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Exploring the genetic diversity of *Edwardsiella ictaluri* in Vietnamese striped catfish (*Pangasianodon hypophthalmus*) farms over a 20-year period

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Since first being identified in 1999, disease outbreaks from *Edwardsiella ictaluri* remain a significant health challenge for the farmed catfish sector in Vietnam. To better understand the population structure of *E. ictaluri* circulating in Vietnamese striped catfish (*Pangasianodon hypophthalmus*) farms, this study aimed to investigate the genetic diversity of 83 *E. ictaluri* isolates recovered from natural disease outbreaks occurring in the Mekong Delta region between 2001 and 2021. Pulsed-field gel electrophoresis resolved isolates into 15 pulsotypes following restriction digest with *speI*, with a Simpson's diversity index of 0.8548. The genetic fingerprints of isolates recovered from recent outbreaks across different provinces were highly shared (100% similarity), suggesting clonal expansion within the production systems situated in the Mekong Delta region. Findings from this study also showed that *E. ictaluri* populations circulating this region have changed over time, as isolates recovered between 2001 and 2011 were genetically distinct from those recovered after 2017. Furthermore, isolates recovered after 2004 and 2010 lacked the virulence gene *traD* and a 5.8 Kb plasmid DNA fragment, respectively. Findings from this study illustrate the need for continued epidemiological monitoring of *E. ictaluri* to ensure prevention and treatment strategies in the Vietnamese striped catfish sector remain robust and effective.

KEYWORDS

***Edwardsiella ictaluri*, Bacillary Necrosis of Pangasius, *Pangasianodon hypophthalmus*, Vietnam, genotyping, plasmids, virulence**

1 Introduction

Edwardsiella ictaluri is described as an intracellular, facultative, Gram-negative rod-shaped bacterium and member of the *Hafniaceae* family (Adeolu et al., 2016). Historically, *E. ictaluri* was described as biochemically homogenous, testing positive for lysine decarboxylase activity and glucose metabolism, however host-dependent differences have expanded this description to include isolates positive for ornithine decarboxylase and cytochrome oxidase activities as well as citrate utilisation and H_2S production (Rogge et al., 2013; Machimbirike et al., 2022). Recently, published genomes of *E. ictaluri* have revealed a genome size ranging from 3.6 – 4.0 Mb, with G+C (%) of 57.0 to 57.6 and the presence of a diverse collection of antimicrobial resistance and virulence genes (Abdelhamed et al., 2018; Machimbirike et al., 2021). The bacterium *E. ictaluri* was initially reported to have a narrow host range (Hawke et al., 1981), however has since been found to infect 44 susceptible hosts, either naturally or experimentally, including striped catfish (*Pangasianodon hypophthalmus*) (Crumlish et al., 2002; Ngoc Phuoc et al., 2020; Machimbirike et al., 2022).

Vietnamese production of striped catfish has intensified in recent years to support the growing demand for aquatic animal protein, increasing from 40,000 tonnes in 1997 to 1.5 million tonnes in 2020, worth $1.7 billion USD (FAO, 2021). However, this intensification has been accompanied by increased incidence of disease outbreaks across the production sector (Hoa et al., 2021). The bacterium *E. ictaluri* was first identified as the aetiological agent of bacillary necrosis of Pangasius (BNP) in striped catfish from Vietnam over two decades ago (Crumlish et al., 2002). The clinical signs of BNP have remained consistent over time, where diseased fish typically show erratic swimming as well as swollen abdomens, and internally present multifocal white lesions on the liver, kidney and spleen (Ngoc Phuoc et al., 2020). Despite its historical presence within the country, recent surveillance of the production sector suggests outbreaks of BNP are on the rise (Hoa et al., 2021). This is particularly concerning given that the disease causes significant economic losses on the farm, affecting all stages of development with up to 90% mortality rates reported (Vu et al., 2019), thereby requiring effective control strategies to combat infection.

Epidemiological investigation of disease outbreaks is vital to inform health management policies, as it provides criteria on which to base surveillance and vaccination programmes. Whilst alternative high-resolution methods are available for bacterial identification (e.g., whole genome sequencing), pulsed-field gel electrophoresis (PFGE) is considered the gold-standard of genotyping methods for molecular characterisation and to determine the genetic relatedness of bacterial isolates during disease outbreaks, due to its low cost, high discriminatory power, reproducibility and ease of interpretation (Duman et al., 2022; Ramadan, 2022; Sciuto et al., 2022). Indeed, this method has been used previously to type several aquatic bacterial pathogens including *Aeromonas salmonicida* subsp. *salmonicida*, *Edwardsiella tarda*, *Flavobacterium psychrophilum*, *Vibrio parahaemolyticus* and *Yersinia ruckeri* (García et al., 2000; Yang et al., 2013; Narayanan et al., 2020; Calvez et al., 2021; Feng et al., 2022). Furthermore, PFGE has been previously applied to understand the genetic diversity of *E. ictaluri* isolates recovered from diseased striped catfish in Vietnam between 2001 and 2004 (Bartie et al., 2012), which found that Vietnamese isolates were genetically distinct from those recovered from channel catfish in United States (Bartie et al., 2012). Geographical or host-dependant genotypes in *E. ictaluri* have also been confirmed through other typing methods, whereby isolates from Southeast Asia can differ in their plasmid DNA and virulence gene profiles compared with American isolates (Rogge et al., 2013; Dong et al., 2019). Since the early studies by Bartie et al. (2012) and Rogge et al. (2013), and subsequent whole genome sequencing of an isolate from Northern Vietnam (Machimbirike et al., 2021) were completed, no further genotyping studies on Vietnamese *E. ictaluri* have been performed to the best of our knowledge. There is therefore a lack of understanding on the genetic diversity that exists in *E. ictaluri* populations currently circulating the striped catfish sector in Vietnam. This information is vital to understand the epidemiological spread of *E. ictaluri* populations in the Vietnamese striped catfish sector and better inform effective control strategies.

Considering the epidemic nature of *E. ictaluri* in Vietnam and scarcity in knowledge surrounding the epidemiology of this species, the aim of this study was to characterise the genetic diversity of *E. ictaluri* recovered from clinical disease outbreaks in Vietnamese farmed striped catfish over a twenty-year period (2001 and 2021). The use of PFGE as well as profiling plasmid DNA and virulence genes was applied to better understand the structure of *E. ictaluri* populations currently circulating striped catfish farms in Vietnam and how these populations have changed over time.

