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Comparative Phenotypic and Genotypic Analysis of *Edwardsiella* Isolates from Different Hosts and Geographic Origins, with Emphasis on Isolates Formerly Classified as *E. tarda*, and Evaluation of Diagnostic Methods

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ABSTRACT *Edwardsiella* spp. are responsible for significant losses in important wild and cultured fish species worldwide. Recent phylogenomic investigations have determined that bacteria historically classified as *Edwardsiella tarda* actually represent three genetically distinct yet phenotypically ambiguous taxa with various degrees of pathogenicity in different hosts. Previous recognition of these taxa was hampered by the lack of a distinguishing phenotypic character. Commercial test panel configurations are relatively constant over time, and as new species are defined, appropriate discriminatory tests may not be present in current test panel arrangements. While phenobiochemical tests fail to discriminate between these taxa, data presented here revealed discriminatory peaks for each *Edwardsiella* species using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) methodology, suggesting that MALDI-TOF can offer rapid, reliable identification in line with current systematic classifications. Furthermore, a multiplex PCR assay was validated for rapid molecular differentiation of the *Edwardsiella* spp. affecting fish. Moreover, the limitations of relying on partial 16S rRNA for discrimination of *Edwardsiella* spp. and advantages of employing alternative single-copy genes *gyrB* and *sodB* for molecular identification and classification of *Edwardsiella* were demonstrated. Last, *sodB* sequencing confirmed that isolates previously defined as typical motile fish-pathogenic *E. tarda* are synonymous with *Edwardsiella piscicida*, while atypical nonmotile fish-pathogenic *E. tarda* isolates are equivalent to *Edwardsiella anguillarum*. Fish-nonpathogenic *E. tarda* isolates are consistent with *E. tarda* as it is currently defined. These analyses help deconvolute the scientific literature regarding these organisms and provide baseline information to better facilitate proper taxonomic assignment and minimize erroneous identifications of *Edwardsiella* isolates in clinical and research settings.

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The *Edwardsiella* genus was first recognized in the 1960s to describe a group of isolates that did not fit within any known group of *Enterobacteriaceae*. Initially referred to simply as "bacterium 1483-1459," this group included representatives of the "Bartholomew" group first isolated from a human patient with enteric fever and acute gastroenteritis (1) and possessed many similarities to the "Asakusa" group reported from snakes in Japan (2–4). Based on phenotypic differences between the 1483-1459 strains and other groups of *Enterobacteriaceae*, the genus was designated *Edwardsiella* and the species *E. tarda* was adopted to represent this previously undescribed group (5).

Prior to 2013, the genus consisted of only 3 taxa, *E. tarda*, *E. ictaluri*, and *E. hoshinae* (6), which represented a diverse group of Gram-negative bacteria infecting a wide range of piscine, reptilian, avian, and mammalian hosts (7). There are limited reports of *E. hoshinae* from a small number of avian and reptilian hosts (8, 9). Conversely, *E. ictaluri* is well studied as the causative agent of enteric septicemia of catfish (ESC) in catfish aquaculture in the southeastern United States (10). Although it is predominantly considered a pathogen of U.S. farm-raised channel catfish, reports have implicated *E. ictaluri* in mortality events in catfish aquaculture in Asia (11–14) and *Pangasius* catfish imported into the Caribbean (15). Moreover, *E. ictaluri* was reported from mortality events in tilapia pond culture in Central America, laboratory populations of zebrafish in the United States, and wild populations of ayu (*Plecoglossus altivelis*) in Japan (16–18).

Comparatively, *Edwardsiella tarda* is cited as the causative agent of edwardsiellosis in fish and has been reported from over 25 fish species and on all seven continents (7, 19, 20). It has also been isolated from reptiles, birds, and mammals; it has moderate zoonotic potential and is likely an opportunistic pathogen in young, elderly, and immunocompromised individuals (7, 21–25). Despite its wide host range, *E. tarda* has mostly been implicated in disease outbreaks in cultured fish and is considered one of the most important bacterial pathogens in global aquaculture (26–28).

Primarily viewed as a pathogen of marine and freshwater fish, *E. tarda* has extensive phenotypic and genetic diversity. In 2012, a comparative phylogenomic study demonstrated that isolates phenotypically identified as *E. tarda* comprised two genetically distinct, polyphyletic groups (29). This work was supported by concurrent investigations utilizing multilocus sequence analysis (MLSA) of *E. tarda* isolates in Asia and Europe, as well as genotypic and phenotypic analyses of *E. tarda* isolates from fish in the United States (30, 31). These studies concluded that isolates historically classified as *E. tarda* actually represented three genetically distinct yet phenotypically indistinguishable species. Further phenotypic characterization, DNA-DNA hybridization, and phylogenetic analyses led to the adoption of *E. piscicida* as a fourth member of the genus in 2013 (6). Expanding on these analyses, polyphasic phenotypic and genomic characterization of *Edwardsiella* isolates from diseased eels and other fishes led to the addition of a fifth species of *Edwardsiella*, *E. anguillarum*, in 2015 (32).

