). The isolates were kindly provided for the present study (Table 3.1). Temerloh represents the upstream region of the river, while Pekan is located downstream, closer to the river estuary.

Table 3.1: List of positive *E. ictaluri* isolates

Month	**Sampling Sites**	
	Pekan	**Temerloh**
March	–	2 isolates
April	1 isolate	–
May	5 isolates	–
June	3 isolates	–
Total	**9 isolates**	**2 isolates**

3.1.2 DNA Extraction

E. ictaluri isolates preserved in glycerol stocks were revived by streaking onto Tryptic Soy Agar (TSA) (Oxoid, United Kingdom) and incubated at 28 ºC for 48 h. Two complete streak lines from each agar plate were inoculated into 15 ml of Brain Heart Infusion (BHI) broth (Oxoid, United Kingdom) and incubated at 28 ºC for 48 h with agitation. Following incubation, bacterial cells were harvested by centrifugation at 17,000 x g for 1 min. The collected cell pellets, approximately 0.3 ml, was subjected to genomic DNA extraction using the G-spin™ Total DNA Extraction Kit (Intron Biotechnology, South Korea) following the manufacturer's protocol, specifically Protocol F for bacterial samples (Mohd Salim et al., 2024).

200 µl of Buffer CL, 20 µl of Proteinase K, and 5 µl of RNase A solution were added to the tube containing pellets. The mixture was vortexed and incubated at 56 ℃ for 6 min using a preheated heat block, with gentle inversion every 2 min to facilitate cell lysis. Subsequently, 200 µl of Buffer BL was added, followed by incubation at 70 ℃ for 2 min. The tube was then centrifuged at 17,000 x g for 5 min to remove un-lysed particles. Approximately 350 – 400 µl of the supernatant was transferred to a new 1.5 ml microcentrifuge tube containing 200 µl of absolute ethanol, mixed by pulse vortexing, and transferred into a Spin Column. After centrifugation at 17,000 x g for 1 min, the filtrate was discarded. The column was then washed sequentially with 700 µl of Buffer WA and Buffer WB, followed by an additional centrifugation step at 17,000 x g for 1 min to dry the membrane. The flow-through and collection tube were discarded, and the spin column was placed into a new 1.5 ml centrifuge tube. Finally, 50 µl of Buffer CE was added directly onto the membrane and incubated at room temperature for 1 min. The column was then centrifuged at 17,000 x g for 1 min to elute the DNA. DNA obtained were then kept at – 20 ℃ until further used.

3.1.3 Polymerase Chain Reaction (PCR) Amplification and Gel Electrophoresis

PCR amplification was conducted using species-specific primers, IVS and IRS, targeting the 16S – 23S rRNA intergenic spacer region (Table 3.2) (Williams et al., 2010; Janda & Duman, 2024). The composition for PCR master mix (Thermo Scientific, USA) was shown in Table 3.3. PCR conditions included an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. The final elongation will be at 72°C for 10 min. After that, 4 µl of PCR product and 2 ul of loading dye (Thermo Scientific, USA) were mixed and electrophoresed through a 1% of agarose gel (First Base, Singapore) containing Gel Red (First Base, Singapore). 1kb DNA Ladder (Thermo ScientificTM GeneRuler, USA) was used. The electrophoresis was run at 100V and 400Amp for 30 min using MT-108 Wide Mini Horizontal Gel Electrophoresis System (Major Science, USA). Then, the gel was observed under Gel Doc XR_ Gel Documentation System (BioRad, California). The band sizes for the present of *E. ictaluri* were 2000-bp.

Table 3.2: Set of species-specific primers (IVS and IRS) used for PCR Amplification

Types	Primers	Target Gene	Product size (bp)	Reference
Species specific	IRS (5'-TAC GCT TTC CTC AGT GAG TGT C-3')	16S-23S rRNA intergenic spacer region	2000	Williams and Lawrence, 2010
	IVS (5'-TTA AAG TCG AGT TGG CTT AGG G-3')			

Table 3.3: The composition of PCR master mix

PCR Component	Volume (µl)	Concentration
PCR Buffer	2	10 x
$MgCl_2$	2	25 mM
IVS	0.5	100 pmol μL^{-1}
IRS	0.5	100 pmol μL^{-1}
Deoxynucleotide triphosphates (dNTPs)	0.5	10 mM
Taq DNA polymerase	0.5	5 μL^{-1}
DNA template	1	50 ng/µL minimum
Distilled water	43	-
Total volume per sample	**50**	**-**

3.1.4 Polymerase Chain Reaction (PCR) Amplification using Universal Primer

PCR amplification using universal primer was conducted to further verify the quality and purity of the extracted genomic DNA sample. The amplification utilized the 27F Forward primer and 1492R Reverse primer (Table 3.4) to amplify the target 16S rRNA regions (Turner et al., 1999). PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72°C for 2 min. The final elongation was at 72 °C for 10 min. After that, 4 µl of PCR product and 2 ul of loading dye (Thermo Scientific, USA) were mixed and electrophoresed through a 1% of agarose gel (First Base, Singapore) containing Gel Red (First Base, Singapore). 1kb DNA Ladder (Thermo ScientificTM GeneRuler, USA) was used. The electrophoresis was run at 100V and 400Amp for 30 min in a MT-108 Wide Mini Horizontal Gel Electrophoresis System (Major Science, USA). Then, the gel was observed under Gel Doc XR_ Gel Documentation System (BioRad, California). The band sizes for the present of *E. ictaluri* were 1500-bp.

Table 3.4: Set of universal primers (27F and 1492R) used for PCR Amplification

Types	Primers	Target Gene	Reference
Universal primer	27F (5'- AGA GTT TGA TCM TGG CTC AG -3')	16S rRNA	Lane, (1991)
	1492R (5'- GGT TAC CTT GTT ACG ACT T -3')		Turner et al., (1999)

3.2 WHOLE GENOME SEQUENCING OF *Edwardsiella ictaluri*

3.2.1 Library Preparation and Nanopore Sequencing

For library construction, 500 ng of high-quality gDNA was using the Oxford Nanopore Ligation Sequencing Kit (LSK114), following the manufacturer's instructions. Sequencing was performed on a Flongle R10.4.1 flow cell mounted on a MinION Mk1B sequencer (Oxford Nanopore Technologies, UK). To ensure data quality, only raw sequencing reads with a length exceeding 1,000 bp were retained for downstream analysis. High accuracy basecalling of the generated POD5 files was conducted using Dorado v0.7.0 with the simplex dna_r10.4.1_e8.2_400bps_sup_v5.0.0 model with integrated adapter trimming.

3.2.2 De Novo Genome Assembly and Quality Assessment

Raw sequencing read statistics were generated using SeqKit v2.3.0 (Shen et al., 2016). De novo genome assembly was carried out using Flye v2.9 in –nano-hq mode to optimize for high-accuracy Nanopore reads (Kolmogorov et al., 2019). The structural integrity and statistical quality of the resulting assemblies were assessed using SeqKit v2.3.0, while genome completeness was evaluated using BUSCO v5.4.3 (Gurevich et al., 2013; Simão et al., 2015). To standardize the assembly, circular contigs were re-oriented to the dnaA gene using Circlator v1.5.5 (Hunt et al., 2015). Additionally, plasmid-derived contigs were identified and characterized using Plasmer v0.1 (Zhu et al., 2023).

3.2.3 Functional Genome Annotation and Antimicrobial Resistance (AMR)

The assembled genomes were annotated locally using Bakta v1.9.3, supported by its comprehensive standard database (Schwengers et al., 2021). Protein-coding sequences (CDS) were predicted using Prodigal v2.6.0 (Hyatt, 2010) with default parameters. For functional characterization, the predicted protein sequences were analyzed via the eggNOG-mapper and KofamKOALA web servers for functional annotation and KEGG orthology assignment (Cantalapiedra et al., 2021; Aramaki et al., 2020). The assembled genomes were further screened against multiple antimicrobial resistance (AMR) databases using ABRicate pipeline (https://github.com/tseemann/abricate).

3.2.4 Phylogenomic Analysis of *Edwardsiella ictaluri*

Phylogenomic analyses were conducted at two hierarchical levels. An inter-species analysis was performed to confirm taxonomic placement within the genus *Edwardsiella*. This was followed by an intra-species analysis to investigate the genomic relatedness of *E. ictaluri* strains from different geographical regions and hosts species.

3.2.4.1 High-resolution Taxonomic Classification and Inter-Species Phylogenomic Analysis of Edwardsiella spp.

High-resolution taxonomic classification was conducted by calculating pairwise Average Nucleotide Identity (ANI) between the assembled genomes and reference genomes from the GTDB release r220 using FastANI v1.33 (Jain et al., 2018; Parks et al., 2020). The top ANI matches for each genome were selected for downstream Species-level phylogenomic analysis using PhyloPhlAn3 (Asnicar et al., 2020). PhyloPhlAn3 identified 400 universally conserved bacterial marker proteins were identified using DIAMOND BLASTP (Buchfink et al., 2021). Each marker protein was aligned individually using MUSCLE v5.1 and trimmed with TrimAl v1.4.rev15 (Capella-Gutiérrez et al., 2009; Edgar, 2022). The trimmed alignments were concatenated, and a maximum-likelihood phylogenomic tree was constructed using FastTree v2.1.11 as implemented in PhyloPhlAn3 (Price et al., 2010).

3.2.4.2 Intra-species Phylogenomic Analysis of Edwardsiella ictaluri strains

Complete genome sequences of *E. ictaluri* together with their associated metadata, such as host and country of origin, were obtained from the NCBI database. Intra-species phylogenomic analysis was performed using GToTree (Lee, 2019) to construct a phylogenomic tree based on whole-genome data. The resulting tree was visualized using the ETE Toolkit (Huerta-Cepas et al., 2010) to infer evolutionary relationships and genomic relatedness among *E. ictaluri* isolates.

3.3 RANDOM AMPLIFIED POLYMORPHIC DNA – POLYMERASE CHAIN REACTION (RAPD-PCR)

3.3.1 Primers Selection

The second objective of this study was to determine the genetic diversity and evolutionary relationships among *E. ictaluri* isolates (Table 3.1) using molecular characterization based on randomly amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR). Seven arbitrary 10-mer RAPD primers (Bioneer, South Korea) were selected for this analysis (Table 3.5). These primers were chosen based on their reported reproducibility and capacity to generate polymorphic banding patterns in bacterial genomic studies. Each primer was used independently to amplify random regions of the bacterial genome, generating distinct DNA fingerprint profiles for each isolate.

Table 3.5: List of primers used in RAPD-PCR analysis of *Edwardsiella ictaluri*

No.	Primers	Primer Sequence	References
1.	OPA-01	5'- CAG GCC CTT C- 3'	Jogi et al., (2023)
2.	OPA-03	5'- AGT CAG CCA C- 3'	Washikur et al., (2025)
3.	OPA-07	5'- GAA ACG GGT G- 3'	Abdulrazaq, (2023).
4.	OPB-10	5'- CTG CTG GGA C- 3'	Dahanayaka et al., (2025)
5.	OPF-01	5'- ACG GAT CCT G- 3'	Caldeira et al., (2009)
6.	OPR-12	5'- ACC AGT GCG T- 3'	Chen et al., (2025)
7.	OPD-08	5'- GTG TGC CCC A- 3'	Younes et al., (2013)

3.3.2 Polymerase Chain Reaction (PCR) Amplification

RAPD-PCR amplification of the extracted DNA (refer 3.1.2) was carried out in a thermal cycler (Hercuvan, United Kingdom) with a total reaction volume of 25 µl (Table 3.6). The PCR cycling condition (Table 3.7) were optimized to promote low-stringency primer annealing, which is characteristic of RAPD amplification.

Table 3.6: The composition of RAPD-PCR master mix

PCR Component	**Volume (µl)**	**Concentration**
PCR Buffer	2.5	10 x
$MgCl_2$	2	25 mM
Deoxynucleotide triphosphates (dNTPs)	0.5	10 mM
Taq DNA polymerase	0.5	5 μL^{-1}
DNA template	1	50 ng/µL minimum
10-mers primers	1	100 pmol μL^{-1}
Distilled water	17.5	-
Total volume per sample	**25**	**-**

Table 3.7: Amplification parameter of RAPD-PCR

Process	Duration	Temperature
Initial denaturation	4 mins	94°C
Denaturation	1 min	94°C
Annealing	1 min	36°C
Extension	1 min	72°C
Final elongation	10 mins	72°C

3.3.3 Gel Electrophoresis

A 1% agarose gel was prepared using 1× TBE running buffer. Following that, 20 µl of the PCR product solution was mixed with 5 µl of loading dye solution (Thermo Scientific, USA) and electrophoresed through 1% of agarose gel (First Base, Singapore) containing Gel Red (First Base, Singapore) at 100 V and 400 Amp for 60 min using an MT-108 Wide Mini Horizontal Gel Electrophoresis System. Then, the gel was visualized and documented using a UVITEC Cambridge Gel Documentation System (UVITEC, United Kingdom).

3.3.4 Random Amplified Polymorphic DNA – Poymerase Chain Reaction (RAPD-PCR) Data Scoring and Binary Matrix Construction

RAPD profiles generated from each primer were scored manually using UVITEC Cambridge gel documentation system (Uvitec, United Kingdom) based on the presence or absence of distinct DNA bands across all isolates. Each visible and reproducible band was scored as “1” (present), and absence of bands as “0” (absent). Faint but consistently reproducible bands were included in the analysis. The scoring results from all primers were

combined to generate a binary data matrix, representing the RAPD fingerprint of each isolate. Redundant or non-informative bands were trimmed to improve data quality. The finalized binary matrix was formatted and entered into NTedit version 1.2a, which was used to prepare and verify the input data for subsequent numerical analysis.

3.3.5 Similarity Analysis and Genetic Distance Calculation

The edited binary matrix was imported into NTSYSpc version 2.10j (Numerical Taxonomy and Multivariate Analysis System) for genetic similarity analysis. Pairwise similarity coefficients among isolates were calculated using the Nei and Li/Dice similarity coefficient, which is widely applied in RAPD-based genetic diversity studies due to its suitability for binary data (Nei & Li, 1979). The resulting similarity matrix was subsequently transformed into a genetic distance matrix, which quantitatively represents the degree of genetic divergence among the isolates. This transformation allows for clearer interpretation of evolutionary relationships and clustering patterns.