2 Materials and methods

2.1 Bacterial isolates

The 83 *E. ictaluri* isolates used in this study (Table S1) were recovered from naturally infected striped catfish between 2001 and 2021 across six provinces in Vietnam (Figure 1). All *E. ictaluri* isolates were recovered from the kidney or liver of fish showing clinical signs of BNP including white spots on the kidney, liver or spleen, during an active disease outbreak. The isolates *E. ictaluri* NCIMB 13272, *E. ictaluri* ATCC 93-146 and *Edwardsiella piscicida* NCIMB 14824, served as an American representative and controls in different assays, respectively (Table S2). All bacterial isolates were recovered from storage on Protect Beads (SWA1147; SLS, UK) at -80°C onto tryptone soya agar (TSA) (CM0131, Oxoid, UK), where they were incubated at 28°C for 72 hours.

2.2 Genomic DNA extraction

Genomic DNA was extracted from bacteria using the method described by Queipo-Ortuño et al. (2008), except final DNA was

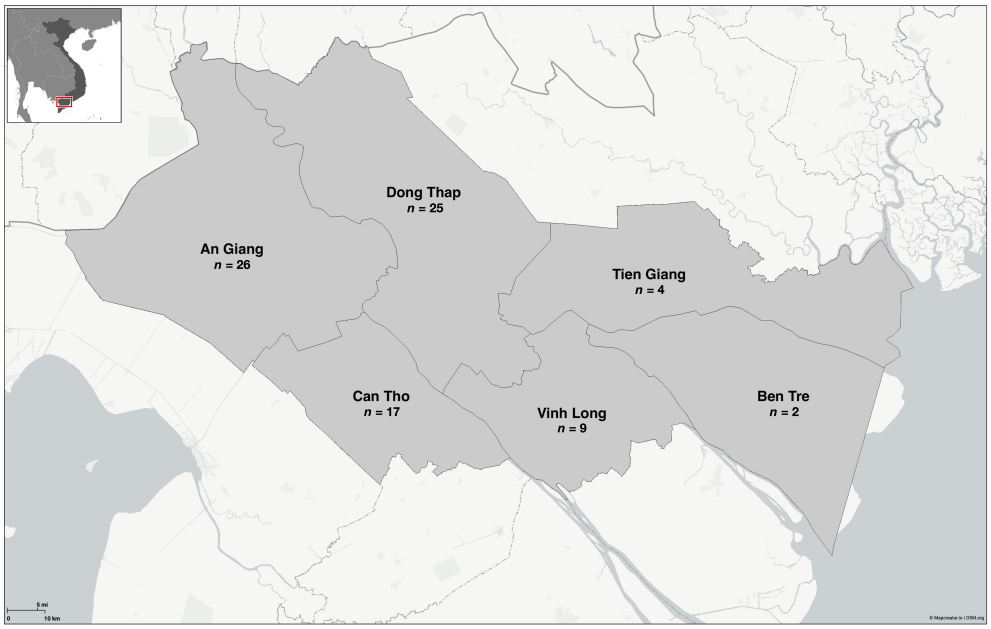

FIGURE 1
Distribution of *Edwardsiella ictaluri* isolates recovered from disease outbreaks in striped catfish (*Pangasianodon hypophthalmus*) farms in the Mekong Delta region of Vietnam. Map created using Mapcreator (https://mapcreator.io).

eluted in 500 µL TE (10 mM tris pH 8.0, 1 mM EDTA pH 8.0). The quality and concentration of extracted DNA samples was measured using the Nanodrop 2000c spectrophotometer (Fisher Scientific, UK) and 10 µL aliquots stored at -20°C, until required.

2.3 Species identification

Primary identification was performed as described by Frerichs and Millar (1993) and biochemical profiles obtained using the commercial API®20E biochemical strips (20100, BioMerieux Ltd, UK), following the manufacturer's instructions, except incubating strips at 28°C for 48 hours before recording results.

All bacterial isolates were identified as *E. ictaluri* by species-specific PCR using the primer set EDi-F (5'- CAGATGAGCGGATTTCACAG –'3) and EDi-R (5'- CGCGCAATTAACATAGAGCC -'3) (Sakai et al., 2009). Briefly, a 10 µL PCR reaction was prepared for each bacterial isolate, which comprised 5 µL 2 X HS mytaQ mastermix (BIO-25045, Bioline, UK), 0.5 µL (5 pM) of each forward and reverse primer, 3 µL nuclease-free water and 10 ng DNA. Genomic DNA from *E. ictaluri* NCIMB 13272 and *E. piscicida* NCIMB 14824 was also included in the analysis, where they served as positive and negative controls, respectively. In addition, a no template control (NTC) was also included to confirm PCR reagents and subsequent reactions were free from microbial DNA contamination. The *E. ictaluri* specific fimbrial gene was then amplified using the PCR conditions; 95°C for two minutes, then 30 x cycles at 95°C for 15 seconds, 65°C for 20 seconds, 72 °C for 30 seconds, with a final extension at 72°C for two minutes. Following PCR, PCR products were visualised on a 1.5% agarose gel containing ethidium bromide (0.06 µg/mL), following electrophoresis at 11 V/cm for 20 minutes. Isolates were identified as *E. ictaluri* based on the presence of a single PCR product with a molecular weight of 470 bp.

2.4 Pulsed-field gel electrophoresis

Macrorestriction analysis of bacterial isolates was performed using PFGE, following the protocol described by Bartie et al. (2012) with minor modifications. Briefly, 24-hour bacterial cultures were washed in 1 mL STE buffer (10 mM tris pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl) before being resuspended in STE buffer to an OD_{600nm} of 0.8, as measured by a cell density meter (Biowave CO 8000; Biochrom, UK). One percent agar plugs were then prepared using equal volumes (100 µL) of bacterial suspension and a 2% PFGE agarose (A2929, Sigma, UK) solution. In addition, bacteria cells were lysed in 3 mL lysis buffer (10 mM tris pH 8.0, 1 mM EDTA pH 8.0, 1% sarcosine, 1 mg/mL proteinase K) at 55°C and 175 rpm for 18 hours. Plug washing, restriction digest of DNA with *speI*-HF (R3133, NEB, UK) and electrophoresis was performed following Bartie et al. (2012). Following PFGE, gels were stained in an ethidium bromide solution (1 µg/mL) for 30 minutes, followed by two consecutive washes in distilled water for 20 minutes and visualised under UV illumination.