Prior to this recent segregation, research documenting phenotypic and genotypic diversity of *E. tarda* resulted in multiple generalized designations to account for the extensive intraspecific variability (28, 33–36). As a result, isolates primarily fell into one of three different categories: (i) typical motile fish-pathogenic *E. tarda*; (ii) atypical nonmotile fish-pathogenic *E. tarda*; and (iii) fish-nonpathogenic *E. tarda* (33, 37). The recent separation of *E. tarda* into three discrete taxa suggests that these designations likely correspond with the recent segregation (32, 38). The work described here employed routine phenotypic and genotypic analyses, coupled with popular microbial identification systems and molecular confirmatory methods, to evaluate current procedures for differentiating the *Edwardsiella* spp. and to link historical records and former *E. tarda* designations to current phylogenomic assignments and contemporary taxonomic nomenclature. In addition, the diverse collection of isolates provided a

unique opportunity to characterize plasmids from a variety of temporally and geographically discrete congeners and identify potential commonalities in plasmids carried by *Edwardsiella* spp. from different origins.

RESULTS

Motility and TSI. The motility and triple sugar iron medium (TSI) results for each isolate are listed in Table 1. The *E. hoshinae* isolate and all *E. piscicida* isolates were motile. Motility was also observed for the three *E. ictaluri* isolates, although dispersion was not as widespread. The observed motility of *E. anguillarum* and *E. tarda* isolates varied by isolate. All *Edwardsiella* isolates tested positive for glucose fermentation. No hydrogen sulfide production was observed in the *E. hoshinae* or *E. ictaluri* isolates; production from *E. anguillarum* isolates was weak. All *E. piscicida* and *E. tarda* isolates were positive for hydrogen sulfide production. Gas production was present in 6/7 *E. anguillarum*, 1/1 *E. hoshinae*, 0/3 *E. ictaluri*, 25/25 *E. piscicida*, and 10/11 *E. tarda* isolates.

Microbial identification systems. The API 20E system correctly identified all PCR-confirmed *E. tarda* and *E. hoshinae* isolates with ≥99% confidence. The three *E. ictaluri* isolates from three different fish hosts all produced an identical API code, in line with previous reports (17, 18), which resulted in an identification of *Escherichia coli* with a 52.7% confidence level (CL). Of the *E. piscicida* isolates tested, 64% (16/25) were identified as *E. tarda* (CL, 96.7% to 99.9%). The remaining 36% (9/25) of *E. piscicida* isolates produced codes that were nondefinitive as they represented multiple species. Similarly, 29% (2/7) of *E. anguillarum* isolates produced nondefinitive ambiguous codes. Of the remaining *E. anguillarum* isolates, 4/7 (57%) were identified as *E. tarda* (CL, 96.7% to 99.4%) and 1/7 (14%) was identified as *Vibrio parahaemolyticus* (CL, 53.2%). API 20E results are consistent with those reported previously for *Edwardsiella* spp. (27, 33, 39) and can be found in Table 2.

The Biolog microbial identification system identified all study isolates as members of *Edwardsiella* (*E. hoshinae*, *E. ictaluri*, or *E. tarda*) (Table 2) with various levels of confidence. The *E. hoshinae* isolate (CL, 98%) and the *E. ictaluri* isolates (CL, 70% to 97%) were both correctly identified. *E. tarda* isolate Edwardsiella 9.2 was identified as *E. ictaluri* (CL, 67%). All other *E. tarda* isolates (10 of 11; 91%) were identified in agreement with PCR results (CL, 62% to 96%). Similarly, *E. piscicida* isolates were identified as either *E. tarda* (17 of 25; 68%; CL, 58% to 95%) or *E. ictaluri* (8 of 25; 32%; CL, 62% to 88%). The *E. anguillarum* isolates also generated multiple codes, resulting in identifications of *E. ictaluri* (4/7; 57%; CL, 68% to 97%), *E. tarda* (2/7; 29%; CL, 70% to 76%), or *E. hoshinae* (1/7; 14%; CL, 69%).