3.3.6 Cluster Analysis and Phylogenetic Tree Construction

Cluster analysis was performed using the Sequential, Agglomerative, Hierarchical and Nested (SAHN) clustering method implemented in NTSYSpc. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was applied to construct a dendrogram based on the genetic distance matrix. UPGMA was selected as it assumes a constant rate of evolution and is commonly used in RAPD studies for illustrating genetic relationships among bacterial isolates. The resulting dendrogram provided a visual representation of

genetic similarity, clustering patterns, and evolutionary relationships among the *E. ictaluri* isolates.

3.4 SANGER SEQUENCING

3.4.1 Sanger Sequencing of Polymerase Chain Reaction (PCR) Products

Purified PCR amplicons obtained as described in Section 3.1 were subjected to bidirectional Sanger sequencing. Sanger sequencing was selected due to its high accuracy and reliability for sequencing specific PCR-amplified regions, particularly conserved ribosomal RNA genes commonly used for bacterial identification (Sanger et al., 1977). Species-specific primers, IVS and IRS, were employed for sequencing, targeting 16S – 23S rRNA intergenic spacer region, respectively (Figure 3.2). Sequencing services were outsourced to Apical Scientific, utilizing the BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), following the manufacturer's standard protocol. Both forward and reverse strands were sequenced for each isolate to ensure maximum sequence accuracy and comprehensive coverage of the target regions. Bidirectional sequencing also minimizes sequencing errors caused by dye blobs, ambiguous peaks, or signal drop-off commonly observed at the ends of single-direction reads.

3.4.2 Sequence Assembly and Quality Control

Raw sequencing chromatograms generated from the forward and reverse reactions were subjected to manual inspection and quality assessment prior to sequence assembly. High-quality sequencing read were expected to begin with a region of single peaks, followed by

a region of double peaks at least 20 bp from the beginning of the sequencing results. To ensure reliability, only sequences with minimal noise and consistent peak patterns were retained for further analysis. Trimming of poor-quality bases at the 5' and 3' ends was performed where necessary. All forward and reverse sequences for the 11 bacterial isolates were aligned and edited using BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999). The ClustalW Multiple Alignment tool embedded within BioEdit was used to align overlapping regions of the forward and reverse reads. The resulting consensus sequences represent the most accurate reconstruction of the target gene regions and were subsequently used for sequence identification and phylogenetic analysis.

3.4.3 Sequence Identification (BLAST)

Confirmation of the taxonomic identity of the isolates was achieved through comparison of the assembled consensus sequences with publicly available nucleotide sequences in the National Center for Biotechnology Information (NCBI) database. Sequence similarity searches were conducted using the Nucleotide BLAST (blastn) algorithm. Isolates were identified based on the percentage of identity, query coverage and E-values relative to existing *E. ictaluri* sequences in the GenBank repository. Each isolate was identified based on three key parameters, percentage sequence identity, query coverage, and E-value. High percentage identity and query coverage, coupled with low E-values, were considered strong indicators of reliable species-level identification. The obtained sequences were specifically compared against existing *E. ictaluri* reference sequences available in the GenBank repository to confirm species assignment.

3.4.4 Phylogenetic Tree Construction and Evolutionary Analysis

Phylogenetic analysis was performed using MEGA (Molecular Evolutionary Genetics Analysis) software version 12 (Kumar et al., 2018) to visualize genetic distances and evolutionary relationships among the *E. ictaluri* isolates. Multiple sequence alignment was conducted using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm, which provides higher alignment accuracy and computational efficiency compared to traditional alignment methods (Edgar, 2004). The aligned nucleotide sequences were used to construct a phylogenetic tree using the Maximum Likelihood (ML) method based on the Tamura-Nei nucleotide substitution model (Felsenstein, 1981). The robustness of the inferred phylogeny was evaluated using standard bootstrap analysis with 1,000 replicates. The initial tree for the ML analysis was generated automatically, and branch optimization was performed using the Nearest-Neighbor Interchange (NNI) heuristic method. Bootstrap support values were mapped onto the tree nodes to assess the reliability of the branching patterns.

3.5 ANALYSIS OF POLYMORPHISM STUDY

Genetic polymorphism among the *E. ictaluri* isolates was assessed using two complementary molecular approaches, RAPD profiling and Sanger sequencing–based phylogenetic analysis. These methods differ in resolution and genomic coverage, allowing polymorphic variation to be assessed at both genome-wide and sequence-specific levels. RAPD-based dendrogram and the sequence-based phylogenetic tree were subsequently compared to evaluate the consistency of the clustering patterns across marker-based and sequence-based approaches. Similar clustering patterns between the two analyses were interpreted as evidence of stable genetic relationships, while differences between the results highlighted variations in sensitivity between genome-wide polymorphic markers and

targeted sequence variation. This combined approach allows a robust evaluation of genetic polymorphism and intra-species variation among *E. ictaluri* isolates.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 MOLECULAR SCREENING

4.1.1 Bacterial isolates

A total of 11 *E. ictaluri* isolates were successfully recovered from glycerol stock cultures and subcultured onto tryptic soy agar (TSA). Following incubation, all isolates exhibited consistent colonial morphology, characterized by translucent, small, and pinpoint colonies. These morphological characteristics are consistent with previous descriptions of *E. ictaluri* growth on TSA and indicate successful revival and viability of the bacterial isolates prior to downstream molecular analyses. As summarised in Table 4.1, the positive isolates were obtained from *P. hypophthalmus* with body weights ranging from 9g to 161g, representing different growth stages. The isolates were recovered from different organs, with the kidney and spleen accounting for the majority of isolates, followed by the brain, while only a single isolate was obtained from the liver. The recovery of *E. ictaluri* from multiple organs reflects its systemic nature and supports its role as a causative agent of BNP.

Table 4.1: Summary of *Edwardsiella ictaluri* isolates from cage-cultured *Pangasianodon hypophthalmus* in the Pahang River.

No.	Sample IDs	Fish Body weight (g)	District	Sampling month (2023)	Organ of isolation
1	PLS 31B	9	Temerloh	March	Brain
2	PLS 31K	9	Temerloh	March	Kidney
3	PTP 274K	24	Pekan	April	Kidney
4	PKA 69S	161	Pekan	May	Spleen
5	PKA 86S	75	Pekan	May	Spleen
6	PTP 324K	46	Pekan	May	Kidney
7	PTP 324S	46	Pekan	May	Spleen
8	PTP 329K	35	Pekan	May	Kidney
9	PTP 354B	53	Pekan	June	Brain
10	PTP 358B	57	Pekan	June	Brain
11	PKA 110L	100	Pekan	June	Liver

Note: Sample IDs denote sampling location, fish number, and organ of isolation. Location codes are as follows: ***PLS*** *= Kampung Lebak Seberang,* ***PTP*** *= Kampung Tanjong Pulai, and* ***PKA*** *= Kampung Acheh. For example,* ***PLS 31B*** *refers to an isolate obtained from P. hypophthalmus collected at Kampung Lebak Seberang, fish number 31, isolated from the brain.*

4.1.2 DNA Extraction

Genomic DNA from all bacterial isolates was successfully extracted using the G-spin™ Total DNA Extraction Kit (Intron Biotechnology, South Korea). The integrity and quality of the extracted DNA were first evaluated by 1% agarose gel electrophoresis. As shown in Figure 4.2, all isolates exhibited distinct, high-molecular-weight DNA with no observable smearing, indicating minimal degradation and good DNA quality. The presence

of intact genomic DNA without fragmentation suggests that the extraction procedure effectively preserved DNA quality and minimized mechanical and enzymatic degradation.

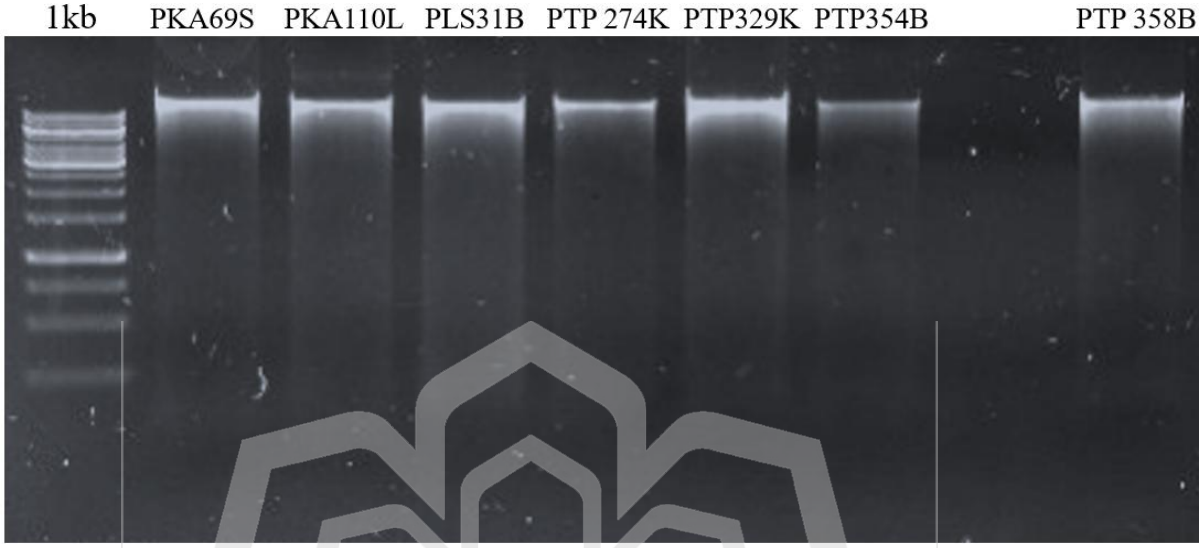


Figure 4.1: Agarose gel electrophoresis (1%) showing genomic DNA of some representative *E. ictaluri* isolates | 1 kb DNA ladder (Thermo Scientific, 1.5 µL).

During DNA extraction and pipetting, excessive physical handling can introduce shear forces that cause breakage of double-stranded DNA molecules (Gautam, 2022). Therefore, extraction protocols that limit vigorous mixing, repeated pipetting, or harsh mechanical disruption are more likely to preserve longer DNA fragments (Oosting et al., 2020). In addition to mechanical stress, enzymatic degradation represents another major factor affecting DNA integrity. DNA degradation can occur rapidly after sample collection due to the activity of endogenous nucleases, particularly endonucleases and exonucleases, which cleave DNA within cells. This process may begin within minutes to hours after sampling and can continue even during storage (Graham et al., 2015; Guo et al., 2018; Oosting et al., 2020). Since enzymatic activity is temperature-dependent, lower temperatures significantly slow down nuclease activity and reduce DNA degradation (David et al., 2022). Thus, maintaining samples at low temperatures during handling and extraction is essential to preserve high-quality genomic DNA. The successful use of the G-spin™ Total DNA Extraction Kit (Intron Biotechnology, South Korea) in this study is

consistent with previous reports demonstrating its effectiveness for extracting genomic DNA from *E. ictaluri* (Mohd Salim et al., 2024).

In addition to gel electrophoresis, DNA concentration and purity were evaluated using NanoDrop spectrophotometry. The DNA concentration of the isolates ranged from approximately 85 to 210 ng/uL, which is sufficient for downstream molecular applications such as PCR and sequencing (Maghini et al., 2021). The A260/A280 ratios ranged between 1.80 and 1.92, while the A260/A230 ratios were between 2.00 and 2.25. An A260/A280 ratio of ~1.8 is generally accepted as indicative of pure DNA, reflecting minimal protein contamination (Koetsier & Cantor, 2019; Nafe, 2025). Similarly, A260/A230 ratios within the range of 2.0–2.2 suggest low levels of organic compounds, phenol, salts, or other extraction-related contaminants (Maghini et al., 2021). The obtained purity values indicate that the extracted genomic DNA was free from significant protein, RNA, or chemical contamination. Collectively, the absence of such deviations in the present study confirms the efficiency of the extraction protocol and the suitability of the DNA for downstream molecular analyses.

4.1.3 Polymerase Chain Reaction (PCR) Amplification Using Species-Specific Primers

Using a set of species-specific primers, all bacterial isolates yielded a distinct band at approximately 2,000 base pairs (Figure 4.3). The presence of a single, clear amplicon of the expected size indicates successful and specific amplification of the target gene (Hadi et al., 2026). Species-specific primers are short nucleotide sequences designed to selectively amplify a DNA region unique to a particular bacterial species and avoid cross-reaction with non-target DNA (Yaman, 2025). This design ensures high specificity, minimizing cross-

reactivity with closely related bacterial species and reducing the likelihood of false-positive amplification (Nhinh et al., 2022).

Figure 4.2: Agarose gel electrophoresis (1%) of PCR amplification products obtained using species-specific primers (IVS and IRS) | 1 kb DNA ladder (Thermo Scientific, 1.5 µL).

In this study, the primers targeted the 16S–23S rRNA intergenic spacer (ITS) region (refer Table 3.2). The ITS region is widely used in bacterial identification because it exhibits greater sequence variability than the highly conserved 16S rRNA gene (Zhang et al., 2024). This increased variability makes it particularly useful for differentiating closely related species within the genus *Edwardsiella*, particularly *E. ictaluri* and *Edwardsiella tarda*. Although the ITS sequences of *E. ictaluri* and *E. tarda* are highly similar, previous studies have demonstrated that subtle sequence differences and variations in restriction sites within this region enable differentiation between the two species (Panangala et al., 2005; Machimbirike et al., 2022). It contains conserved flanking regions that allow stable primer binding, ensuring consistent amplification across isolates (Iwen et al., 2002; Panangala et

al., 2005). Therefore, the ITS region provides a suitable balance between conservation and variability, enhancing the diagnostic specificity of PCR-based identification. The clear 2,000 bp amplicon obtained from all isolates in this study further confirms that the primers successfully targeted a species-specific of *E. ictaluri* (Williams and Lawrence, 2010). Thus, based on the band intensity and consistency, four isolates exhibiting strong and well-defined amplicons were selected for further amplification using universal primers to enable subsequent sequencing and phylogenetic analysis.