2.5 Plasmid profiles

Plasmid DNA was extracted from 17-hour cultures using the QIAprep® Spin Miniprep Kit (27104, Qiagen, UK), following the manufacturer's instructions with minor modifications. Briefly, the QIAprep® columns were washed in 500 µL buffer PB. In addition,

plasmid DNA was eluted in 100 µL pre-heated buffer EB. Following extraction, 250 ng plasmid DNA was digested with *EcoRI*-HF (R3101, NEB, UK) (Dong et al., 2019) and visualised on a 0.8% agarose gel in 1 X TAE (40 mM tris, 20 mM acetic acid, 1 mM EDTA) and ethidium bromide (0.25 µg/mL), following electrophoresis at 5 V/cm for 90 minutes. Plasmid DNA was visualised alongside a 1 Kb DNA ladder (Fisher Scientific, UK) under UV illumination.

2.6 Virulence gene profiles

The presence of seven putative virulence genes (*eseJ*, *traD*, *hcp*, *fldA*, *wzx*, *fhuC* and *ureE*) was detected in this study using primer pairs listed in Table 1. Briefly, a 10 µL PCR reaction was prepared for each bacterial isolate, which comprised 5 µL 2 X HS mytaQ mastermix, 0.5 µL (5 pM) of each forward and reverse primer, 3 µL nuclease-free water and 10 ng DNA. Primer sets were designed against the genome of *E. ictaluri* ATCC 93-146 (Accession: NC_012779.2) using the NCBI primer design software (Ye et al., 2012). Genomic DNA from *E. ictaluri* ATCC 93-146 and *E. piscicida* NCIMB 14824 was also included in the analysis, where they served as positive and negative controls, respectively. In addition, a NTC was also included to confirm PCR reagents and subsequent reactions were free from microbial DNA contamination. Virulence genes were amplified in DNA samples using the following PCR conditions: 95°C for two minutes, then 30 x cycles at 95°C for 15 seconds, 62-64°C for 15 seconds and 72°C for 30 seconds, followed by a final elongation stage at 72°C for two minutes. Following PCR, PCR products were visualised on a 1.5% agarose gel containing ethidium bromide (0.06 µg/mL), following electrophoresis at 11 V/cm for 20 minutes. The annealing temperature and product size of each virulence gene is given in Table 1.

2.7 Data analysis

Genetic fingerprints were analysed by GelJ (Version 2) (Heras et al., 2015). Fingerprints for each bacterial isolate were clustered based on the Dice Coefficient with tolerance set to 1%. A dendrogram was then generated to visualise clusters using the unweighted pair group method with arithmetic mean or "UPGMA" algorithm. Isolates that had DNA fingerprints with similarities of more than 86% were grouped into the same pulsotype and considered closely or possibly related (Feng et al., 2022). Isolates which had identical DNA fingerprints were termed clonal isolates. To determine the genetic diversity of the bacterial isolate collection from Vietnam, Simpson's Diversity Index (SDI) was calculated based on pulsotypes detected and with a confidence interval (CI) of 95% (Carriço et al., 2006). A binary matrix of presence/absence data was generated for plasmid DNA band and virulence gene datasets to determine similarities between isolates based on Euclidean distance and complete linkage (UPGMA) using Rstudio 1.4.1717. Dendograms and heatmaps for plasmid DNA and virulence gene data were generated using Rstudio 1.4.1717 and "factoextra" and "ggplot2" packages. The distribution of plasmid DNA bands and virulence genes were compared between PFGE

TABLE 1 Primer sets used to detect *Edwardsiella ictaluri* virulence genes in this study.

Gene	Target	Virulence Factor[a]	Genome Position	Primer Name	Forward & Reverse (5′ - ′3)	Product (bp)	Ta[b] (°C)
eseJ	Effector protein (Type III secretion system)	Effector Delivery Systems	(957432…961520)	eseJ-F	ATCAGAACGGCACATCCCTG	440	64
				eseJ-R	CATGTCCTTCACCCGGAACA		
traD	Conjugative transfer system coupling protein (Type IV secretion system)	Effector Delivery Systems	(338471…340561)	traD-F	TGGAGATGAGCGATTCACGG	344	64
				traD-R	GATCCGGGCCTCAATATCCG		
hcp	Effector protein (Type VI secretion system)	Effector Delivery Systems	(3306400…3306879)	hcp-F	CACTCCGCCTGGGTTCTATC	306	64
				hcp-R	TGCTACTCATGCCGTTAGGC		
fldA	Flavodoxin (hydrogen peroxide resistance)	Immune Modulation	(2788772…2789299)	fldA-F	CGGTAAACTGGTTGCCCTGT	265	64
				fldA-R	CTCTGTGCTGAGCTGCTTGA		
wzx	O antigen transporter	Immune Modulation	(1294396…1294806)	wzx-F	ACGATGAGTGTTGGCGCTTA	411	62
				wzx-R	GAACAGCGGGAAACCAGAGA		
fhuC	ABC transporter ATP-binding protein (iron uptake)	Nutritional/ Metabolic Factor	(705425…706198)	fhuC-F	CGAGGTCCTGGTTATCGAACA	656	64
				fhuC-R	TTTGGTACAGCTCGGACAGG		
ureE	Urease accessory protein	Stress Survival	(1981667…1982344)	ureE-F	AACCCATCGCTTTACTGGCA	194	62
				ureE-R	TATGAACATGGCGGTGTCCC		

[a]Assigned from the Virulence Factor Database (Chen et al., 2005).
[b]Annealing temperature.

groups, province and year using the Chi-square (X^2) or Fisher's exact tests on JMP Pro software (Version 17.0.0), with significance determined when $p < 0.05$.

3 Results

3.1 Species identification

All isolates were confirmed to be *E. ictaluri* and possessed the fimbrial gene as detected by species-specific PCR (Figure S1). All isolates were described as being Gram-negative rods, non-motile, negative for cytochrome oxidase and fermentative by the OF test (Table S1). Eighty-one (98%) isolates were positive for lysine decarboxylase and glucose metabolism on the API20E system, giving the numerical profile 4004000 (Table S1). Two (2%) isolates, isolated in 2003 and 2017, were found to also be positive for citrate utilisation in addition to lysine decarboxylase and glucose metabolism, giving a numerical profile of 4204000 (Table S1).