The BBL Crystal Enteric/Nonfermentor identification kit also correctly identified the *E. hoshinae* isolate (CL, 99.9%) and all *E. tarda* isolates (CL, 70.3% to 99.9%). Of the three *E. ictaluri* isolates, only S97-773 (isolated from a diseased catfish) produced a code present in the BBL database, which identified it as *E. tarda* (78.6%). The *E. piscicida* isolates produced a variety of codes, all resulting in an identification of *E. tarda* from the BBL database with confidence levels ranging between 70.3% and 99.9%. Of the seven *E. anguillarum* isolates, four (57%) produced identical codes, which resulted in an identification of *Burkholderia gladioli* (CL, 94.4%). The remaining three *E. anguillarum* isolates produced similar codes resulting in an identification of *E. tarda* (CL, 70.3% to 99.9%). BBL Crystal codes are consistent with those reported previously for *Edwardsiella* (31, 38) and listed in Table 2.

The matrix-assisted laser desorption ionization (MALDI) identification score for each isolate, based on the Bruker Biotyper RTC v. 3.1 and microbial peptide mass spectrum database V5.0.0.0 (Bruker Daltonics, Billerica, MA), is displayed in Table 2. The Bruker MALDI-time of flight (TOF) method correctly identified all the *E. tarda*, *E. ictaluri*, and *E. hoshinae* isolates examined with an identification score above 2.0. All *E. piscicida* and *E. anguillarum* isolates tested were identified as *Edwardsiella tarda* with a score above 2.0. However, unique species-specific peptide mass peaks (*m*/*z*) at 7,628, 8,793, and 4,252 were observed in the spectral profiles for *E. anguillarum*, *E. piscicida*, and *E. tarda*, respectively (Fig. 1).

TABLE 1 Motility and TSI analysis of the isolates used in the current study

Isolate	Motility[a,b]	TSI[b]
E. anguillarum		
EA181011	−	K/A + gas + H2S (weak)
LADL05-105	+	K/A + gas + H2S (weak)
43472	+ (weak)	K/A + gas + H2S (weak)
43664	+ (weak)	K/A + H2S (weak); no gas
43473	+	K/A + gas + H2S (weak)
43659	+	K/A + gas + H2S (weak)
43651	+	K/A + gas + H2S (weak)
E. hoshinae ATCC 35051	+	A/A + gas
E. ictaluri		
11-149A	+ (weak)	K/A
RUSVM-1	+ (weak)	K/A
S97-773	+ (weak)	K/A
E. piscicida		
PB 07-309	+	K/A + gas
NFAVS-1	+	K/A + gas + H2S
Fr373.2	+	K/A + gas + H2S
HL1.1	+	K/A + gas + H2S
HL25.1	+	K/A + gas + H2S
HL32.1	+	K/A + gas + H2S
WFE1	+	K/A + gas + H2S
S11-285	+	K/A + gas + H2S
C1490	+	K/A + gas + H2S
CMT 8211-1	+	K/A + gas + H2S
REDS 81911-E	+	K/A + gas + H2S
RBR8.1	+	K/A + gas + H2S
SC 09-03	+	K/A + gas + H2S
ACC69.1	+	K/A + gas + H2S
CAQ 8.10	+	K/A + gas + H2S
CAQ 10.10	+	K/A + gas + H2S
CAQ 3.9	+	K/A + gas + H2S
A15-02670	+	K/A + gas + H2S
43628	+	K/A + gas + H2S
43662	+	K/A + gas + H2S (weak)
43644	+	K/A + gas + H2S
43475	+	K/A + gas + H2S
43658	+	K/A + gas + H2S
43468	+	K/A + gas + H2S
43656	+	K/A + gas + H2S
E. tarda		
Edwardsiella 9.1	+	K/A + gas + H2S
Edwardsiella 9.2	+	K/A + gas + H2S
Edwardsiella 9.3	+	K/A + gas + H2S
Edwardsiella 9.4	+	K/A + gas + H2S
FL95-01	+	K/A + gas + H2S
070720-1 3A	+	K/A + gas + H2S
070720-1 2HLDOM	+	K/A + gas + H2S
43657	−	K/A + gas + H2S
43650	+	K/A + gas + H2S
43627	+	K/A + gas + H2S
43663	−	K/A + H2S

[a]+, positive result; −, negative result.
[b]K/A, glucose fermentation only; A/A, glucose and lactose and/or sucrose fermentation; gas, gas production; H2S, sulfur reduction; (weak), positive result less robust than that observed in other samples.

Fatty acid methyl ester (FAME) analysis. The major fatty acid constituents of the *Edwardsiella* isolates were 14:0, 16:0, 17:0 cyclo, summed feature 3 (16:1 w7c/16:1 w6c and 16:1 w6c/16:1 w7c), and summed feature 8 (18:1 w7c and 18:1 w6c). Fatty acid analysis results are displayed in Table 3.