4.1.4 Polymerase Chain Reaction (PCR) Amplification using Universal Primers

PCR amplification with universal primers 27F and 1492R (refer Table 3.4), targeting the 16S rRNA gene, resulted in a single, clean amplicon of approximately 1,500 bp for all four selected isolates, indicating successful and specific amplification (Figure 4.4). The absence of non-specific bands or smearing suggests that the genomic DNA was of high purity and free from significant contaminants that could inhibit Taq polymerase activity or interfere with amplification efficiency (Yar'adua & Yusuf, 2024; Anwari et al., 2024). Universal primers differ from species-specific primers in both design and application. Species-specific primers are developed to selectively amplify DNA regions unique to *E. ictaluri*, enabling rapid and targeted pathogen confirmation (Williams and Lawrence, 2010; Machimbirike et al., 2022). In contrast, universal primers anneal to conserved regions of the bacterial 16S rRNA gene and amplify nearly the full-length ~1,500 bp sequence. Because the 16S rRNA gene is universally present in bacteria and contains both conserved and hypervariable regions, it serves as a reliable molecular marker for taxonomic identification and phylogenetic analysis (Yang et al., 2016; Bertolo et al., 2024).

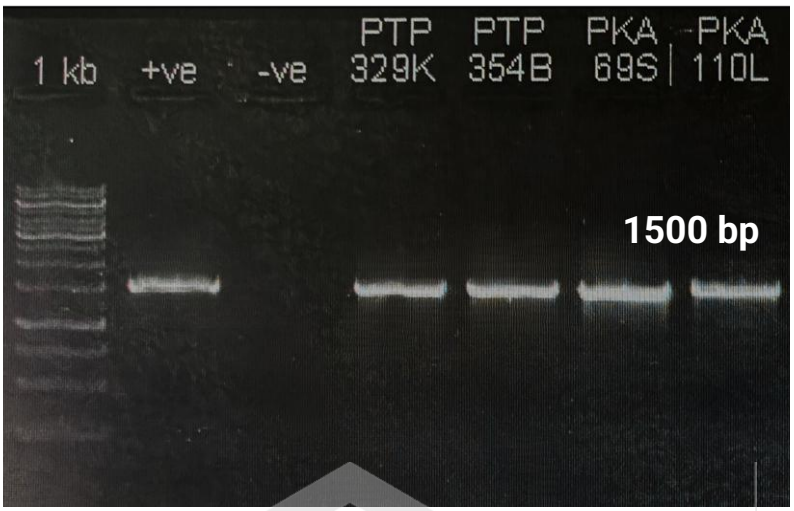


Figure 4.3: Agarose gel electrophoresis (1%) of PCR amplification products obtained using universal primers (27F and 1492R) | 1 kb DNA ladder (Thermo Scientific, 1.5 µL).

Successful amplification of a single ~1,500 bp product indicates that the DNA template originated from a single dominant bacterial species rather than mixed microbial populations (Sunthornthummas et al., 2025). The presence of a single, distinct band without additional amplicons further confirms the molecular purity of the isolates. Therefore, amplification of the 16S rRNA gene represents a critical validation step prior to Sanger sequencing, as it enables sequence comparison with reference databases for definitive species confirmation while simultaneously verifying the absence of contaminating bacterial DNA (Al-Mozan, 2023; Silva et al., 2023).

The fundamental components of the PCR cocktail were identical for both species-specific and universal primers amplifications. Both reactions contained PCR buffer, $MgCl_2$, dNTPs, primers, Taq DNA polymerase, template DNA, and nuclease-free water (Mohd Salim et al., 2025). By maintaining an identical reagent profile, the reaction chemistry was kept constant, allowing for a direct methodological comparison between the two amplification systems. While the chemistry remain unchanged, minor adjustments to the cycling conditions were implemented to accommodate the unique thermodynamic properties of each primer set. The universal primer assay employed a slightly longer initial

denaturation step and a lower annealing temperature of 48°C, compared to the 50°C species-specific primers (Refer Sections 3.1.3 and 3.1.4).

These modifications reflect differences in primer melting temperature (T_m) and the requirement for stable binding across more diverse template regions. The extended denaturation step likely enhanced the accessibility of conserved binding sites within the genomic DNA, thereby facilitating more efficient amplification of the 16S rRNA region (Leese et al., 2021). Interestingly, despite the ITS-targeted product (2,000 bp) was longer than the 16S amplicon (1,500 bp), a uniform extension time of one minute at 72°C proved effective for both. This is consistent with the known processivity of Taq DNA polymerase, which typically synthesizes approximately 1 kb of DNA per minute under optimal conditions (Asif et al., 2021). The fact that only minimal optimization of cycling parameters was required, while the cocktail composition remained untouched, confirm that amplification differences were primarily driven by primer specificity and target gene characteristics rather than alterations in reaction chemistry.

Among these four isolates, PTP 354B (hereafter referred to as PTP_1) was selected for Sanger sequencing because it was isolated from brain tissue. Infections involving brain tissue indicate systemic dissemination and invasive progression of edwardsiellosis (Armwood et al., 2022; De Rose et al., 2026). *E. ictaluri* the causative agent of bacillary necrosis of Pangasius (BNP) and enteric septicemia of catfish (ESC) , is known to cause septicaemia and can invade multiple internal organs, including the kidney, liver, spleen, and brain (Miyazaki & Plumb, 1985; Machimbirike et al., 2022). Recovery of the isolate from brain tissue therefore suggests an advanced stage of systemic infection and potentially higher virulence. Consequently, PTP_1 represents a clinically relevant isolate suitable for molecular confirmation and further genomic characterization.

4.2 WHOLE GENOME SEQUENCING

4.2.1 Nanopore Sequencing

The raw whole-genome sequencing (WGS) data yielded a total of 82,796 reads, resulting in 623,704,324 bases, with an average read length of 7,533 bp (Table 4.2). This relatively long average read length is indicative of long-read sequencing technology and is advantageous for genome assembly. The N50 read length of 10,726 bp further supports the presence of high-quality long reads, indicating that at least 50% of the total bases are contained in reads of this length or longer (Thrash et al., 2020). This raw sequencing output is consistent with previous WGS studies of *E. ictaluri*, which report approximately ~6.0–7.0 Mb genome size (Jin et al., 2022).

Table 4.2: Sequencing statistics

Sample	Number of reads	Total bases	Average length	N50	GC (%)
PTP_1	82,796	623,704,324	7,533	10,726	57.04

4.2.2 Genome Assembly and Quality Check

The genomic assembly of PTP_1 revealed two contigs, consisting of a circular chromosome and a plasmid, with a total genome size of 3,854,526 bp (Table 4.3). This genome size falls within the reported range for *E. ictaluri*, which typically varies between approximately 3.6–4.0 Mb among previously published strains and generally comprises a single chromosome accompanied by one or more plasmids carrying diverse set of ARGs and virulence genes (Payne et al., 2025). The slightly larger genome size observed in PTP_1 may be attributed to strain-specific genomic variation, including the presence of accessory genes, or plasmid-associated sequences (Lemieux et al., 2023). Genome size variation among isolates is

common and may reflect adaptation to environmental pressures, host specificity, and the acquisition of mobile genetic elements (Saini et al., 2024).

Table 4.3: Genomic statistics

Sample	Number of contig	Total bases	N50	GC (%)
PTP_1	2	3854526	3761778	57.39

The GC content of PTP_1 (57.39%) is highly consistent with previously reported *E. ictaluri* genomes, which generally exhibit GC values around 57.0 and 57.7% (Machimbirike et al., 2022; Payne et al., 2025). The GC content represents the proportion of guanine and cytosine nucleotides in the genome (Chen et al., 2024). The close similarity to reference strains supports the taxonomic identity of PTP_1 and suggests the absence of major contamination or large-scale genomic rearrangements. The N50 value of 3,761,778, represents approximately 97% of the genome, suggesting that most of the sequence is contained within a single dominant contig. Such high contiguity reflects a near-complete assembly with minimal fragmentation (Thrash et al., 2020). Furthermore, BUSCO analysis yielded a completeness score of 100%, indicating that all expected single-copy orthologous genes were successfully recovered (Figure 4.5). This confirms that the assembly is highly complete and contains the essential conserved gene set required for a high-quality bacterial genome (Sarfraz et al., 2024; Farooq & Rafique, 2025). Comparatively, the complete genome sequence of *E. ictaluri* strain 93-146 from the United States is 3,812,315 bp in length, comprising 3,783 predicted protein-coding genes, with an average GC content of 57.4% (Williams et al., 2012). Similarly, isolates recovered from diseased *P. hypophthalmus* in the Mekong Delta, Vietnam, sequenced using Oxford Nanopore MinION technology, exhibit genome sizes ranging from 3.55 to 3.75 Mbp with GC contents between 57.0 and 57.7% (Erickson et al., 2024). Together, these comparisons further support that

the genomic features of PTP_1 are consistent with previously characterized *E. ictaluri* strains while highlighting minor variations that may reflect strain-level diversity.

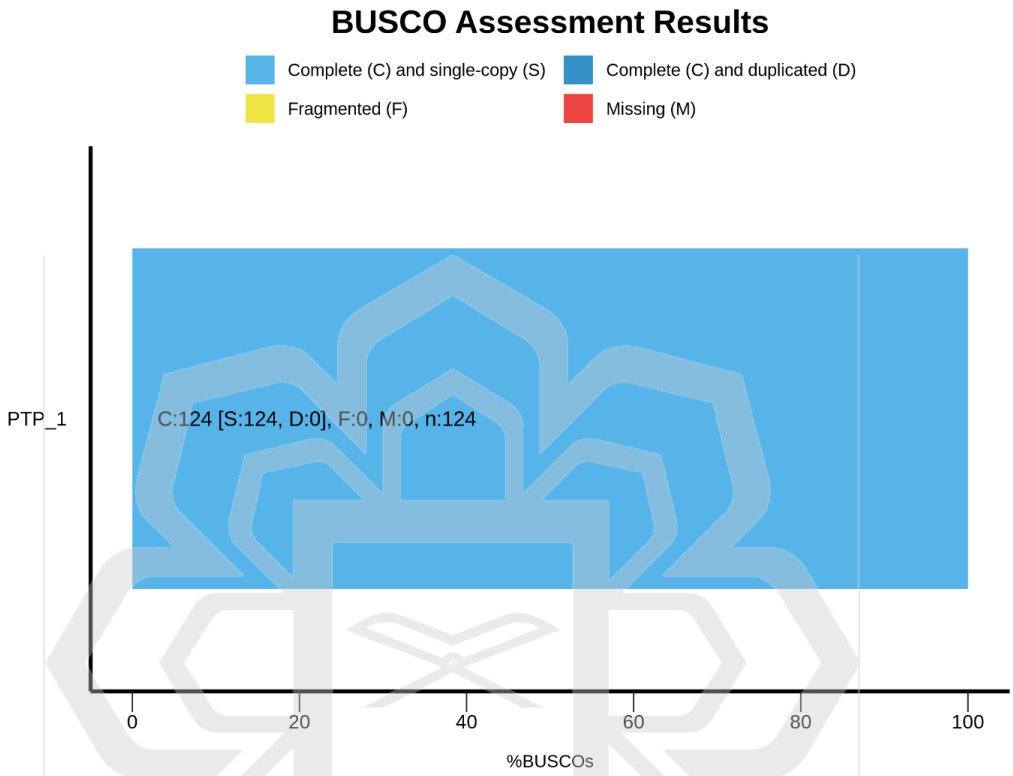


Figure 4.5: BUSCO evaluation of genome completeness based on the bacteria odb10 protein markers

The detection of a plasmid suggests the presence of extrachromosomal genetic material that may carry accessory genes, including those associated with virulence, antimicrobial resistance, or environmental adaptation (Rodriguez-Beltran et al., 2021; Harris et al., 2023). Plasmid presence varies among *E. ictaluri* isolates, therefore, differences in plasmid content may contribute to strain-specific pathogenicity or ecological fitness (Benz & Hall, 2023). If the plasmid content differs from previously reported isolates, this could indicate recent acquisition events or local evolutionary adaptation. Overall, the genome size, GC content, and completeness metrics of PTP_1 are comparable to previously published *E. ictaluri* strains, while the presence of a plasmid may represent strain-specific

genetic features with potential biological significance. The high assembly quality makes this genome highly suitable for downstream comparative genomics and functional analyses.

Table 4.4: Top genomic matches from the GTDB R220 database

Accession_Number	Species Name	% ANI	% coverage
RS_GCF_000264785.1	*Edwardsiella_ictaluri*	99.69	95.10
RS_GCF_008801815.1	*Edwardsiella_anguillarum*	92.99	63.47
RS_GCF_001896205.1	*Edwardsiella_piscicida*	92.24	69.28
RS_GCF_003113495.2	*Edwardsiella_tarda*	84.05	22.88
RS_GCF_000474215.1	*Edwardsiella_hoshinae*	82.85	16.61

Table 4.4 presents the top genomic matches of the PTP_1 strains based on comparison with reference genomes in the Genome Taxonomy Database (GTDB) R220, a curated framework designed to standardize bacterial taxonomy based on whole-genome phylogeny (Parks et al., 2020). Only genomes with >50% alignment coverage and ≥80% ANI were retained to ensure robust and meaningful taxonomic inference. Average Nucleotide Identity (ANI) has become the gold standard for bacterial species delineation, replacing traditional DNA–DNA hybridization methods (Richter & Rosselló-Móra, 2009; Hu et al., 2022). PTP_1 demonstrated the highest similarity to *E. ictaluri*, with an ANI of 99.69% and 95.10% genomic coverage (Table 4.4). This value is substantially above the species-level cutoff, providing strong genomic confirmation that PTP_1 belongs to *E. ictaluri*. ANI values above 99% typically indicate extremely close relatedness, often reflecting strains within the same clonal lineage or recent evolutionary divergence (Rodriguez-R et al., 2024).