3.2 Pulsed-field gel electrophoresis

Eighty-four *E. ictaluri* isolates were successfully typed by PFGE using *speI*, which yielded at least 21 well-resolved DNA fragments ranging in size from 20 – 540 Kb (Figure 2). Isolates were distributed into three distinct clusters based on their DNA fingerprint (≤ 75% similarity). Within these clusters, 13 pulsotypes (≥ 86% similarity) and three singletons were identified (Figure 2). Cluster I was represented by the American type isolate (Pulsotype A), which was distinct from the Vietnamese isolates that clustered into pulsotypes B – D and E – P, within Clusters II and III, respectively (Figure 2). Cluster II was represented by 10% of isolates (n = 8) recovered from multiple provinces in Vietnam between 2001 and 2003 (Figure 2). The remaining Vietnamese isolates (89%; n = 75) representing disease outbreaks across all surveyed provinces between 2001 and 2021 were distributed into Cluster III (Figure 2). Within Cluster III, the DNA fingerprints of isolates recovered between 2001 and 2011 (pulsotypes H - L) were found to group separately from those of isolates recovered after 2017 (pulsotypes E – G and M - P) (Figure 2). The SDI of Vietnamese isolates in this study was 0.8548 (95% CI 0.8545 – 0.8551). Furthermore, the SDI was found to reduce over time as *E. ictaluri* isolates recovered between 2001 and 2011 had an SDI of 0.7503 (95% CI of 0.7497 – 0.7509), whereas those recovered after 2017 had an SDI of 0.6598 (95% CI of 0.6595 – 0.6600). Clonal isolates were detected in several pulsotypes including M & P, which comprised clonal isolates recovered from An Giang and Dong Thap in 2019 and 2020 (Figure 2). Genetically distinct isolates (< 86% similarity) recovered from the same farm in the same year were also detected on several occasions, including An Giang in 2002, Can Tho in 2002 and 2020, Dong Thap in 2021, Tien Giang in 2020 and Vihn Long in 2003 and 2020 (Figure 2).

Pulsotype P was the most prevalent of all types, accounting for 27% (n = 23) of all isolates, closely followed by K (23%; n = 19) (Tables 2, 3). An Giang, Dong Thap and Can Tho were the most represented provinces surveyed, contributing 31% (n = 26), 30% (n = 25) and 20% (n = 17) of Vietnamese isolates investigated, respectively (Table 2). Within these provinces, pulsotype K was found to dominate An Giang and Can Tho, accounting for 29% (n = 10) and 41% (n = 7) of isolates within this pulsotype, respectively, whereas pulsotype P was found to dominate Dong Thap (44%; n = 11) (Table 2). On further investigation, pulsotype K was detected between 2001 and 2006, whereas pulsotype P was only detected after 2017 (Table 3).

3.3 Plasmid profiles

The *EcoRI* digestion of plasmid DNA was successfully performed on all *E. ictaluri* isolates screened and revealed 30 distinct plasmid DNA patterns across the collection (Figure 3). All isolates had at least one plasmid DNA band, however variability was noted between isolates in both the number (range: 1 – 8 bands) and size of plasmid DNA bands (range: 2.3 – more than 20 Kb) detected (Figure 3). No conserved plasmid DNA band was detected across the isolate collection (Figure 3). Within the Vietnamese isolates, a plasmid DNA band at 3.8 Kb was the most frequently detected, found in 98% (n = 81) of isolates screened, followed by a 4.8 Kb plasmid DNA band (52%; n = 43) (Figure 3). Forty (48%) Vietnamese isolates were found to harbour both 3.8 Kb and 4.8 Kb plasmid DNA bands (Figure 3). The American type isolate was found to have a distinct plasmid restriction profile, with two plasmid DNA bands observed at 4.8 Kb and 5.8 Kb (Figure 3). A similar sized plasmid DNA band at 5.8 Kb was also detected in 39% (n = 32) of Vietnamese isolates, although both year of recovery ($p < 0.0001$) and PFGE cluster ($p = 0.0002$) was found to influence the detection of this band across the isolate collection. In fact, the 5.8 Kb plasmid DNA band was only detected in those isolates recovered between 2001 and 2009 and was more commonly associated with isolates within PFGE Cluster II compared with Cluster III (Figure 3).

3.4 Virulence gene profiles

The presence of seven genes, representing suspected virulence factors in *E. ictaluri*, were screened by PCR with bands at the expected molecular size considered positive results (Figure S2). Four virulence gene profiles were identified across the isolate collection in this study, where profile III was most frequently detected (61%; n = 51) (Table 4). Further, virulence gene profiles I and III were more associated with isolates in PFGE Cluster III, whereas profiles II and IV were more associated with PFGE Cluster II ($p < 0.0001$) (Figure 4). All isolates possessed the genes *hcp*, *fldA*, *wzx*, *fhuC* and *ureE* but there was variation in whether isolates harboured the *eseJ* and *traD* genes (Figure 4). A total of 56 (67%) isolates, were positive for carrying *eseJ* (Figure 4). Whilst the proportion of isolates harbouring *eseJ* was higher in An Giang, Ben Tre, Dong Thap and Vinh Long ($p = 0.0306$), the presence of *eseJ* did not relate with specific PFGE clusters or year of recovery ($p > 0.05$) (Figure 4). The gene *traD* was only detected in 12% of isolates (n = 10) and was more associated with isolates

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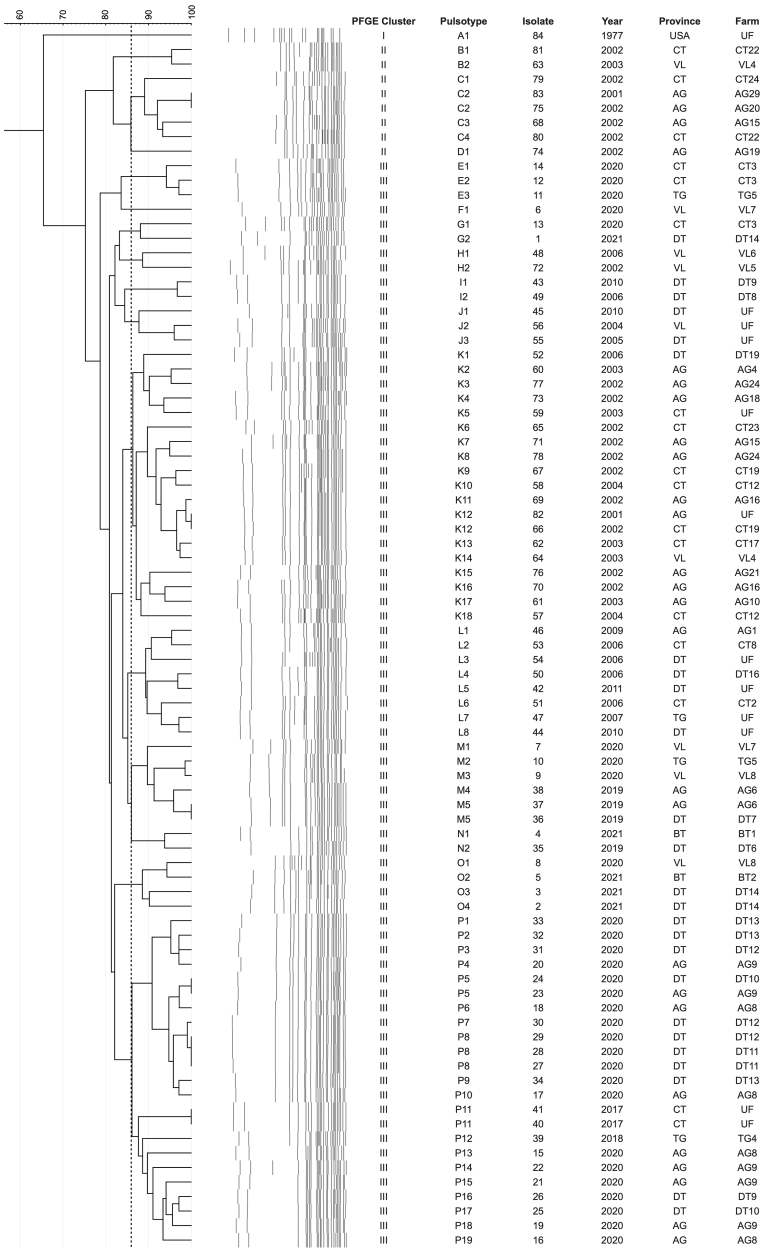