Antimicrobial susceptibility profiles. The MICs of 39 antimicrobial compounds were tested for all 47 *Edwardsiella* isolates in the current study, resulting in a range of

TABLE 2 Antimicrobial identification system results for isolates analyzed in the current study[a]

	API 20E			Biolog		BBL Crystal Enteric/Nonfermentor			MALDI-TOF	
Isolate	Code	ID	CL (%)	ID	CL (%)	Code	ID	CL (%)	ID	CS
E. piscicida										
PB 07-309	6364000[b]			*E. tarda*	65	2403010113	*E. tarda*	99.9	*E. tarda*	2.23
NFAVS-1	6764000[b]			*E. tarda*	81	2002010113	*E. tarda*	99.5	*E. tarda*	2.12
Fr373.2	4744000	*E. tarda*	99.4	*E. tarda*	87	2003010113	*E. tarda*	99.2	*E. tarda*	2.18
HL1.1	4744000	*E. tarda*	99.4	*E. tarda*	83	2003110113	*E. tarda*	70.3	*E. tarda*	2.25
HL25.1	4344000	*E. tarda*	99.4	*E. ictaluri*	67	2003110113	*E. tarda*	70.3	*E. tarda*	2.21
HL32.1	4744000	*E. tarda*	99.4	*E. tarda*	58	2003110113	*E. tarda*	70.3	*E. tarda*	2.24
WFE1	4764000	*E. tarda*	96.7	*E. tarda*	59	2003100113	*E. tarda*	98.7	*E. tarda*	2.23
S11-285	4744000	*E. tarda*	99.4	*E. tarda*	95	2403110113	*E. tarda*	99.9	*E. tarda*	2.25
C1490	6764000[b]			*E. tarda*	68	2002000113	*E. tarda*	99.9	*E. tarda*	2.13
CMT 8211-1	6764000[b]			*E. tarda*	86	2003000113	*E. tarda*	99.7	*E. tarda*	2.24
REDS 81911-E	4764000	*E. tarda*	96.7	*E. tarda*	58	2003100113	*E. tarda*	98.7	*E. tarda*	2.21
RBR8.1	6564000[b]			*E. ictaluri*	62	2003000113	*E. tarda*	99.7	*E. tarda*	2.26
SC 09-03	4764000	*E. tarda*	96.7	*E. tarda*	94	0403010113	*E. tarda*	99.9	*E. tarda*	2.20
ACC69.1	6564000[b]			*E. tarda*	62	2003000113	*E. tarda*	99.7	*E. tarda*	2.18
CAQ 8.10	6565000[b]			*E. ictaluri*	80	2002000113	*E. tarda*	99.9	*E. tarda*	2.22
CAQ 10.10	4564000	*E. tarda*	97.4	*E. ictaluri*	81	2003000113	*E. tarda*	99.7	*E. tarda*	2.18
CAQ 3.9	4544000	*E. tarda*	99.9	*E. ictaluri*	62	2002000113	*E. tarda*	99.9	*E. tarda*	2.25
A15-02670	4344000	*E. tarda*	99.4	*E. tarda*	81	2003110113	*E. tarda*	70.3	*E. tarda*	2.24
43628	4764000	*E. tarda*	96.7	*E. tarda*	83	2003110113	*E. tarda*	70.3	*E. tarda*	2.24
43662	6364000[b]			*E. tarda*	83	2003110113	*E. tarda*	70.3	*E. tarda*	2.24
43644	6764000[b]			*E. tarda*	74	2003100113	*E. tarda*	98.7	*E. tarda*	2.18
43475	4744000	*E. tarda*	99.4	*E. tarda*	62	2003110113	*E. tarda*	70.3	*E. tarda*	2.28