Other members of the genus, including *Edwardsiella anguillarum*, *Edwardsiella piscicida*, *Edwardsiella tarda*, and *Edwardsiella hoshinae*, exhibited lower ANI values and reduced coverage, falling below the accepted species threshold. This clear genomic separation is consistent with previous whole-genome studies demonstrating that species within the genus *Edwardsiella* are genetically distinct despite phenotypic similarities (Abayneh et al., 2013; Divya et al., 2021). The sharp ANI drop between *E. ictaluri* and

other *Edwardsiella spp*. reinforces that PTP_1 is not a borderline or transitional strain. High genomic coverage (95.10%) indicates that the majority of the PTP_1 genome aligns with the reference *E. ictaluri* genome. Lower coverage values in other species suggest greater genomic divergence and fewer homologous regions. Coverage, together with ANI, strengthens taxonomic confidence by demonstrating both sequence identity and genome-wide similarity (Jain et al., 2018). Overall, the exceptionally high ANI (99.69%) and strong genomic coverage (95.10%) conclusively confirm that PTP_1 belongs to *Edwardsiella ictaluri*. The clear separation from other *Edwardsiella* species demonstrates precise taxonomic resolution and validates the reliability of the whole-genome dataset for downstream comparative and evolutionary analyses.

4.2.3 Analysis of Genome Assembly and Antimicrobial Resistance (AMR)

There is a total of 3746 genes were identified, comprising 3,620 protein-coding genes and 126 RNA genes. This gene count is consistent with previously published *E. ictaluri* genomes, which typically range between approximately 3,235–3,641 predicted coding sequences within genome sizes of ~3.6–3.9 Mb (Machimbirike et al., 2022). A coding capacity of ~3,600 genes is characteristic of facultative pathogenic Gram-negative bacteria, reflecting sufficient metabolic flexibility for survival in both aquatic environments and host tissues (Ramli et al., 2021). If the gene count were substantially higher, this could indicate acquisition of accessory genes through horizontal gene transfer, often associated with increased adaptability or virulence potential (Arnold et al., 2022). Conversely, significantly fewer genes might suggest genome streamlining or host-restricted adaptation, which is not evident in PTP_1 (Segers et al., 2017; Gossett et al., 2023). Similarly, he presence of 126 RNA genes, including rRNA and tRNA operons, further supports genome completeness and functional stability. Adequate representation of RNA genes is essential for efficient protein synthesis and cellular regulation, particularly in rapidly proliferating pathogens (Cui et al., 2022).

Functional classification using the Clusters of Orthologous Genes (COG) database revealed that Category E (amino acid transport and metabolism), Category J (translation, ribosomal structure and biogenesis), and Category X (Mobilome: prophages, transposons) were the most abundant. High representation of Category J is typical of bacterial genomes, as ribosomal proteins and translation-related genes are essential housekeeping components required for cellular growth (Jiao et al., 2023; Kiraz & Ozcan, 2025). Enrichment in Category E reflects metabolic adaptability, enabling the pathogen to utilise diverse amino acid sources during infection and environmental persistence (Zhang et al., 2025). Notably, the substantial presence of Category X genes suggests the occurrence of mobile genetic elements. Prophages and transposons contribute to genomic plasticity and may facilitate horizontal gene transfer, promoting virulence evolution and antimicrobial resistance development (Das et al., 2022). Similar mobilome enrichment has been reported in comparative genomic analyses of *Edwardsiella* species, highlighting the role of mobile elements in strain diversification (Erickson et al., 2024).

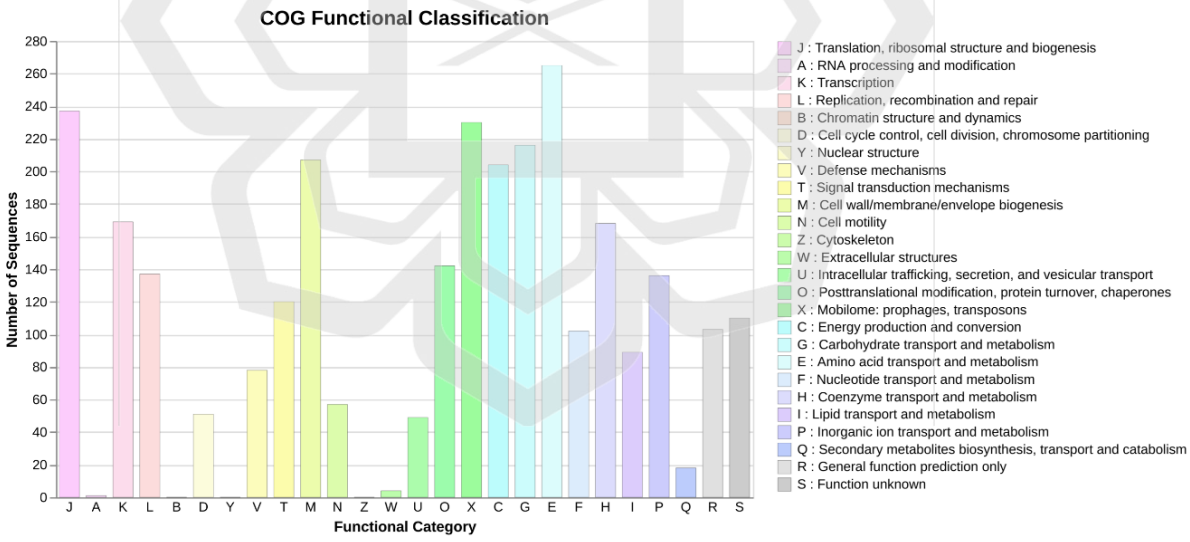


Figure 4.5: Distribution of genes categorized by Cluster of Orthologous Groups (COG) classification.

Antimicrobial resistance (AMR) prediction for the PTP_1 strain was conducted based on the Comprehensive Antibiotic Resistance Database (CARD) identified the presence of the cAMP receptor protein (CRP) gene with 99.68% sequence identity (Table 4.5) (Darji & Joshi, 2025). CRP is a highly conserved global transcriptional regulator widely distributed among diverse group of bacteria (Papenfort & Melamed, 2023). CRP functions as a cAMP-dependent transcription factor that regulates genes involved in carbon metabolism, stress response, membrane transport, and also virulence (Krol et al., 2023; Haque et al., 2025). Beyond metabolic regulation, CRP has been implicated in modulating antimicrobial susceptibility through control of efflux pump systems, outer membrane proteins, and global stress-adaptation pathways (Dawan & Ahn, 2022; Liu et al., 2025). Alterations in CRP activity have been shown to influence resistance phenotypes toward fluoroquinolones, β-lactams (including penams), and macrolides indirectly by affecting gene expression networks associated with membrane permeability and multidrug efflux systems (Zahari et al., 2023).

Table 4.5: Identification of Antibiotic Resistance Genes and Virulence Factors

Gene coordinate	AMR Gene	%ID	Database	Antibiotic
contig_001-chr:1654923-1655553(-)	CRP	99.68	card	Fluoroquinolone; macrolide; penam

CRP is widely recognized for modulating diverse metabolic and physiological pathways, enabling bacteria to respond efficiently to fluctuating environmental conditions (Krol et al., 2023). Recent findings by Zhao et al. (2024) reported that CRP functions through a coordinated dual regulatory mechanism alongside the bacterial alarmone guanosine tetra- and penta- phosphate, collectively known as (p)ppGpp. This interaction

allows bacteria to adjust metabolic activity and activate stress-response systems during unfavorable conditions, such as nutrient limitation or environmental stress. Stress-adapted cells can exhibit reduced antibiotic susceptibility due to slowed metabolism, altered membrane permeability, and enhanced efflux activity (Rojas-Andrade & Hochbaum, 2025). The presence of CRP in PTP_1 is particularly relevant in the context of aquaculture practices. Some antimicrobials are applied in aquaculture systems through medicated feed or direct addition into water for disease prevention and treatment. While this approach can effectively control bacterial outbreaks, excessive or inappropriate antibiotic use increases the risk of AMR development in aquatic bacteria, primarily through the selection and dissemination of antimicrobial resistance genes (ARGs) (Singh et al., 2022). Consequently, antimicrobial-resistant bacteria and their associated genes may accumulate in the aquatic environment, contributing to biological contamination (Grenni, 2022).

Fluoroquinolones, particularly enrofloxacin, are widely used in the global aquaculture industry due to their broad spectrum activity and effectiveness against both Gram-positive and Gram-negative bacterial pathogens (Afroze et al., 2025). However, their use has been restricted or banned in several regions since 2012 due to concerns regarding antimicrobial resistance development and environmental persistence (Dao, 2022). The detection of fluoroquinolone-associated resistance determinants in the PTP_1 strain despite such regulatory measures, suggests the long-term persistence of resistance-associated genes in bacterial populations, potentially driven by historical antibiotic exposure or co-selection pressure from other antimicrobial agents. In addition, the association of CRP with resistance to macrolide and penam antibiotics indicates a broader multidrug resistance regulatory capability. This finding highlights the adaptive potential of *E. ictaluri* to survive under antimicrobial stress conditions, posing a challenge to disease management strategies in aquaculture systems. Comparable observations have been reported in previous studies. For instance, *E. ictaluri* isolates from the Mekong River, Vietnam were generally susceptible to several antibiotics' classes, including florfenicol, penicillins, quinolones, fluoroquinolones, aminoglycosides, and tetracyclines, but exhibited resistance to macrolides (Machimbirike et al., 2022).

4.2.4 Complete Genome Map and Structural Characteristics of *Edwardsiella ictaluri* PTP_1

The complete circular genome map of *E. ictaluri* PTP_1 (Figure 4.7), provides a comprehensive overview of its genomic architecture and annotated genomic features. Protein-coding sequences (CDSs) are distributed along both forward and reverse strands, which is typical of bacterial circular chromosomes. This bidirectional distribution reflects the organization of genes relative to the origin and terminus of replication, ensuring efficient transcription and replication processes (Saponaro, 2022; Okoń, 2024). Similar strand distribution patterns have been reported in previously published *E. ictaluri* reference genomes, indicating that PTP_1 exhibits conserved chromosomal organization without major structural rearrangements (Erickson et al., 2024).

Functional classification based on COG categories demonstrates that CDSs are predominantly involved in metabolism, cellular processes and signalling, information storage and processing, as well as mobilome-related functions (Das et al., 2022). The dominance of metabolism-related genes is consistent with the ecological adaptability of *E. ictaluri*, which must survive in aquatic environments and within host tissues (Dong & Li, 2025). Genes associated with amino acid transport and metabolism, carbohydrate metabolism, and energy production are essential for nutrient acquisition in host-associated niches (Zhang et al., 2025). Comparable functional distributions have been observed in other *E. ictaluri* strains, where metabolic versatility supports both environmental persistence and pathogenicity (Machimbirike et al., 2022).

Genes classified under cellular processes and signalling include those related to cell wall/membrane biogenesis, motility, secretion systems and stress response. In *E. ictaluri*, these functions are particularly important for host colonisation, immune evasion and intracellular survival in fish macrophages (Dumpala, 2010; Nguyen et al., 2025). Information storage and processing genes such as those involved in transcription, translation and DNA replication, represent conserved housekeeping components necessary for bacterial viability (Joshi et al., 2022). The proportional distribution of these functional categories in PTP_1 appears consistent with previously characterised *E. ictaluri* genomes,

suggesting no major functional gene loss or expansion (Payne et al., 2025). The genome map also highlights non-coding RNA regions, including rRNA and tRNA genes, which are essential for protein synthesis and translational efficiency (George et al., 2024). The number and distribution of these RNA genes are comparable to other *E. ictaluri* strains, reflecting evolutionary conservation of core translational machinery (Lu et al., 2025). Variations in rRNA operon copy number in bacteria can influence growth rate and adaptation capacity, thus, their conserved arrangement further supports genomic stability in PTP_1.

The inner rings representing GC content and GC skew provide additional insights into nucleotide composition and replication dynamics. The overall GC content of *E. ictaluri* is approximately 57–59%, which is consistent with previously reported genomes of this species (Payne et al., 2025). This moderate GC composition indicates genomic stability and balanced nucleotide usage (Ruden, 2025). Similarly, GC skew analysis allows prediction of the origin (oriC) and terminus (ter) of replication, as shifts in GC skew polarity are characteristic of bidirectional replication in bacteria (Hubert, 2022). The presence of a clear GC skew pattern in PTP_1 indicates intact replication architecture without major chromosomal rearrangements.

Importantly, the absence of gaps or fragmented regions confirms that PTP_1 was assembled into a complete circular chromosome, demonstrating high assembly quality and structural integrity. Complete circular assemblies are critical for accurate genome annotation, comparative genomics and evolutionary analysis, as fragmented assemblies may obscure structural variations or misrepresent gene organisation (Minhas & Iqbal, 2025). Overall, the genome map not only presents structural features but also confirms that PTP_1 share conserved genomic architecture, functional composition and nucleotide characteristics with other *E. ictaluri* strains, while providing a reliable foundation for downstream comparative and evolutionary investigations.