PFGE Cluster	Pulsotype	Isolate	Year	Province	Farm
I	A1	84	1977	USA	UF
II	B1	81	2002	CT	CT22
II	B2	63	2003	VL	VL4
II	C1	79	2002	CT	CT24
II	C2	83	2001	AG	AG29
II	C2	75	2002	AG	AG20
II	C3	68	2002	AG	AG15
II	C4	80	2002	CT	CT22
II	D1	74	2002	AG	AG19
III	E1	14	2020	CT	CT3
III	E2	12	2020	CT	CT3
III	E3	11	2020	TG	TG5
III	F1	6	2020	VL	VL7
III	G1	13	2020	CT	CT3
III	G2	1	2021	DT	DT14
III	H1	48	2006	VL	VL6
III	H2	72	2002	VL	VL5
III	I1	43	2010	DT	DT9
III	I2	49	2006	DT	DT8
III	J1	45	2010	DT	UF
III	J2	56	2004	VL	UF
III	J3	55	2005	DT	UF
III	K1	52	2006	DT	DT19
III	K2	60	2003	AG	AG4
III	K3	77	2002	AG	AG24
III	K4	73	2002	AG	AG18
III	K5	59	2003	CT	UF
III	K6	65	2002	CT	CT23
III	K7	71	2002	AG	AG15
III	K8	78	2002	AG	AG24
III	K9	67	2002	CT	CT19
III	K10	58	2004	CT	CT12
III	K11	69	2002	AG	AG16
III	K12	82	2001	AG	UF
III	K12	66	2002	CT	CT19
III	K13	62	2003	CT	CT17
III	K14	64	2003	VL	VL4
III	K15	76	2002	AG	AG21
III	K16	70	2002	AG	AG16
III	K17	61	2003	AG	AG10
III	K18	57	2004	CT	CT12
III	L1	46	2009	AG	AG1
III	L2	53	2006	CT	CT8
III	L3	54	2006	DT	UF
III	L4	50	2006	DT	DT16
III	L5	42	2011	DT	UF
III	L6	51	2006	CT	CT2
III	L7	47	2007	TG	UF
III	L8	44	2010	DT	UF
III	M1	7	2020	VL	VL7
III	M2	10	2020	TG	TG5
III	M3	9	2020	VL	VL8
III	M4	38	2019	AG	AG6
III	M5	37	2019	AG	AG6
III	M5	36	2019	DT	DT7
III	N1	4	2021	BT	BT1
III	N2	35	2019	DT	DT6
III	O1	8	2020	VL	VL8
III	O2	5	2021	BT	BT2
III	O3	3	2021	DT	DT14
III	O4	2	2021	DT	DT14
III	P1	33	2020	DT	DT13
III	P2	32	2020	DT	DT13
III	P3	31	2020	DT	DT12
III	P4	20	2020	AG	AG9
III	P5	24	2020	DT	DT10
III	P5	23	2020	AG	AG9
III	P6	18	2020	AG	AG8
III	P7	30	2020	DT	DT12
III	P8	29	2020	DT	DT12
III	P8	28	2020	DT	DT11
III	P8	27	2020	DT	DT11
III	P9	34	2020	DT	DT13
III	P10	17	2020	AG	AG8
III	P11	41	2017	CT	UF
III	P11	40	2017	CT	UF
III	P12	39	2018	TG	TG4
III	P13	15	2020	AG	AG8
III	P14	22	2020	AG	AG9
III	P15	21	2020	AG	AG9
III	P16	26	2020	DT	DT9
III	P17	25	2020	DT	DT10
III	P18	19	2020	AG	AG9
III	P19	16	2020	AG	AG8

FIGURE 2
Cluster analysis of *speI* macrorestriction profiles of 84 *Edwardsiella ictaluri* isolates. Dendogram was generated using DICE and UPGMA algorithms with band tolerance set to 1%. Pulsotypes were defined by a cut-off value of ≥ 86% similarity (dashed line). For each isolate, PFGE cluster, pulsotype, year of recovery, province and farm are shown. Provinces include An Giang (AG), Ben Tre (BT), Can Tho (CT), Dong Thap (DT), Tien Giang (TG), United States of America (USA) and Vinh Long (VL). UF, unknown farm.

recovered between 2001 and 2003 ($p = 0.0188$) and PFGE cluster II ($p < 0.0001$) (Figure 4). Six (7%) isolates including the American type isolate were found to harbour all seven virulence genes screened (Figure 4) and for Vietnamese isolates were more likely to be associated with PFGE Cluster II ($p < 0.0001$).

4 Discussion

Exploring strain diversity of bacterial pathogens over time is critical to the development and maintenance of efficacious prevention strategies. In this study, the PFGE fingerprints of isolates from Vietnam clustered away from the type isolate recovered from United States, supporting previous work which suggest that American and Asian *E. ictaluri* isolates are genetically distinct (Bartie et al., 2012). Furthermore, results from the present study demonstrated low genetic diversity in *E. ictaluri* populations recovered from recent outbreaks of BNP in the Mekong Delta region of Vietnam, when compared with populations from historical outbreaks. These findings also support previous work by Bartie et al. (2012), who successfully typed 47 *E. ictaluri* isolates from disease outbreaks in the same region between 2001 and 2004

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TABLE 2 Distribution of pulsotypes of *Edwardsiella ictaluri* isolates recovered from disease outbreaks in striped catfish (*Pangasianodon hypophthalmus*) farms in different provinces of Vietnam between 2001 and 2021.