43658	4744000	*E. tarda*	99.4	*E. ictaluri*	88	2003110113	*E. tarda*	70.3	*E. tarda*	2.14
43468	6744000	*E. tarda*	99.4	*E. ictaluri*	69	2003110113	*E. tarda*	70.3	*E. tarda*	2.23
43656	4344000	*E. tarda*	99.4	*E. ictaluri*	67	2003110113	*E. tarda*	70.3	*E. tarda*	2.17
E. anguillarum										
EA181011	4744000	*E. tarda*	99.4	*E. tarda*	70	2002010113	*E. tarda*	99.5	*E. tarda*	2.15
LADL05-105	4344100	*V. parahaemolyticus*	53.2	*E. ictaluri*	68	2403014113	*E. tarda*	99.9	*E. tarda*	2.17
43472	6744100	*E. tarda*	99.4	*E. tarda*	76	2003114113	*B. gladioli*	94.4	*E. tarda*	2.31
43664	4764000	*E. tarda*	96.7	*E. hoshinae*	69	2003114113	*B. gladioli*	94.4	*E. tarda*	2.29
43473	6744100	*E. tarda*	99.4	*E. ictaluri*	76	2003110113	*E. tarda*	70.3	*E. tarda*	2.23
43659	6745100[b]			*E. ictaluri*	97	2003114113	*B. gladioli*	94.4	*E. tarda*	2.20
43651	6345100[b]			*E. ictaluri*	86	2003114113	*B. gladioli*	94.4	*E. tarda*	2.26
E. hoshinae ATCC 35051	4744120	*E. hoshinae*	99.9	*E. hoshinae*	98	0443014013	*E. hoshinae*	99.9	*E. hoshinae*	2.26
E. ictaluri										
11-149A	4004000	*E. coli*	52.7	*E. ictaluri*	97	2003010023[c]			*E. ictaluri*	2.31
RUSVM-1	4004000	*E. coli*	52.7	*E. ictaluri*	72	2002000103[c]			*E. ictaluri*	2.02
S97-773	4004000	*E. coli*	52.7	*E. ictaluri*	70	2002000113	*E. tarda*	78.6	*E. ictaluri*	2.32
E. tarda										
Edwardsiella 9.1	4744000	*E. tarda*	99.4	*E. tarda*	62	2002000113	*E. tarda*	99.9	*E. tarda*	2.33
Edwardsiella 9.2	4744000	*E. tarda*	99.4	*E. ictaluri*	67	0403110013	*E. tarda*	99.9	*E. tarda*	2.33
Edwardsiella 9.3	4744000	*E. tarda*	99.4	*E. tarda*	94	0402000013	*E. tarda*	99.9	*E. tarda*	2.29
Edwardsiella 9.4	4744000	*E. tarda*	99.4	*E. tarda*	71	2402000013	*E. tarda*	99.9	*E. tarda*	2.49
FL95-01	4744000	*E. tarda*	99.4	*E. tarda*	96	2002010113	*E. tarda*	99.5	*E. tarda*	2.39
070720-1 3A	6744000	*E. tarda*	99.4	*E. tarda*	72	2003110113	*E. tarda*	70.3	*E. tarda*	2.43
070720-1 2HLDOM	6744000	*E. tarda*	99.4	*E. tarda*	72	2002000113	*E. tarda*	99.9	*E. tarda*	2.26
43657	6744000	*E. tarda*	99.4	*E. tarda*	96	2003110113	*E. tarda*	70.3	*E. tarda*	2.34
43650	4744000	*E. tarda*	99.4	*E. tarda*	96	2003110113	*E. tarda*	70.3	*E. tarda*	2.34
43627	4744000	*E. tarda*	99.4	*E. tarda*	96	2003010113	*E. tarda*	99.2	*E. tarda*	2.47
43663	6744000	*E. tarda*	99.4	*E. tarda*	94	2003010113	*E. tarda*	99.2	*E. tarda*	2.30