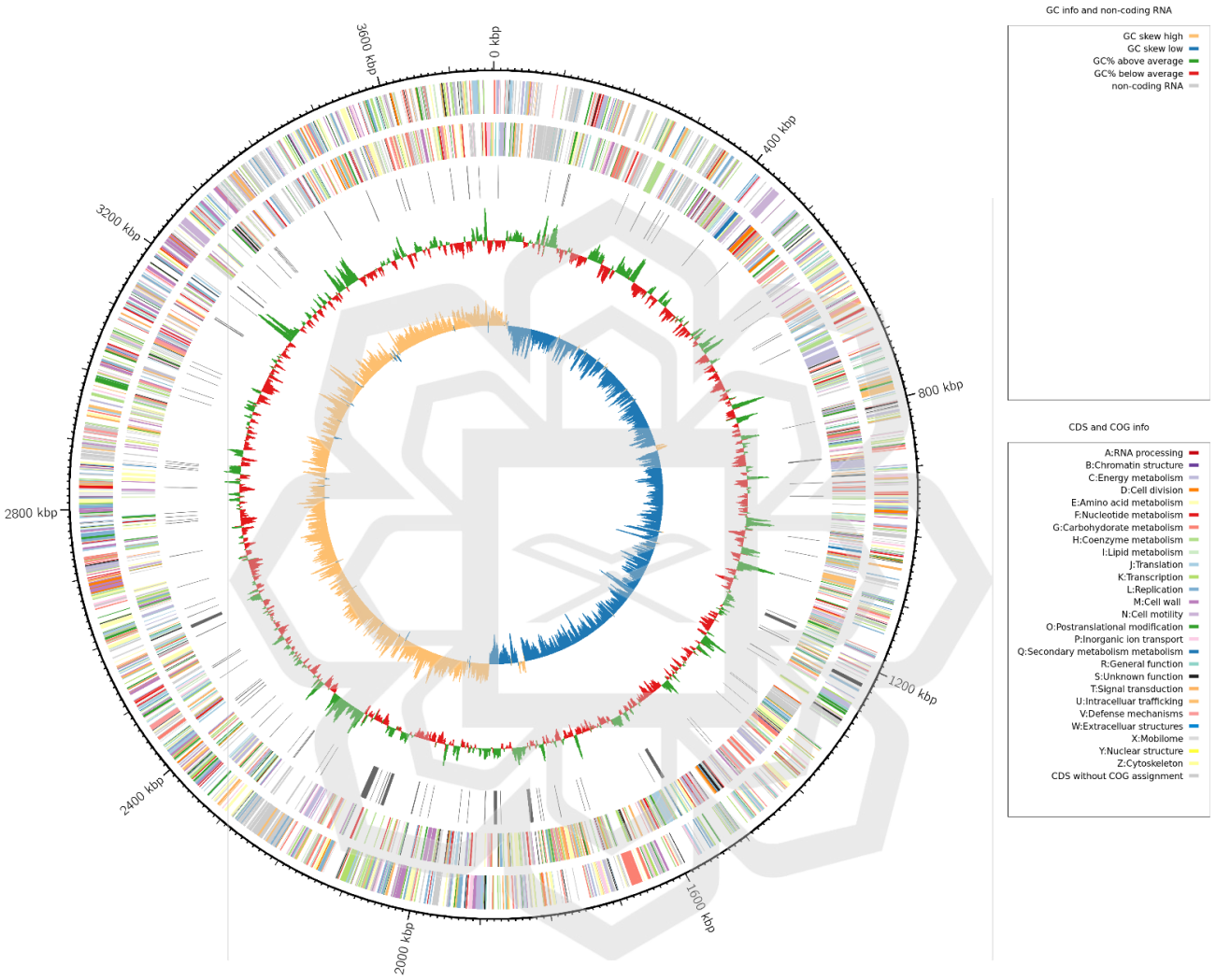


Figure 4.6: The circular genome map of *E. ictaluri* PTP_1

4.2.5 Phylogenomic Analysis of *Edwardsiella ictaluri* PTP_1

4.2.5.1 High-resolution Taxonomic Classification and inter-species Phylogenomic Analysis of Edwardsiella spp.

A species-level phylogenomic analysis was performed to confirm the taxonomic placement of the isolates within the genus *Edwardsiella* and to discriminate among closely related species, including *E. ictaluri*, *E. tarda, E. hoshinae, E. piscicida* and *E. anguillarum*. The resulting phylogenomic tree (Figure 4.8) clearly demonstrated that PTP_1 clustered together with reference strains of *E. ictaluri*, confirming its taxonomic identity at the species level. In phylogenomic analysis, clustering on the same branch indicates high whole-genome similarity and shared evolutionary ancestry (Shakya et al., 2020). When isolates group on the same terminal branch, it means they are genetically very closely related and likely share a recent common ancestor. However, if they share a larger branch, this indicates they still belong to the same species but may represent different strains or sub-lineages (Wailan et al., 2019).

The strong bootstrap support values observed at the nodes separating PTP_1 and other *E. ictaluri* strains further reinforce the robustness of this classification. Bootstrap analysis is a statistical resampling method used to assess the reliability of phylogenetic tree branches. In simple terms, the dataset is resampled hundreds or thousands of times, and the tree is reconstructed repeatedly (Challa & Neelapu, 2019; Simon, 2022). The bootstrap value represents how often a particular branch appears across these replicates. Values above 70% are generally considered reliable, while values exceeding 90–95% indicate very strong statistical confidence (Mokhtar et al., 2023). Therefore, the high bootstrap values of 100 observed in this study indicate that the clustering of PTP_1 with *E. ictaluri* is not random or artefactual but is strongly supported by genome-wide data.

Importantly, PTP_1 was clearly separated from *E. tarda, E. hoshinae, E. piscicida* and *E. anguillarum*, which formed distinct and independent clades with *E. ictaluri*. Similar results can be observed in the previous study (da Costa et al., 2022). Although these species belong to the same genus and share certain phenotypic similarities, whole-genome phylogenomics allows higher resolution discrimination compared to single-gene approaches like 16S rRNA (Paul, 2023). The clear branching separation indicates substantial genomic divergence between species, supporting their taxonomic distinction. Overall, the phylogenomic tree not only

confirms the taxonomic identity of PTP_1 as *E. ictaluri* but also demonstrates clear genomic boundaries between *Edwardsiella* species. The strong bootstrap support, distinct clade separation, and branch length patterns collectively validate the reliability of the classification and highlight the evolutionary relationships within the genus.

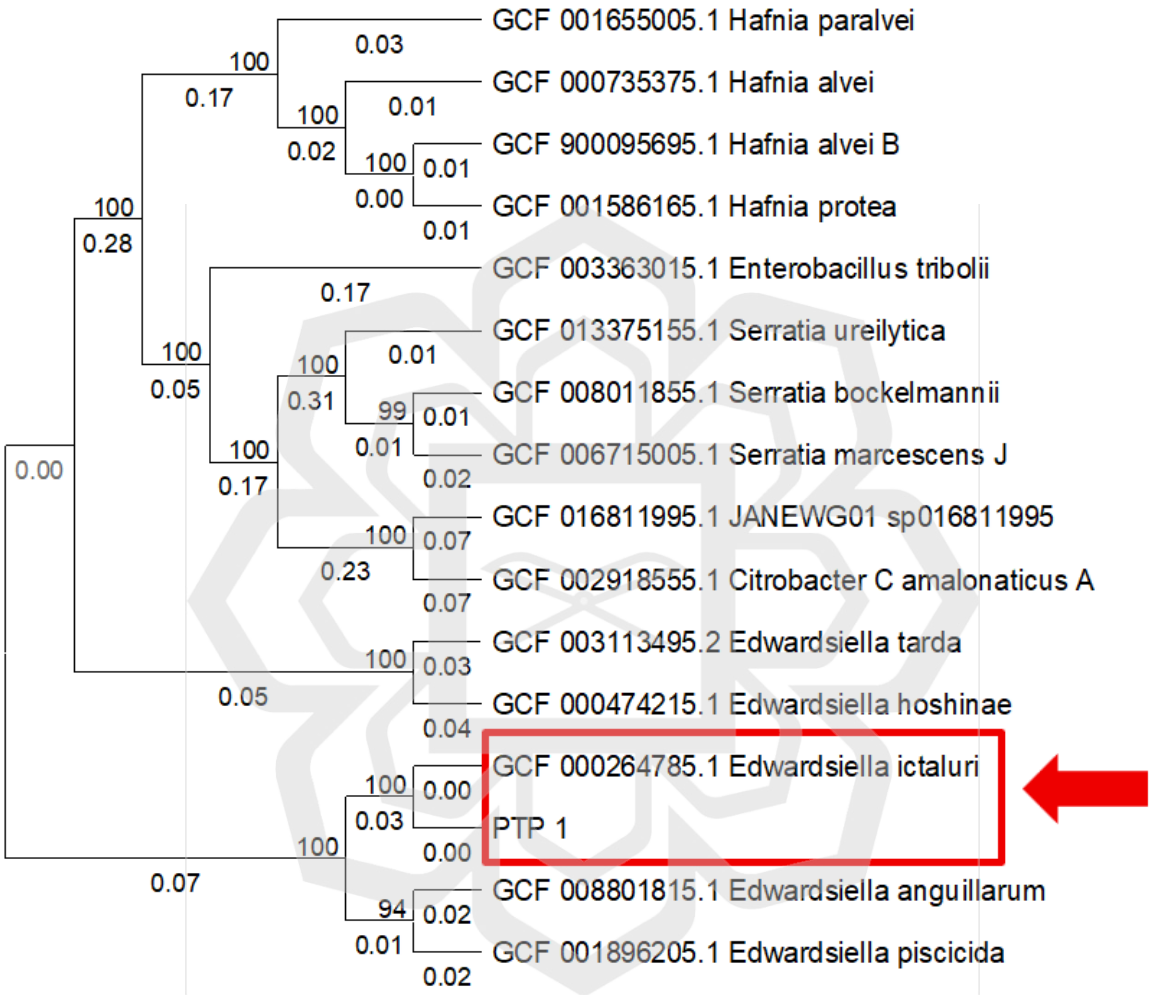


Figure 4.7: Phylogenomic tree of *E. ictaluri* PTP_1 among *Edwardsiella spp.*

4.2.5.2 Intra-species Phylogenomic Analysis of Edwardsiella ictaluri strains

Next, to localise the PTP_1 lineage within the broader *E. ictaluri population*, a comprehensive intra-species phylogenomic analysis was performed by analysing PTP_1 alongside 80 additional complete *E. ictaluri* genomes retrieved from the NCBI database (Figure 4.9). The resulting tree topology revealed clear clustering patterns corresponding to geographical origins and previously reported lineages. Notably, strain PTP_1 was positioned between clusters comprising Vietnamese and Chinese isolates, suggesting a close genomic relationship with strains circulating in these regions (Figure 4.10). This intermediate placement may reflect regional dissemination patterns, historical transmission events, or shared evolutionary ancestry within Asian aquaculture systems.

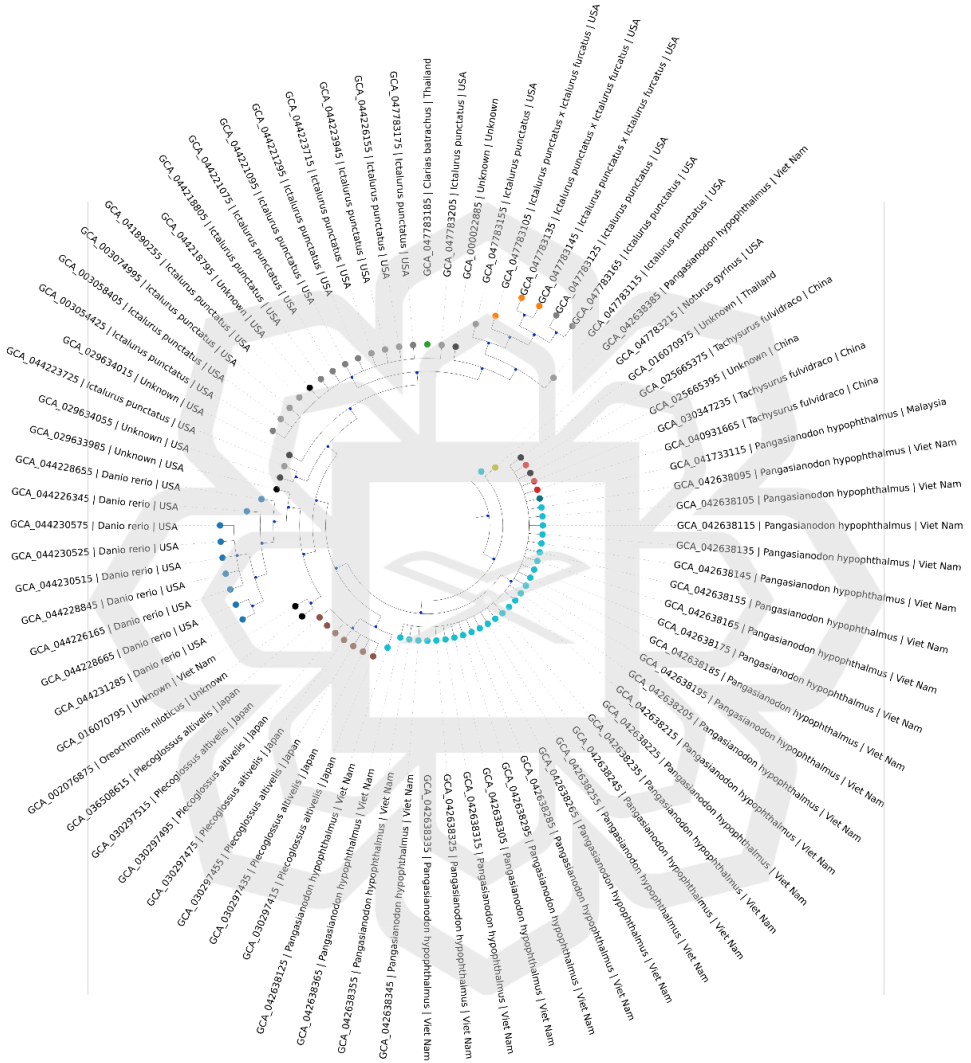


Figure 4.8: Intra-species phylogenomic tree of *E. ictaluri* strains

Figure 4.9: Zoomed-in view of the phylogenomic tree indicating the clustering of PTP_1 (GCA_041733115 | *Pangasianodon hypophthalmus* | Malaysia)

Comparative genomic analysis also revealed that the Malaysian *E. ictaluri* strain is most closely related to strains from China and Vietnam, as evidenced by a large shared core genome comprising 3,246 genes (Figure 4.11). In addition, the Malaysian strain also shared more accessory genes with the Vietnamese strain (55 genes) than with the Chinese strain (21 genes). In contrast, only a limited number of strain-specific genes were identified in each country (Malaysia: 51 genes; China: 41 genes; Vietnam: 29 genes). The strain-specific genes identified in the Malaysian isolate include those encoding outer membrane protein C precursor (OMP), ATP-binding protein, acid shock protein, and hemolysin secretion protein, which may be associated with stress adaptation and virulence. Overall, these findings indicate a high degree of genomic conservation among *E. ictaluri* strains across the regions, with relatively minor genetic variations. This conserved genomic pattern suggests that the isolates may share a common ancestral lineage, with observed differences likely arising from microevolutionary processes driven by local environmental conditions or host adaptation (Fu et al., 2022; Tonkin-Hill et al., 2025).