Pulsotype	AG	BT	CT	DT	TG	VL	Total	
A	-	-	-	-	-	-	0	0%
B	-	-	1	-	-	1	2	2%
C	3	-	2	-	-	-	5	6%
D	1	-	-	-	-	-	1	1%
E	-	-	2	-	1	-	3	4%
F	-	-	-	-	-	1	1	1%
G	-	-	1	1	-	-	2	2%
H	-	-	-	-	-	2	2	2%
I	-	-	-	2	-	-	2	2%
J	-	-	-	2	-	1	3	4%
K	10	-	7	1	-	1	19	23%
L	1	-	2	4	1	-	8	10%
M	2	-	-	1	1	2	6	7%
N	-	1	-	1	-	-	2	2%
O	-	1	-	2	-	1	4	5%
P	9	-	2	11	1	-	23	28%
Total	26	2	17	25	4	9	83	-
	31%	2%	20%	30%	5%	11%	-	-

Provinces include An Giang (AG), Ben Tre (BT), Can Tho (CT), Dong Thap (DT), Tien Giang (TG) and Vinh Long (VL).
-: No data.

using the same restriction enzyme. In their study, isolates resolved into 16 pulsotypes, corresponding to an SDI of 0.8936 (95% CI of 0.8874 – 0.8999), when typed using *speI* and applying a similar clustering threshold of ≥ 86% (data not shown). Findings from the current study identified a low genetic diversity of *E. ictaluri* overall, irrespective of outbreak period, as the SDI of the complete Vietnamese collection in this study was lower than the SDIs of other aquatic bacterial pathogen populations including *E. tarda* (SDI = 0.897) and *Y. ruckeri* (SDI = 0.93), when typed using *speI* or other restriction enzymes, respectively (Yang et al., 2013; Calvez et al., 2015). These findings are promising from the perspective of developing effective control strategies, as high pathogen diversity can be a major challenge for the development of cross-protective vaccines (Kennedy et al., 2020; Munang'andu et al., 2020).

The genomic profiles presented in this study identified genetic changes in the *E. ictaluri* populations circulating within the Vietnamese catfish farms. The mechanisms behind the genetic shifts identified within this bacterial population have not been elucidated in this study, but the virulence profile data may provide insight into changes in the pathogenesis from the varied pulsotypes. The gene *traD*, encoded within the T4SS, was only detected in isolates recovered before 2004 and similar omissions were reported by Rogge et al. (2013), who were unable to detect *virD4*, a homologue of *traD*, in *E. ictaluri* isolates recovered from Vietnam between 2006 and 2011. The T4SS is an important virulence system for bacterial pathogens, as the genes encoded mediate the translocation of macromolecules such as DNA and proteins across the cell envelope into eukaryotic or prokaryotic host cells (Mary et al., 2018). Indeed, the T4SS has been demonstrated to play a role in the virulence of other aquatic bacterial pathogens including *E. piscicida* and *Y. ruckeri*, through the transfer of plasmid DNA as well as secretion of proteins that promote immune evasion, respectively (Liu et al., 2017; Liu et al., 2022). Although its function in *E. ictaluri* is not presently known, *traD/virD4* has been identified as an ATP-ase "coupling protein" functioning in substrate recruitment in other bacterial species (Grohmann et al., 2018). In addition, this gene has also been implicated in F pilus assembly in *E. tarda* (Verjan-Garcia et al., 2015), thus similar functions may exist in *E. ictaluri*. Nonetheless, findings from this study suggest a shift in T4SS-driven virulence pathways for *E. ictaluri* isolates recently recovered from diseased striped catfish in Vietnam. These shifts may be driven by host-dependent evolution of *E. ictaluri* genotypes as reported by Machimbirike et al. (2021). This would certainly be supported by findings from previous studies, which found host-dependent variability in virulence factors including *traD/virD4* in channel catfish, Nile tilapia (*Oreochromis niloticus*), striped catfish and zebrafish (*Danio rerio*) (Griffin et al., 2016; Nhinh et al., 2022).

TABLE 3 Distribution of pulsotypes of *Edwardsiella ictaluri* isolates recovered from disease outbreaks in striped catfish (*Pangasianodon hypophthalmus*) farms in Vietnam between 2001 and 2021.

Pulsotype	2001	2002	2003	2004	2005	2006	2007	2009	2010	2011	2017	2018	2019	2020	2021	Total	
A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0%
B	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	2	2%
C	1	4	-	-	-	-	-	-	-	-	-	-	-	-	-	5	6%
D	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1%
E	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	3	4%
F	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	1%
G	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1	2	2%
H	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	2	2%
I	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	2	2%
J	-	-	-	1	1	-	-	-	1	-	-	-	-	-	-	3	4%
K	1	10	5	2	-	1	-	-	-	-	-	-	-	-	-	19	23%
L	-	-	-	-	-	4	1	1	1	1	-	-	-	-	-	8	10%
M	-	-	-	-	-	-	-	-	-	-	-	-	3	3	-	6	7%
N	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	2	2%
O	-	-	-	-	-	-	-	-	-	-	-	-	-	1	3	4	5%
P	-	-	-	-	-	-	-	-	-	-	2	1	-	20	-	23	28%
Total	2	17	6	3	1	7	1	1	3	1	2	1	4	29	5	83	-
	2%	20%	7%	4%	1%	8%	1%	1%	4%	1%	2%	1%	5%	35%	6%	-	-

-: No data.