[a]Abbreviations: CL, confidence level; CS, confidence score; ID, identification.
[b]Unacceptable profile in API, multiple species identifications possible.
[c]Profile not in BBL database, unable to provide an identification.

intraspecific and interspecific variation for each antimicrobial compound (Tables 4 and 5). However, no discriminatory antimicrobial compound was identified. For many of the carbapenems, cephalosporins, and macrolides, the MICs for different isolates within each *Edwardsiella* species were largely consistent. Greater variation among MICs was

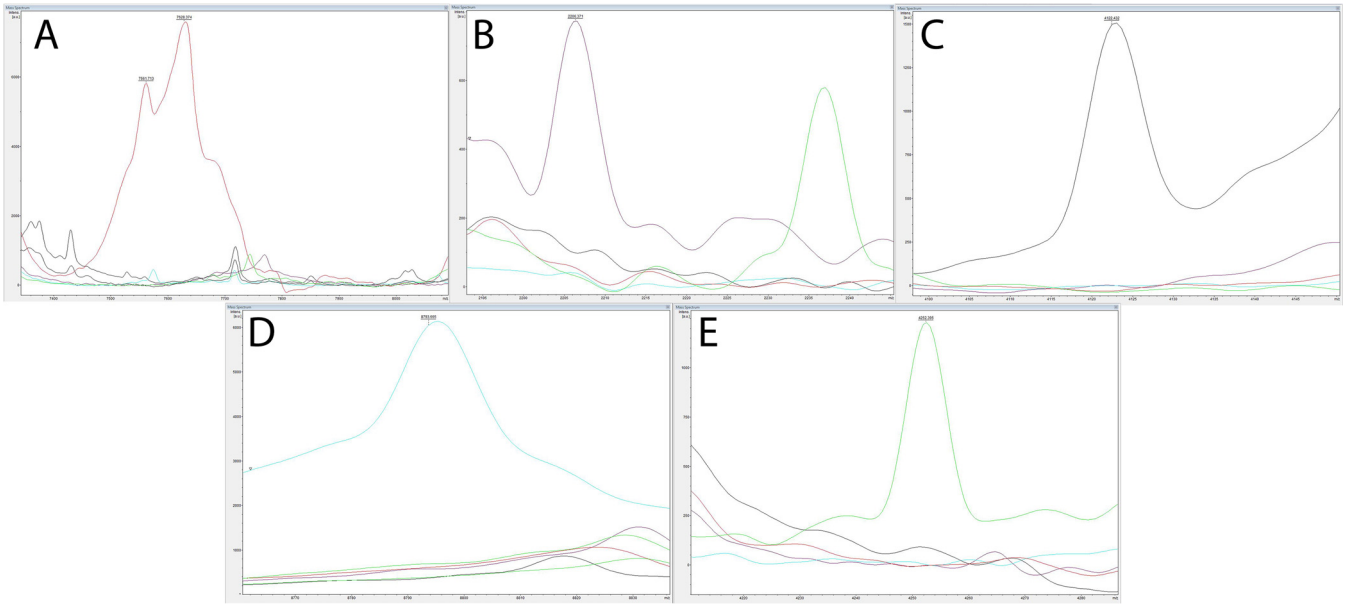

FIG 1 Unique peptide mass peaks generated from *E. anguillarum* (red) (A), *E. hoshinae* (purple) (B), *E. ictaluri* (black) (C), *E. piscicida* (blue) (D), and *E. tarda* (green) (E) using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) technology.

present for aminoglycosides and tetracyclines. The susceptibility of *E. piscicida* isolates to amoxicillin was more variable than that of the other *Edwardsiella* spp., with MICs ranging from ≤0.5 to 4 mg/liter. Similarly, the patterns of susceptibility of *E. anguillarum* isolates to penicillin displayed a greater degree of intraspecific variation than did those of the other *Edwardsiella* spp. The antimicrobial susceptibility profiles generated from this analysis were generally consistent with the putative antibiotic resistance function of plasmid-carried open reading frames (ORFs) (see Fig. 5, 6, and 7 and also Tables S1 and S2 in the supplemental material).

Phylogenetic analysis. Within groups, partial 16S rRNA sequences (1,062 bp) displayed high intraspecific similarity (99.90% to 100%). However, 16S rRNA had low discriminatory power among *Edwardsiella* congeners, with 99.15% to 99.91% interspecific similarity among them (Table 6; Fig. S1). Conversely, *gyrB* (1,800 bp) and *sodB* (461 bp) displayed high discriminatory power among *Edwardsiella* congeners (84.02% to 95.88% and 83.95% to 97.16%, respectively) while at the same time maintaining high intraspecific similarity (99.47% to 100% and 99.72% to 100%, respectively) (Fig. 2 and

TABLE 3 Mean percent fatty acid composition for *Edwardsiella* spp. analyzed in the current study

	Mean (SD) fatty acid content of species:				
Fatty acid	*E. hoshinae*[a]	*E. ictaluri*	*E. piscicida*	*E. anguillarum*	*E. tarda*
12:0	1.80	1.62 (0.08)	0.97 (0.18)	1.05 (0.16)	2.45 (0.18)
13:0	2.21	0.41 (0.15)	1.23 (0.49)	0.67 (0.18)[b]	0.69 (0.22)
14:0	9.41	11.15 (0.40)	15.06 (1.13)	14.10 (1.01)	11.22 (0.98)
16:0	22.51	28.29 (2.07)	26.65 (1.49)	30.83 (1.91)	29.24 (0.85)
17:0 cyclo	11.12	8.84 (3.91)	21.47 (5.02)	15.07 (3.11)	13.83 (2.79)
17:0	1.79	0.48 (0.17)	1.24 (0.36)	0.72 (0.37)	0.86 (0.27)
18:1 w9c	1.14	1.57 (0.16)	1.47 (0.22)	1.36 (0.16)	1.52 (0.12)
18:0	1.24	1.65 (0.44)	1.12 (0.21)	1.10 (0.17)	1.28 (0.16)
19:0 cyclo w8c	0.61	2.02 (1.44)	1.12 (0.50)	1.37 (0.40)	1.52 (0.44)
Summed feature 2	4.00	4.57 (0.52)	4.83 (0.46)	4.54 (0.35)	4.35 (0.26)
Summed feature 3	27.40	31.07 (3.58)	15.68 (5.70)	22.89 (4.29)	23.71 (3.43)
Summed feature 5	0.90	1.28 (0.08)	1.10 (0.21)	0.85 (0.16)	0.80 (0.06)
Summed feature 8	11.75	5.67 (1.97)	5.99 (1.01)	4.54 (0.56)	7.61 (1.09)

[a]Standard deviation could not be calculated; only 1 *E. hoshinae* isolate was analyzed.
[b]Fatty acid was present in only 5 of 7 *E. anguillarum* isolates analyzed.