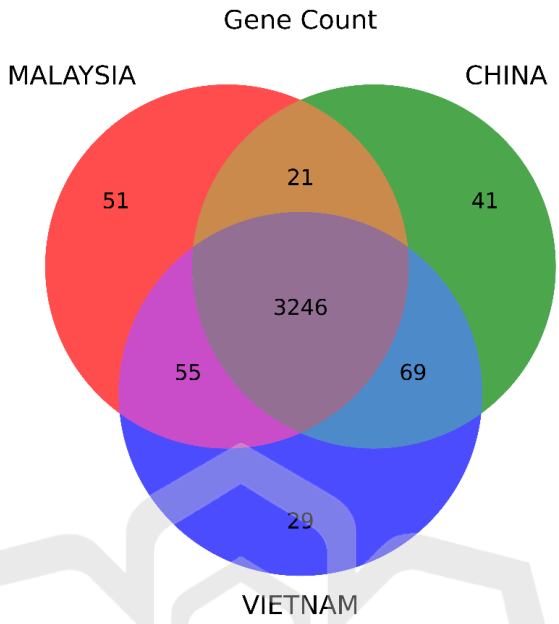


Figure 4.10: Venn diagram showing the gene count of *E. ictaluri* strains from Malaysia (GCA_041733115), China (GCA_040931665), and Vietnam (GCA_042638095).

The close genetic relatedness among Malaysian, Chinese, and Vietnamese strains strongly supports the hypothesis of transboundary movement of *E. ictaluri* within Asian aquaculture networks. Aquaculture trade plays a significant role in the regional dissemination of aquatic pathogens (Avunje, 2021). The import and export of live fish, particularly fingerlings and broodstock, have been identified as the primary pathways for cross-border disease transmission (Mackinnon et al., 2023). The high genomic similarity observed in this study may therefore reflect repeated introductions of closely related strains through commercial fish trade between Malaysia, China, and Vietnam. Furthermore, the limited number of strain-specific genes and the overall high genetic similarity may indicate clonal expansion of a dominant lineage circulating across Asian aquaculture systems. In intensively farmed species, the movement of infected but asymptomatic fish can facilitate silent dissemination of pathogens across borders before disease outbreaks become clinically evident.

Evidence from regional outbreak investigations further reinforces this hypothesis. In Northern Vietnam, among 26 documented disease outbreaks between 2019 and 2021, 19 were traced to imported seeds, while only seven originated from domestic sources (Nhinh et al., 2022). This finding highlights the substantial role of seed trade in disease dissemination. Overall, the genomic evidence presented in this study aligns with epidemiological data indicating that aquaculture trade is a major driver of pathogen dissemination in Southeast and East Asia. The high level of genomic conservation among Malaysian, Chinese, and Vietnamese *E. ictaluri* strains underscores the urgent need for coordinated regional surveillance, stricter movement controls, and improved hatchery certification systems to safeguard the sustainability of the aquaculture industry.

4.2.6 Whole Genome Sequencing Data

Whole-genome sequencing data generated in this study are availale in the NCBI Sequence Read Archive repository under the accession number CP168653.1.

4.3 RANDOM AMPLIFIED POLYMORPHIC DNA – POLYMERASE CHAIN REACTION (RAPD-PCR)

4.3.1 Primers Selection

Figure 4.11: RAPD-PCR amplification profile of *E. ictaluri* sample PTP329K generated using seven arbitrary 10-mer primers | 1kb DNA ladder (Thermo Scientific, 1.5 µL)

Figure 4.12 shows the RAPD-PCR amplification profiles of *E. ictaluri* sample PTP329K generated using seven arbitrary 10-mer primers (OPA1, OPA3, OPA7, OPB10, OPD8, OPF1, and OPR12). These primers were selected based on their documented reproducibility, discriminatory capacity, and prior successful application in bacterial genetic diversity studies. OPA1 and OPA3 are commonly used in RAPD analyses and have demonstrated high reproducibility in bacterial classification studies involving freshwater fish pathogens (Damasco et al., 1996; Nur-Nazifah et al., 2011; Mehraj et al., 2017). OPA7, OPD8 and OPF1 have been similarly been applied in microbial diversity studies and are known to generate clear, polymorphic and well-resolved banding patterns across a broad

fragment size range (Susilo & Setyaningsih, 2018; Hartati & Cahyono, 2023; Wojciechowska-Koszko et al., 2023). OPB10 was included due to its effectiveness in profiling Gram-negative bacteria, consistent with the characteristics of *E. ictaluri*, while OPR12 is recognized for producing distinct larger fragments that enhance isolate discrimination (Hesami et al., 2015). Collectively, these primers provide balanced genome coverage and reliable polymorphism detection.

All primers successfully produced amplification products, confirming their suitability for RAPD analysis in this isolate. The resulting banding patterns varied among the primers in terms of number, size and, intensity of amplified fragments. indicating differences in primer binding frequency and genomic target distribution (Ray et al., 2022; Ihwan & Uslan, 2025). Such variation is expected in RAPD analysis because short arbitrary primers anneal randomly to complementary sequences across the genome under low-stringency conditions (Cao et al., 2022). Amplification occurs when two primer binding sites are present in opposite orientations within an amplifiable distance, generating fragments that represent anonymous genomic regions (Nair, 2023). Therefore, mutations, insertions, deletions or genomic rearrangements that alter primer binding sites or inter-primer spacing can influence the presence or absence of bands, enabling RAPD to detect genome-wide polymorphisms (Pérez-Álvarez et al., 2025).

In this study, all reactions were performed using a uniform annealing temperature of 36°C (Table 3.7). The use of a single annealing temperature is sufficient in RAPD-PCR because all primers are short (10 bases) and designed to function under low-stringency conditions, typically 35–40°C (Al-Yassiry & Al-Alwani, 2022). Once optimized, these conditions allow consistent amplification across different primers. The clear, well-resolved and reproducible bands observed, with fragment sizes spanning a wide molecular weight range relative to the 1 kb DNA ladder, indicate high-quality DNA templates and optimized PCR conditions (Tanny et al., 2025). The presence of multiple polymorphic bands further demonstrates underlying genetic variability and highlights the strong discriminatory potential of the selected primers for polymorphism analysis and phylogenetic assessment.

4.3.2 Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) Band Scoring and Binary Matrix Construction

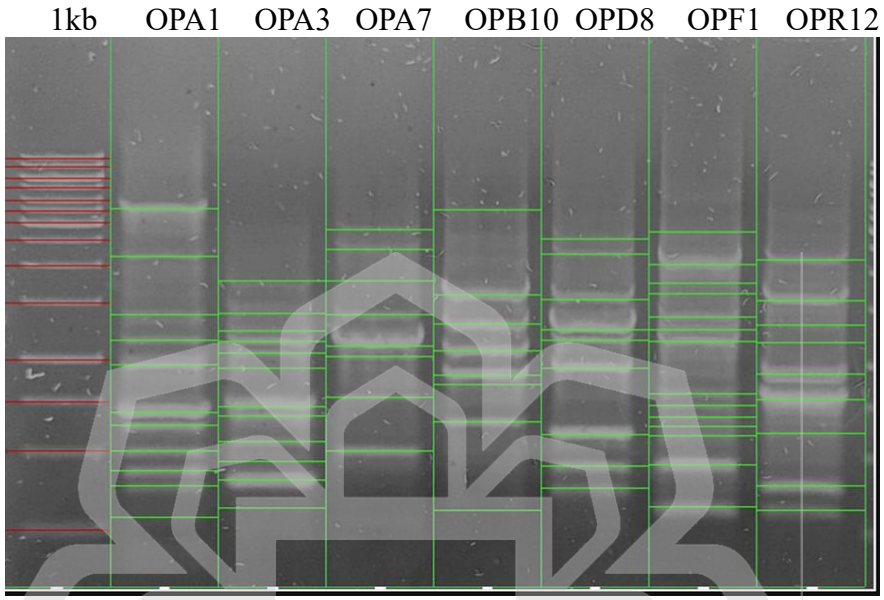


Figure 4.12: Band scoring using UVITEC Cambridge gel documentation system

Based on the presence or absence of amplified fragments, a complete RAPD-PCR fingerprint was obtained for each of the eleven *E. ictaluri* isolates. Only clearly visible and consistently reproducible bands were retained for analysis to ensure reliability, although faint but reproducible bands were also included to capture additional polymorphic variation (Figure 4.13). The combined banding data from all primers were subsequently converted into a binary matrix, in which the presence and absence of bands were scored as "1" and "0", respectively (Figure 4.14). The use of a binary scoring system is standard in RAPD analysis because the technique detects dominant markers rather than codominant alleles (Tingey et al., 1994; Amiteye, 2021). RAPD does not differentiate between homozygous and heterozygous states, instead, it simply records whether a specific DNA fragment is amplified (Bhaskar & Sharon, 2022). Therefore, binary coding provides a straightforward and objective method for transforming gel banding patterns into numerical data suitable for

statistical analysis, such as similarity coefficient calculation, cluster analysis and dendrogram construction.

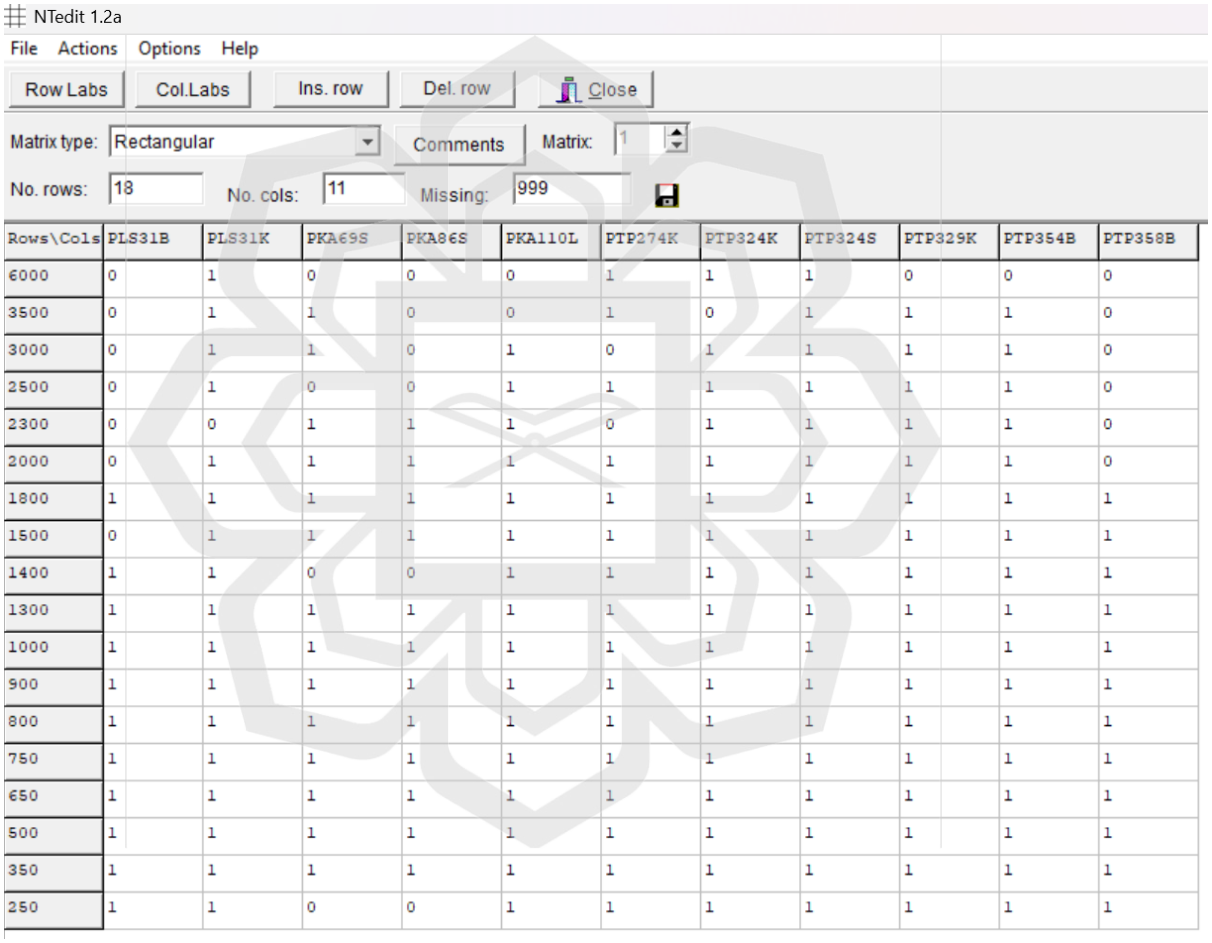
	A	AG	AH	AI	AJ	AK	AL	AM	AN	AO	AP	AQ	AR	AS	AT	AU	AV	AW	AX
1	Size (bp)	PKA110L							PTP274K							PTP324K			
2		OPB10	OPD8	OPF1	OPR12	OPA1	OPA3	OPA7	OPB10	OPD8	OPF1	OPR12	OPA1	OPA3	OPA7	OPB10	OPD8	OPF1	OPR12
3	250	0	0	1	0	1	0	1	0	0	1	1	1	1	1	1	0	0	0
4	350	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	1	0
5	500	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1
6	650	1	1	1	1	1	1	0	1	1	0	1	1	1	0	1	1	1	1
7	750	0	0	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1
8	800	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	1	1	0
9	900	0	0	0	1	0	0	1	1	0	0	1	1	1	0	0	1	1	1
10	1000	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1
11	1300	1	1	1	0	1	1	1	0	1	1	0	1	0	1	1	1	1	0
12	1400	0	0	1	0	0	0	1	1	0	1	0	1	0	1	0	0	0	0
13	1500	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
14	1800	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1
15	2000	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	1	1
16	2300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
17	2500	1	0	1	1	0	0	1	0	0	1	0	0	0	1	0	1	0	1
18	3000	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	0
19	3500	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
20	6000	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1

Figure 4.13: Compilation of binary data matrix

4.3.3 Cluster Analysis and Phylogenetic Tree Construction

Following that, the binary matrix represented the RAPD fingerprint of each isolate was subsequently formatted and entered into NTedit version 1.2a prior to numerical analysis (Figure 4.15). The binary data matrix shows that several band sizes, particularly at 1800, 1300, 1000, 900, 750, 650, 500 and 350 bp, were consistently scored as "1" across all eleven *E. ictaluri* isolates. The uniform presence of these bands indicates that they are monomorphic fragments shared by all isolates (Sahil, 2026). Such conserved bands likely represent stable genomic regions within the species and may correspond to core genetic elements that are highly preserved among *E. ictaluri* strains (Gonzalez-Diaz et al., 2022).

In RAPD analysis, monomorphic bands contribute to overall similarity values but do not provide discriminatory power for differentiating isolates (Hamood & Guda, 2025). Instead, they reflect genetic homogeneity and species-level conservation. The presence of multiple shared bands suggests that the isolates belong to the same species and may share a common evolutionary origin or clonal lineage (Mishra et al., 2026). This finding is consistent with the phylogenomic analysis, which also confirmed their close genetic relationship.