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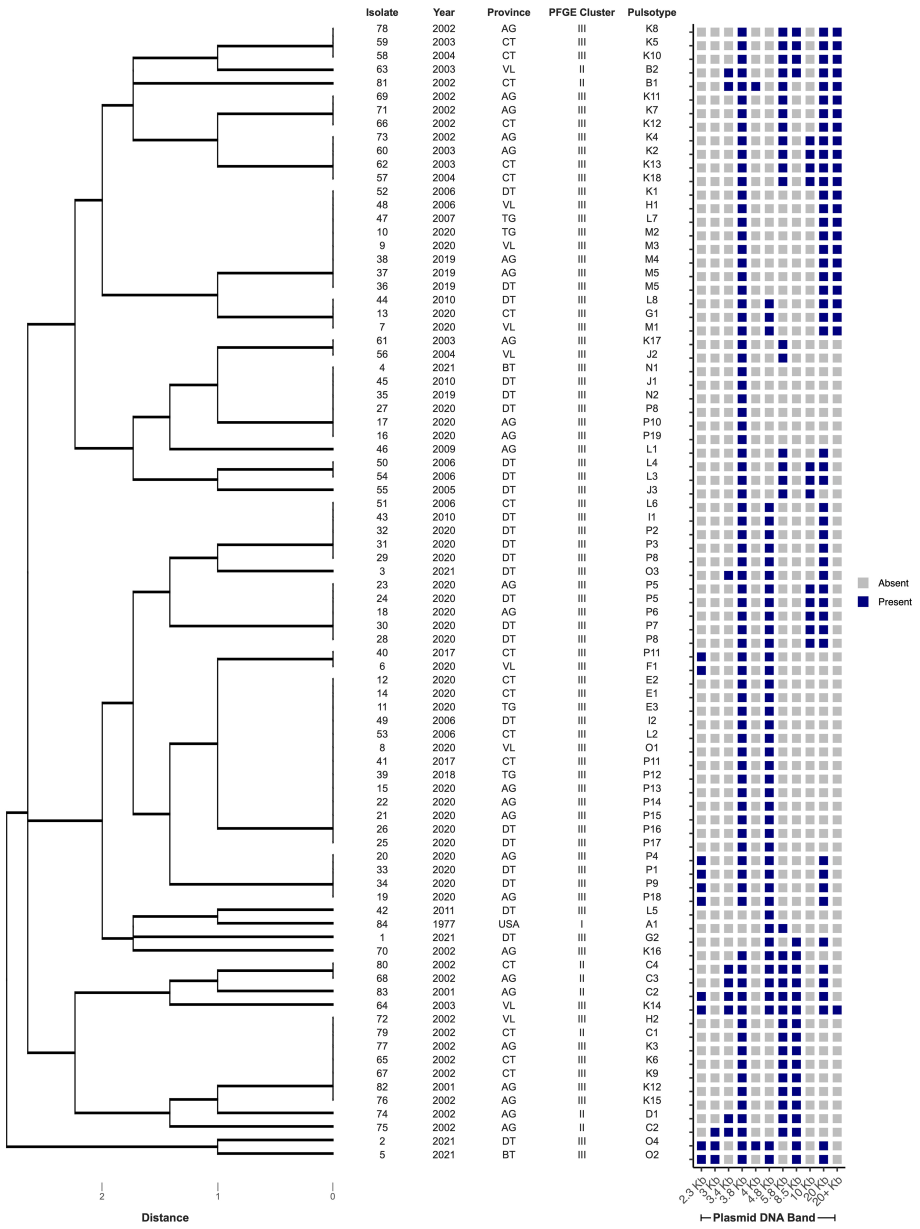

FIGURE 3
Cluster analysis of plasmid restriction DNA profiles of 84 *Edwardsiella ictaluri* isolates following digestion by *EcoRI*. Dendograms were generated using Euclidean distance and UPGMA. Provinces include An Giang (AG), Ben Tre (BT), Can Tho (CT), Dong Thap (DT), Tien Giang (TG), United States of America (USA) and Vinh Long (VL).

The PFGE of *E. ictaluri* isolates in this study found that a number of outbreaks from different provinces in 2019 and 2020 originated from a single population or a common source of contamination, as these isolates shared identical DNA fingerprints following restriction digest with *speI*. Furthermore, clonal isolates were more commonly shared between neighbouring provinces. Together, these findings would indicate either a single source of infection or a cross-contamination between farms and suggest that similar clones are circulating this region. These findings reflect the structure of the striped catfish sector within Vietnam, as clonal isolates were frequently identified in An Giang and Dong Thap provinces, where hatchery and nursery systems are concentrated (Hasan and Shipton, 2021), therefore the movement of fish stocks may be encouraging the spread of pathogenic populations including *E. ictaluri*. Likewise, production within open systems whereby water sources are shared, for example on the Mekong River, as well as the emergence of large scale vertically integrated companies across the Mekong Delta (Phu et al., 2016; Hasan and Shipton, 2021), may also account for the detection of clonal isolates from distant provinces. If anthropogenic factors are driving the spread of *E. ictaluri* populations across the sector, then biosecurity measures may help to mitigate the ongoing BNP epidemic in Vietnam. However, more

TABLE 4 Virulence gene profiles of *Edwardsiella ictaluri* isolates recovered from disease outbreaks in striped catfish (*Pangasianodon hypophthalmus*) farms in Vietnam between 2001 and 2021.

Gene	Profile I	Profile II	Profile III	Profile IV
eseJ	-	-	+	+
traD	-	+	-	+
hcp	+	+	+	+
fldA	+	+	+	+
wzx	+	+	+	+
fhuC	+	+	+	+
ureE	+	+	+	+
Total	23	4	51	5
	28%	5%	61%	6%

Virulence genes detected by PCR. -: gene absent; +: gene present.

work is required to investigate the spatiotemporal transmission of *E. ictaluri* in the region, especially under different active and passive transmission (e.g., through water currents) mechanisms.

In this study, subpopulations were also found to be circulating several farms across the Mekong Delta in the same year, as isolates recovered from these sites were clustered into different pulsotypes with varying genomic fingerprints. The presence of genetic subpopulations circulating the same fish farm has also been reported for *F. psychrophilum* in rainbow trout production systems in France (Calvez et al., 2021). In the present study, all isolates were recovered from diseased fish, therefore it is unlikely that the subpopulations detected were associated to the health status (e.g., carrier vs diseased) of fish. However, if fish originated from different production systems (e.g., ponds), the occurrence of different pulsotypes within these farms may therefore represent local adaptations of the subpopulations to the environmental niche within the individual production system. Local adaptation of bacterial pathogens is highly likely if these production systems experienced low level disease outbreaks which had been tolerated or poorly controlled. In fact, local adaptation in aquatic bacterial populations has previously been reported in other aquatic pathogenic species (Du et al., 2019; Fu et al., 2021), thus the same may also be true for *E. ictaluri*. These findings present a challenge for ongoing vaccination programs and current work is exploring the influence of genotypic shifts in relation to circulating antigenic profiles.