TABLE 4 Antimicrobial susceptibilities to single compounds of *Edwardsiella* isolates analyzed in the current study[a]

		No. of strains with MIC (mg/liter):									
Antibiotic (range, mg/liter)	**Taxon**	**≤0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**≥256**
Aminoglycosides											
Amikacin (8–32)	All strains				47						
Gentamicin (0.5–8)	*E. anguillarum*	5	1								
	E. hoshinae		1								
	E. ictaluri	2	1								
	E. piscicida	23	2								
	E. tarda	5	6								
Neomycin (2–32)	All strains		47								
Spectinomycin (8–64)	*E. anguillarum*				7						
	E. hoshinae				1						
	E. ictaluri				1		1	1			
	E. piscicida				22		3				
	E. tarda				8		2		1		
Streptomycin (8–1,024)	All strains				45			1			1
Tobramycin (2–8)	All strains		47								
Carbapenems											
Doripenem (0.5–4)	All strains	47									
Ertapenem (0.25–8)	All strains	47									
Imipenem (0.5–8)	All strains	47									
Meropenem (0.5–8)	All strains	47									
Cephalosporins											
Cefepime (4–32)	All strains			47							
Cefazolin (1–16)	*E. anguillarum*	4		3							
	E. hoshinae	1									
	E. ictaluri	3									
	E. piscicida	13		10	2						
	E. tarda	9		2							
Ceftazidime (1–16)	All strains	46		1							
Ceftiofur (0.25–4)	All strains	47									
Ceftriaxone (0.5–32)	All strains	47									
Macrolides											
Erythromycin (0.12–4)	*E. anguillarum*			1		6					
	E. hoshinae			1							
	E. ictaluri					3					
	E. piscicida			1		24					
	E. tarda			1		10					
Tylosin tartrate (2.5–20)	All strains					1	1	45			
Penicillins											
Amoxicillin (0.25–16)	*E. anguillarum*	1	6								
	E. hoshinae	1									
	E. ictaluri	3									
	E. piscicida	1	9	11	4						
	E. tarda	10	1								
Ampicillin (8–16)	All strains				47						
Penicillin (0.06–8)	*E. anguillarum*			1	3	2	1				
	E. hoshinae	1									
	E. ictaluri	2		1							
	E. piscicida				4	10	11				
	E. tarda			6	3	2					
Piperacillin (16–32)	All strains					46				1	
Quinolones											
Ciprofloxacin (0.5–2)	All strains	46	1								
Enrofloxacin (0.12–2)	All strains	46	1								
Levofloxacin (1–8)	All strains	47									
Tetracyclines											
Minocycline (1–8)	*E. anguillarum*	1			3	3					
	E. hoshinae	1									
	E. ictaluri	3									

(Continued on next page)

TABLE 4 (Continued)

Antibiotic (range, mg/liter)	Taxon	No. of strains with MIC (mg/liter): ≤0.5	1	2	4	8	16	32	64	128	≥256
	E. piscicida	5		10	6	1	3				
	E. tarda	11									
Oxytetracycline (0.25–8)	*E. anguillarum*	2	4	1							
	E. hoshinae			1							
	E. ictaluri	3									
	E. piscicida	10	8	2		1	4				
	E. tarda	2	6	1			2				
Tetracycline (0.25–8)	*E. anguillarum*	2	5								
	E. hoshinae	1									
	E. ictaluri	2	1								
	E. piscicida	18	2	1			4				
	E. tarda	6	3				2				
Other											
Aztreonam (1–16)	All strains	46						1			
Clindamycin (0.5–4)	*E. anguillarum*	1				6					
	E. hoshinae			1							
	E. ictaluri					3					
	E. piscicida	1			5	19					
	E. tarda				7	4					
Florfenicol (1–8)	All strains	47									
Nitrofurantoin (32–64)	All strains						46			1	
Novobiocin (0.5–4)	*E. anguillarum*	2				5					
	E. hoshinae					1					
	E. ictaluri			1		2					
	E. piscicida			1	5	19					
	E. tarda	3	3	1	1	3					
Sulfadimethoxine (32–256)	*E. anguillarum*										7
	E. hoshinae										1
	E. ictaluri										3
	E. piscicida										25
	E. tarda						4		1		6
Sulfathiazole (32–256)	*E. anguillarum*										7
	E. hoshinae										1
	E. ictaluri										3
	E. piscicida										25
	E. tarda								1	1	6
Tigecycline (1–8)	*E. anguillarum*	6		1							
	E. hoshinae	1									
	E. ictaluri	3									
	E. piscicida	22		3							
	E. tarda	11									