Rows\Cols	PLS31B	PLS31K	PKA69S	PKA86S	PKA110L	PTP274K	PTP324K	PTP324S	PTP329K	PTP354B	PTP358B
6000	0	1	0	0	0	1	1	1	0	0	0
3500	0	1	1	0	0	1	0	1	1	1	0
3000	0	1	1	0	1	0	1	1	1	1	0
2500	0	1	0	0	1	1	1	1	1	1	0
2300	0	0	1	1	1	0	1	1	1	1	0
2000	0	1	1	1	1	1	1	1	1	1	0
1800	1	1	1	1	1	1	1	1	1	1	1
1500	0	1	1	1	1	1	1	1	1	1	1
1400	1	1	0	0	1	1	1	1	1	1	1
1300	1	1	1	1	1	1	1	1	1	1	1
1000	1	1	1	1	1	1	1	1	1	1	1
900	1	1	1	1	1	1	1	1	1	1	1
800	1	1	1	1	1	1	1	1	1	1	1
750	1	1	1	1	1	1	1	1	1	1	1
650	1	1	1	1	1	1	1	1	1	1	1
500	1	1	1	1	1	1	1	1	1	1	1
350	1	1	1	1	1	1	1	1	1	1	1
250	1	1	0	0	1	1	1	1	1	1	1

Figure 4.14: Data entry into NTedit 1.2a

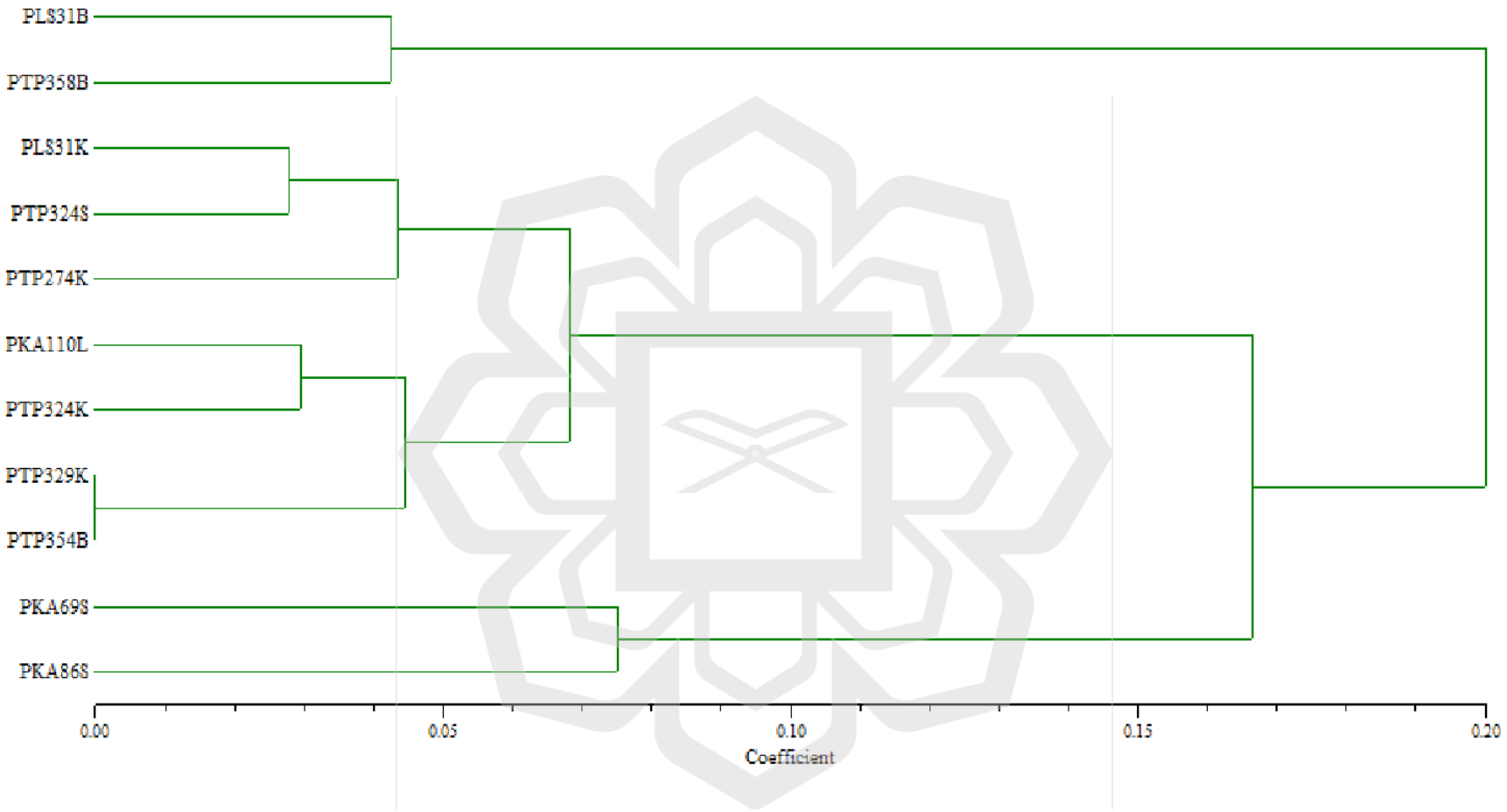


Figure 4.15: Phylogenetic tree shows the degree of relatedness among *E. ictaluri* isolates using primer OPA-1, OPA-3, OPA-7, OPB-10, OPD-8, OPF-1, OPR-12.

The phylogenetic analysis (Figure 4.16) revealed the tightly clustered isolates, indicating high genetic similarity as reflected by low genetic distance coefficients, regardless of their geographical origin (Scarrone et al., 2025). This pattern suggests limited genetic divergence among the isolates, which may be indicative of recent dissemination or a shared evolutionary background. Notably, isolates PTP358B from Pekan and PLS31B from Temerloh clustered very closely, suggesting that they likely belong to the same strain or share a common lineage that has spread across different locations. Similar clustering of genetically related bacterial isolates across geographically distinct aquaculture sites has been reported in Malaysian systems. For example, *Aeromonas dhakensis* was detected across four separate tilapia farms in Malaysia, indicating the presence of a common pathogen population in different production systems (Lim *et al.*, 2025). This occurrence is often associated with anthropogenic activities such as the movement of infected fingerlings, shared water sources, and interconnected river systems that facilitate pathogen transmission (Sakai et al., 2009; Uma et al., 2025). In addition, previous studies have emphasized that inadequate biosecurity measures and limited disease monitoring in aquaculture systems also contribute significantly to the widespread dissemination of pathogenic bacteria (Mzula et al., 2021). Strengthened extension services and effective transfer of knowledge to farmers have been highlighted as essential components in improving farm management and fish health practices.

Although most isolates displayed a high degree of genetic similarity, minor genetic variations were observed, as isolate PKA 110L clustered separately from isolates PKA 69S and PKA 86S despite originating from the same farm in Pekan. This genetic divergence within a single farm may reflect micro-evolutionary changes occurring over time, potentially driven by mutations, genetic recombination, or the activity of mobile genetic elements (Touchon et al., 2020). Similar patterns have been reported by Payne et al. (2025), who demonstrated temporal changes in plasmid profiles and virulence-associated genes among *E. ictaluri* isolates collected from *P. hypophthalmus* farms in the Mekong Delta over a 20-year period. Although the Vietnamese isolates remained closely related overall, detectable genetic shifts were observed across sampling years, indicating ongoing microevolution within aquaculture environments. In addition, variations in infection stage,

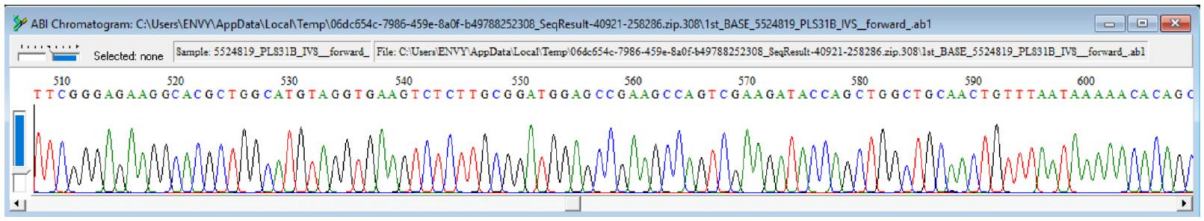
host immune pressure, and environmental conditions such as water quality and antimicrobial exposure may also contribute to localized genetic diversification within a single farm (Van Belkum et al., 2001; Mohammed et al., 2025).

4.4 SANGER SEQUENCING

4.4.1 Sanger Sequencing Output

Bidirectional Sanger sequencing of PCR amplicons targeting the 16S – 23S rRNA intergenic spacer (ITS) region was successfully obtained for all 11 *E. ictaluri* isolates. High-quality forward and reverse chromatograms were generated, characterized by clear, well-resolved, and evenly spaced peaks with minimal background noise (Figure 4.17). Notably, the chromatograms displayed single, distinct peaks at each nucleotide position, indicating the presence of a single dominant DNA template without mixed or overlapping signals (Al-Shuhaib & Hashim, 2023). In Sanger sequencing, a single peak at a given position represents the incorporation of one specific fluorescently labelled dideoxynucleotide, reflecting a homogeneous nucleotide base at that site (Saini et al., 2023). The absence of double or overlapping peaks suggests that the PCR amplification was specific and free from contamination, non-specific amplification, or mixed bacterial populations (Al-Shuhaib & Hashim, 2023).

PLS31B_FORWARD



PLS31B_REVERSE

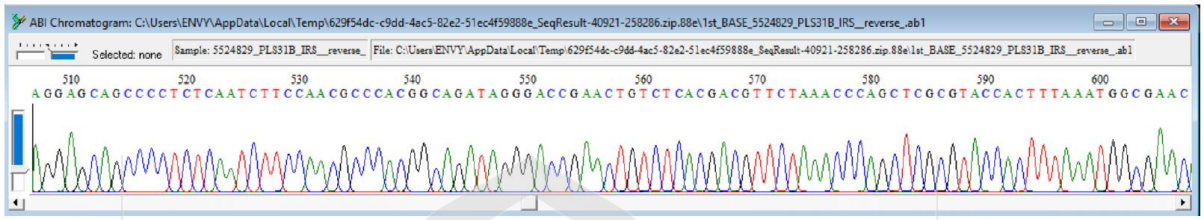


Figure 4.16: Forward and reverse ABI chromatogram result for *E. ictaluri* sample PLS31B

4.4.2 Sequence Assembly and Quality Control

The forward and reverse sequences for each isolate were subsequently aligned using the ClustalW algorithm to generate consensus sequences. Representative alignments and the graphic view of the aligned reads are shown in Figure 4.18 and 4.19.

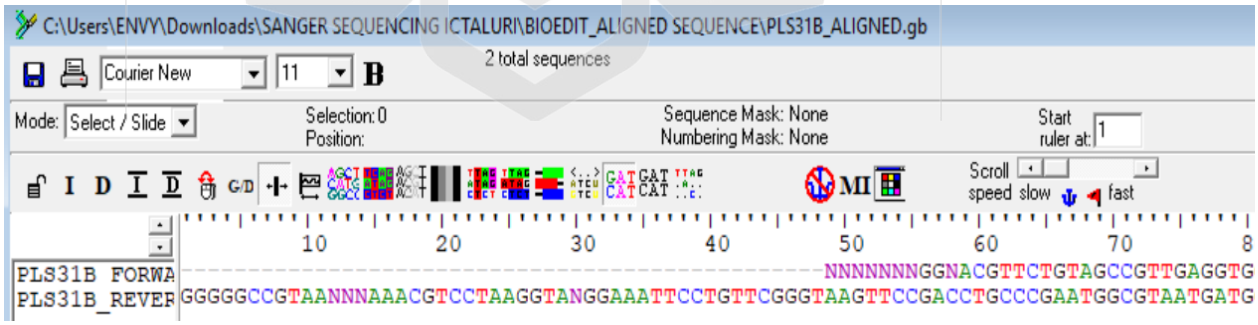


Figure 4.17: Aligned forward and reverse reads for *E. ictaluri* sample PLS31B using ClustalW

Figure 4.18: Graphic view of the aligned reads of *E. ictaluri* sample PLS31B

The successful bidirectional Sanger sequencing of the 16S – 23S rRNA intergenic spacer region for all 11 *E. ictaluri* isolates demonstrates the reliability of the PCR amplification and sequencing approach employed in this study. The generation of high-quality forward and reverse chromatograms with well-resolved peaks and minimal background noise indicates that the target region was sequenced with high accuracy, reducing the likelihood of base-calling errors (Al-Shuhaib & Hashim, 2023). Additionally, bidirectional sequencing further enhanced sequence confidence by resolving ambiguous nucleotides through overlap between complementary reads, which is particularly important when analyzing closely related bacterial isolates (Rodrigues et al., 2023).

Alignment of the forward and reverse sequences using the ClustalW algorithm enabled the construction of accurate consensus sequences for each isolate (Sharma et al., 2024). The clear and consistent alignment patterns observed across isolates suggest that the 16S–23S rRNA intergenic spacer region is sufficiently conserved to enable reliable sequence assembly, while retaining sufficient variability for discrimination at the strain or subpopulation level (Garcia-Martinez et al., 1996; Tokajian et al., 2016). The quality and consistency of the aligned sequences provided a robust foundation for subsequent sequence identification and phylogenetic analyses, ensuring that observed genetic differences among isolates reflect true biological variation rather than technical artefacts.

4.4.3 Sequence Identification using BLASTn Analysis

Nucleotide BLAST (BLASTn) analysis against the NCBI GenBank database confirmed the taxonomic identity of all 11 isolates as *E. ictaluri.* BLASTn functions by comparing a query nucleotide sequence against sequences deposited in the GenBank database to identify regions of local similarity (Zhang & Madden, 1997; Sayers et al., 2022). The algorithm aligns the query sequence with reference sequences and calculates statistical parameters such as percentage identity, query coverage, and E-value (Expected value) (Sharma et al.,

2024). In this study, each assembled consensus sequences showed high query coverage and extremely low E-values when compared with reference *E. ictaluri* sequences, indicating statistically significant and biologically meaningful matches.