Considerable variation was noted in the plasmid profiles of Vietnamese *E. ictaluri* isolates in this study. No Vietnamese isolate was found to share the same plasmid profile as the American type isolate, which was found to harbour two plasmid bands at approximately 4.8 Kb and 5.8 Kb in size and likely the pEI1 (ca. 4.8 Kb) and pEI2 (ca. 5.6 Kb) plasmids detected previously in *E. ictaluri* isolates from channel catfish (Hawke et al., 2013). Similar sized plasmid bands were only found in 52% and 39% of the Vietnamese isolates, respectively. These findings follow that by Rogge et al. (2013), in which geographical differences were noted in plasmid DNA profiles between isolates from Vietnam and USA. Furthermore, in this study, the 5.8 Kb plasmid band was only detected in Vietnamese isolates recovered before 2010 suggesting a change in circulating plasmid populations within the region overtime, although sequencing of detected plasmid bands would be required to confirm this. Most isolates in this study were found to harbour two plasmid bands at 3.8 Kb and 4.8 Kb, with the former being detected in 98% of Vietnamese isolates. A similar sized plasmid has also been reported in *E. ictaluri* from striped catfish in Vietnam (4.0 Kb) as well as zebrafish from United States (3.9 Kb) (Rogge et al., 2013; Griffin et al., 2016). Furthermore, in the work by Griffin et al. (2016), a 3.9 Kb plasmid detected from zebrafish isolates was found to be a derivative of the channel catfish pEI2 plasmid, therefore, the plasmids at 3.8 and 4.8 Kb detected in Vietnamese *E. ictaluri* isolates in this study could be homologous to the pEI2 and pEI1 plasmids, respectively. Considerably larger plasmids (> 8 Kb) were also detected in several isolates in this study. Previous work by Bartie et al. (2012) also detected large plasmids (> 7 Kb) in *E. ictaluri* isolates recovered from Vietnam. However, it is difficult to compare these findings with our own, as the aforementioned study did not use restriction digestion to linearise extracted plasmids, therefore profiles may contain other forms (e.g., nicked or supercoiled) of plasmid DNA.

5 Conclusions

In conclusion, findings from this study demonstrate clonal expansion of *E. ictaluri* populations across several provinces within the Mekong Delta region of Vietnam, likely associated with anthropogenic factors. Hence, improving biosecurity strategies may offer an effective approach to mitigating ongoing BNP challenges in the sector. Furthermore, our data also shows that the *E. ictaluri* populations circulating the striped catfish sector have undergone genetic changes overtime, as evidenced in the observed changes in pulsotype, plasmid DNA and virulence gene profiles of

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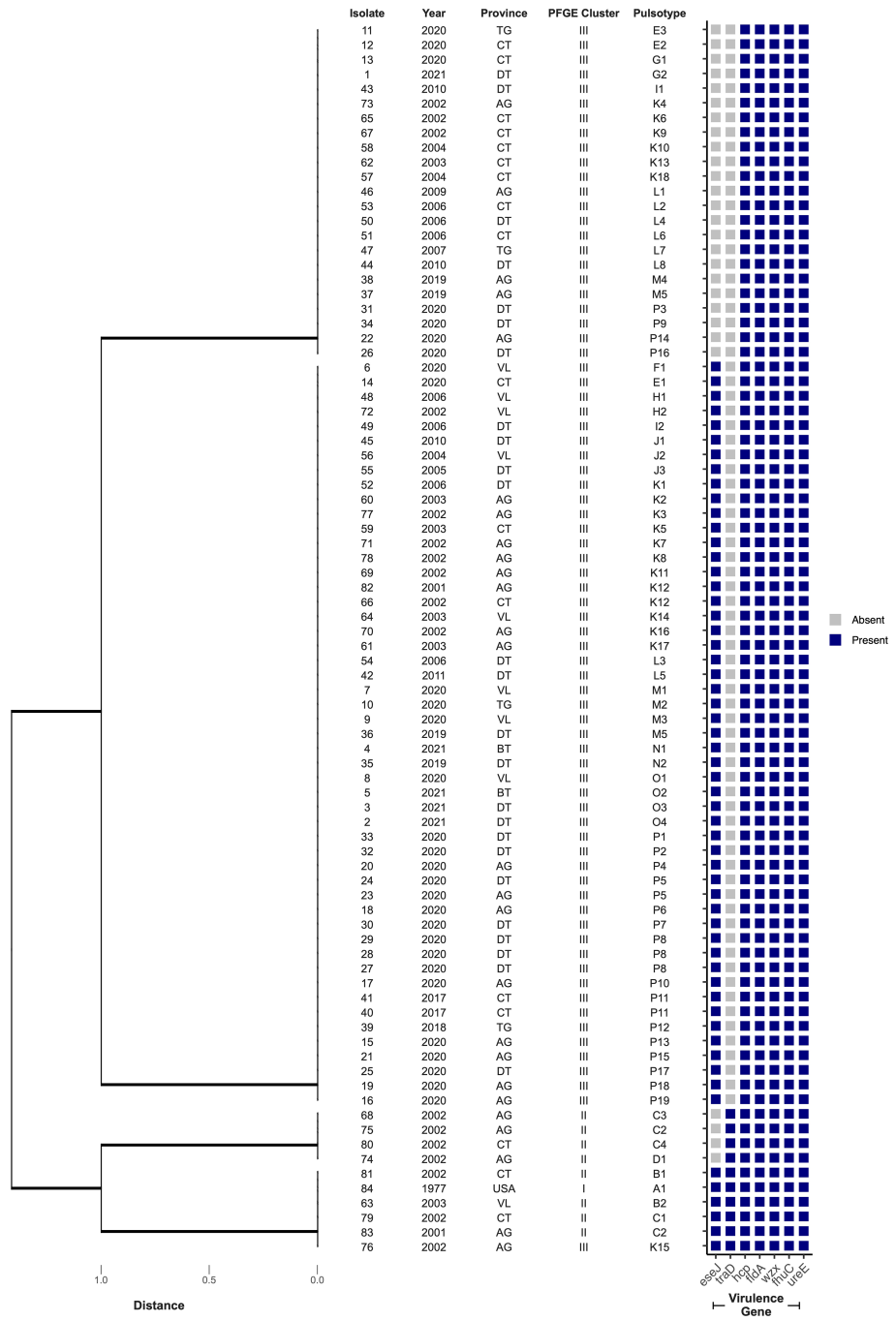

FIGURE 4
Cluster analysis of virulence gene profiles of 84 *Edwardsiella ictaluri* isolates. Dendograms were generated using Euclidean distance and UPGMA. Provinces include An Giang (AG), Ben Tre (BT), Can Tho (CT), Dong Thap (DT), Tien Giang (TG), United States of America (USA) and Vinh Long (VL).

isolates recovered from disease outbreaks between 2001 and 2021. These findings highlight the current and continued needs for surveillance of *E. ictaluri* in Vietnam, as any genetic changes may confer changes in antigenic proteins, thereby compromising the effectiveness of available vaccines to promote protection. Effective vaccination programs will be vital to the striped catfish sector to protect against future BNP outbreaks and curb any development of antibiotic resistance in *E. ictaluri* within the Mekong Delta region.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

CJP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. KG: Methodology, Writing – review & editing. VHP: Methodology, Writing – review & editing. NNP: Methodology, Writing – review & editing. TTD: Methodology, Writing – review & editing. LHP: Conceptualization, Funding acquisition, Writing – review & editing. MC: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1270968/full#supplementary-material

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