For bacterial identification using ribosomal regions, percentage identity thresholds are commonly used to support species-level assignment. For highly conserved genes such as 16S rRNA, ≥98.7–99% identity is typically considered indicative of the same species (Bondoso et a., 2013; Sanz-Sáez et al., 2020). However, the 16S–23S rRNA intergenic spacer (ITS) region is more variable and therefore may exhibit slightly lower identity values while still representing the same species (Yu et al., 2020). The observed identity range of 95.33% to 97.89% relative to the whole-genome reference strain is therefore acceptable for ITS-based identification, particularly when supported by near-complete query coverage. High query coverage indicates that almost the entire sequence length aligns with the reference, reducing the likelihood of partial or misleading matches (Kim et al., 2020).

The E-value obtained in this study was 0.0 for all eleven isolates. The E-value represents the expected number of matches occurring by chance in a database of a given size (Mao, 2025). Lower E-values indicate more statistically significant alignments, with values approaching zero suggesting that the similarity is not due to random chance (Samal et al., 2021). In bacterial identification studies, E-values close to 0.0 are considered highly reliable (Solomon et al., 2025). The negligible E-values obtained for all eleven isolates therefore provide strong statistical support for their classification as *E. ictaluri*. Importantly, BLASTn does not simply compare percentage identity alone, it evaluates alignment length, mismatches, gaps and statistical probability simultaneously. The combination of high sequence similarity, near-complete query coverage and extremely low E-values collectively strengthens confidence in species-level identification.

Edwardsiella ictaluri 93-146, complete genome	Edwardsiella ict...	2200	17390	96%	0.0	97.96%	3812301	CP001600.2
Edwardsiella ictaluri strain NaF-IIUM chromosome, complete genome	Edwardsiella ict...	2200	17385	96%	0.0	97.89%	3761778	CP168652.1
Edwardsiella ictaluri strain MS-17-156 chromosome, complete genome	Edwardsiella ict...	2200	17374	96%	0.0	97.89%	3837027	CP028813.1
Edwardsiella ictaluri strain Ei-96cfu2 chromosome, complete genome	Edwardsiella ict...	2200	17379	96%	0.0	97.89%	3754380	CP152203.1

Figure 4.19: Result of BLASTn analysis against the NCBI GenBank database

4.4.4 Phylogenetic Relationships Among *Edwardsiella ictaluri* Isolates

Phylogenetic analysis based on the aligned 16S–23S rRNA intergenic spacer (ITS) sequences was performed using the Maximum Likelihood method in MEGA version 12. The resulting tree illustrated that all 11 *E. ictaluri* isolates clustered within a single well-defined lineage, clearly separated from the outgroup strain, *E. tarda* (Figure 4.21). The use of an outgroup is essential in phylogenetic reconstruction because it provides a reference point for rooting the tree and determining the direction of evolutionary divergence (Zou et al., 2024). In this analysis, MEGA was used to infer evolutionary relationships, while *E. tarda* served as the outgroup. An outgroup should be closely related but clearly outside the ingroup lineage (DeSalle et al., 2023). The clear separation between the *E. ictaluri* isolates and the outgroup confirms that the isolates share a more recent common ancestor with each other than with *E. tarda*, thereby validating their species-level classification.

The relatively long branch separating the *E. ictaluri* cluster from the outgroup (branch length ≈ 0.31) indicates substantial genetic divergence. In Maximum Likelihood trees, branch length represents the estimated number of nucleotide substitutions per site (Yang et al., 1994; Kapli et al., 2020). Thus, a branch length of 0.31 suggests approximately 31% sequence divergence in the analyzed ITS region between *E. ictaluri* and *E. tarda*. In phylogenetic study, values above 0.1–0.2 generally reflect considerable evolutionary distance in bacterial marker genes (Tamura et al., 2021). If the branch length were substantially higher than 0.31 (for example >0.5), it would indicate even greater sequence

divergence, possibly reflecting more distantly related taxa or saturation effects where multiple substitutions have occurred at the same site (Duchêne et al., 2022). Therefore, the observed value of 0.31 supports the suitability of the ITS region as a discriminatory marker for closely related *Edwardsiella* species, as ITS regions typically evolve faster than conserved genes such as 16S rRNA and provide higher resolution at the species or subspecies level (Church et al., 2020).

Within the *E. ictaluri* cluster, branch lengths were short (0.00–0.03), indicating minimal genetic distance among isolates. Short branch lengths correspond to very few nucleotide substitutions per site, demonstrating high sequence conservation (Nei & Kumar, 2000; Lucaci et al., 2023). In bacterial phylogenetics, intra-species branch lengths close to zero commonly reflect highly similar or nearly identical sequences (Chan et al., 2021). Bootstrap values between 67–82% further support the stability of internal nodes. Bootstrap analysis evaluates how consistently a particular grouping appears when the dataset is resampled multiple times (Challa & Neelapu, 2019; Simon, 2022). Values above 70% are generally considered reliable, while values exceeding 90–95% indicate very strong statistical confidence (Mokhtar et al., 2023). Therefore, the observed bootstrap range indicates that the clustering pattern is statistically reliable.

Although all isolates formed a single primary lineage, th sub-clustering was observed within the *E. ictaluri* clade. For instance, PKA110L and PTP324K grouped closely together with strong bootstrap support, indicating a high degree of genetic similarity between these two isolates. This close relationship is evidenced by their very short branch distance, reflecting near-zero nucleotide substitutions per site, as well as their shared internal node supported consistently across bootstrap resampling (Lemoine & Gascuel, 2024). Such features are standard indicators of genetic similarity in phylogenetic analysis, where short pairwise distances and moderate to high bootstrap values signify closely related sequences (Nei & Kumar, 2000; Lam et al., 2024). Similarly, PTP354B NAF-IIUM clustered tightly with PTP329K and PLS31K, further suggesting minimal sequence variation among these isolates within the ITS region. In contrast, isolates such as PKA69S and PTP274K appeared slightly basal within the cluster. However, a basal position does not imply species-level divergence, rather, it reflects minor nucleotide differences that position

these isolates closer to the ancestral node of the lineage (Thomas et al., 2020). These subtle branching differences likely reflect minor sequence variations within the ITS region. Nevertheless, the overall tree topology confirms that the isolates are genetically homogeneous, with only limited intra-species divergence detected among the studied strains.

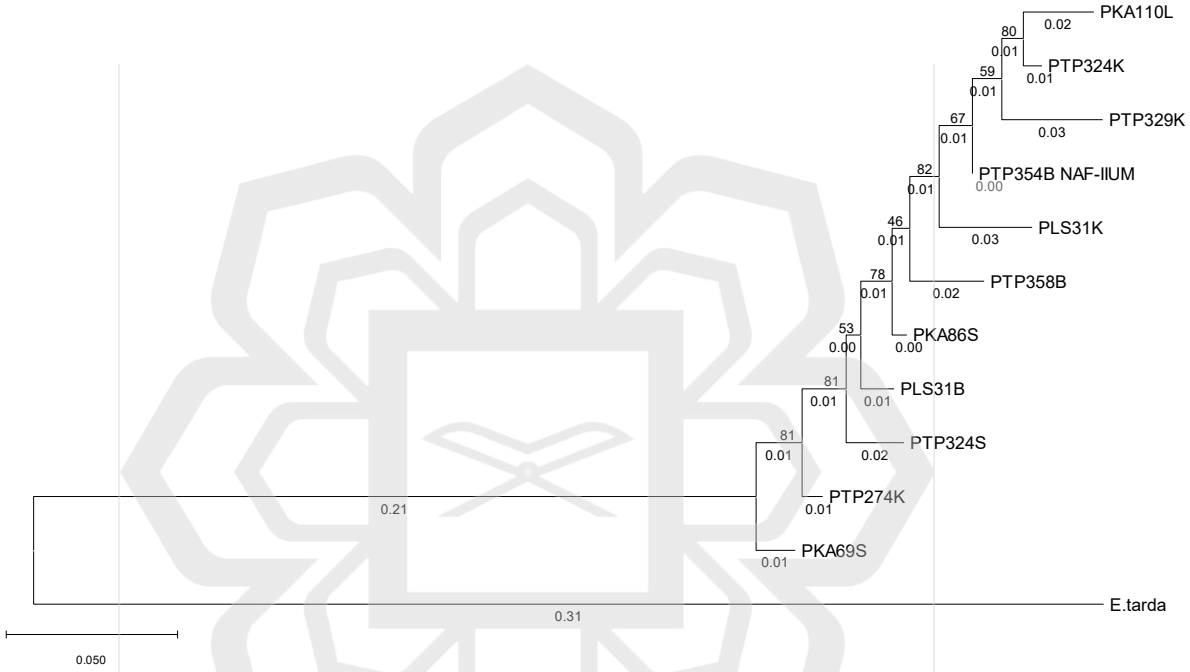


Figure 4.20: Phylogenetic tree of *E. ictaluri* isolates constructed using MEGA

4.5 ANALYSIS OF POLYMORPHISM STUDY

Overall, genetic polymorphism studies provide a fundamental framework for integrating molecular data into aquaculture practices. By identifying genetic variation at the strain and population levels, polymorphism analysis supports selective breeding programs through the selection of broodstock with desirable traits such as disease resistance, faster growth rates, and environmental tolerance (Megahed, 2020; Kashyap et al., 2024). For example, detecting specific genetic markers associated with resistance to *Edwardsiella ictaluri* infection enables hatcheries to prioritise genetically resilient broodstock, thereby reducing mortality rates and reliance on antibiotics (Nguyen, 2021; Saba et al., 2026). Over time, marker-assisted selection can enhance the overall genetic quality and productivity of cultured fish populations.

In addition to selective breeding, polymorphism studies significantly improve disease surveillance and outbreak management (Tiwari et al., 2025). Molecular fingerprinting techniques, such as RAPD-PCR, allow rapid differentiation between closely related bacterial strains during disease outbreaks (Ador et al., 2021). This enables tracing of infection sources, identification of transmission routes between farms, and detection of potential clonal expansion within aquaculture systems (Nguyen, 2024). Early detection of genetically distinct or emerging strains can facilitate timely biosecurity interventions, reduce economic losses and prevent large-scale dissemination (Aly & Fathi, 2024).

Furthermore, polymorphism research enhances understanding of host–pathogen interactions by revealing genetic diversity within pathogenic populations (Ekroth et al., 2021). Variations detected in virulence-associated regions, regulatory genes, or adaptive genomic segments may explain differences in pathogenicity, host specificity, or environmental persistence (Belcher et al., 2021). Understanding these variations helps clarify why certain strains cause severe outbreaks while others remain less virulent. This knowledge supports the development of targeted vaccines, improved diagnostic tools, and more precise treatment strategies (Azad et al., 2020).

The integration of rapid screening methods such as RAPD-PCR with sequence-based approaches, including ITS sequencing and phylogenomic analysis, further strengthens the application of polymorphism studies. RAPD provides a cost-effective and rapid preliminary assessment of genome-wide diversity, while sequencing-based methods offer higher resolution and confirmatory accuracy (Babu et al., 2020). Together, these complementary techniques enable comprehensive strain characterisation, balancing speed, affordability, and precision. Such integrated molecular strategies are increasingly important in modern aquaculture, where sustainable disease management, reduced antimicrobial usage, and genetic improvement are critical for long-term industry resilience and productivity.

CHAPTER FIVE

CONCLUSION

5.1 CONCLUSION

In conclusion, both objectives of this study were successfully achieved, providing a comprehensive molecular and genomic characterization of *E. ictaluri* isolated from cage-cultured *P. hypophthalmus* in Pahang River. The first objective, which was to confirm the taxonomic identity of the isolate using whole-genome sequencing, was successfully accomplished. PCR amplification, BLASTn analysis, phylogenetic reconstruction, and phylogenomic analysis consistently identified PTP_1 as *E. ictaluri*, with strong bootstrap support confirming its species-level classification. The genome was successfully assembled into a complete circular chromosome, indicating high assembly quality. Comparative genomic analysis revealed close relatedness of PTP_1 to *E. ictaluri* strains from China and Vietnam, supported by a large shared core genome of 3,246 genes. Functional annotation identified genes associated with metabolism, cellular processes, information storage and processing, and mobilome-related elements. These features reflect the adaptive and pathogenic potential of the isolate, including the presence of regulatory and antimicrobial resistance–associated genes.

The second objective was to evaluate genetic polymorphism and strain-level variation among 11 isolates of *E. ictaluri* using RAPD-PCR and Sanger sequencing. RAPD analysis successfully revealed detectable intraspecies genetic diversity among the isolates, indicating strain-level variation that may influence virulence and outbreak dynamics. Meanwhile, Sanger sequencing confirmed species identity of all isolates and identified minor nucleotide variations, supporting the RAPD findings. Both methods revealed that the 11 isolates clustered closely together, regardless of their geographical origin. Overall, this study provides valuable insights into the molecular epidemiology, genetic diversity, and

genomic architecture of *E. ictaluri*. The integration of whole genome study and polymorphism analysis strengthens our understanding of pathogen evolution, transmission potential, and disease persistence in aquaculture systems.

5.2 FUTURE RECOMMENDATION

Future research should expand comparative genomic analyses by including a larger number of *E. ictaluri* isolates from different geographical regions and host species to better understand strain evolution, transmission pathways, and genetic diversification patterns. Integration of spatial and temporal epidemiological data would further enhance understanding of pathogen dissemination within aquaculture systems. The application of genomic surveillance combined with environmental monitoring could help detect emerging strains before large-scale outbreaks occur. In addition, transcriptomic or proteomic studies are recommended to investigate gene expression profiles under different environmental stress conditions, allowing deeper insight into virulence regulation and host–pathogen interactions. Particular attention should be given to the characterization of outer membrane proteins (OMPs), as detailed investigation of OMP structure, function, and expression may provide valuable insights into pathogenic mechanisms and identify potential targets for vaccine development, diagnostic biomarkers, or novel antimicrobial strategies. Finally, functional validation of predicted virulence and antimicrobial resistance genes through in vitro or in vivo experiments would also strengthen the biological interpretation of genomic findings. The findings from this study contribute to improved disease surveillance strategies, selective breeding programs for enhanced resistance, and evidence-based health management practices, ultimately supporting sustainable aquaculture production.

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