[a]Antimicrobial susceptibilities were determined by the broth microdilution method using the Sensititre GN4F and Avian1F plates, according to the manufacturer's protocol. Numbers in the lowest concentration of the antibiotic represent the maximal MIC at this concentration. An MIC higher than the highest concentration tested is cited in the subsequent higher concentration step.

Table 6; also Fig. S2). *E. anguillarum* and *E. piscicida* shared the highest similarity with one another, with 95.88% at *gyrB* and 97.16% at *sodB*. Conversely, *E. hoshinae* and *E. ictaluri* were the most divergent, with 84.02% identity at *gyrB* and 83.95% at *sodB*. Intragenomic 16S rRNA gene heterogeneity for each *Edwardsiella* species ranged from 0.0 to 0.6% (Table 7).

Genetic fingerprinting. Similar to the phylogenetic analysis, repetitive extragenic palindromic PCR (rep-PCR) profiles for *Edwardsiella* isolates formed five distinct clusters representing the five taxa of *Edwardsiella*, regardless of primer set. Of the four primer sets evaluated, the BOX and GTG_5 primers demonstrated the smallest amount of intraspecific variability (Fig. 3), with the BOX primer generating the most consistent patterns within groups. UPGMA (unweighted pair group method using arithmetic averages) analysis based on the BOX primer placed these five clusters within two larger phylogroups. In line with previous reports, *E. piscicida*, *E. anguillarum*, and *E. ictaluri* formed one cluster, and the other group contained *E. tarda* and *E. hoshinae* isolates. The genetic profiles of *E. anguillarum*, *E. ictaluri*, and *E. piscicida* all shared greater than 90%

TABLE 5 Antimicrobial susceptibilities to combinatory compounds of *Edwardsiella* isolates analyzed in the current study[a]

Antibiotic and MIC (mg/liter)	No. of strains with MIC by taxon:					
	All strains	*E. anguillarum*	*E. hoshinae*	*E. ictaluri*	*E. piscicida*	*E. tarda*
Ampicillin-sulbactam						
≤4/2	47					
8/4						
≥16/8						
Trimethoprim-sulfamethoxazole						
≤0.5/9.5		4	1	1		10
1/19						
2/38		2			9	
≥4/76		1			8	1
Ticarcillin-clavulanic acid						
≤8/2	47					
16/2						
32/2						
≥64/2						
Piperacillin-tazobactam						
≤8/4	47					
16/4						
32/4						
64/4						
≥128/4						

[a]Antimicrobial susceptibilities were determined by the broth microdilution method using the Sensititre GN4F and Avian1F plates, following the manufacturer's protocol. Numbers in the lowest concentration of the antibiotic represents the maximal MIC at this concentration. An MIC higher than the highest concentration tested is cited in the subsequent higher concentration step.

similarity within their respective taxa. The profiles of *E. tarda* isolates demonstrated the greatest intraspecific variability, with 60% to 96.4% similarity among isolates (Fig. S3).

mPCR. The multiplex real-time PCR (mPCR) assay was repeatable and reproducible, with linear dynamic ranges covering at least 5 orders of magnitude. Disproportionately large quantities of nontarget DNA had no marked effect on amplification efficiency; dilution curves and amplification plots were comparable when run with each *Edwardsiella* target genomic DNA (gDNA) alone or in the presence of nontarget gDNA (Table 8; Fig. 4) with a quantifiable limit of ~100 copies of target DNA. Reaction efficiencies

TABLE 6 Percent similarity matrix between *Edwardsiella* spp. across 1,062 bp of the 16S rRNA locus, 1,800 bp of the *gyrB* locus, and 461 bp of the *sodB* locus

Locus and species	% similarity for species:				
	E. anguillarum	*E. hoshinae*	*E. ictaluri*	*E. piscicida*	*E. tarda*
16S rRNA					
E. anguillarum	100.00				
E. hoshinae	99.15	100.00			
E. ictaluri	99.91	99.25	100.00		
E. piscicida	99.72	99.05	99.63	99.99	
E. tarda	99.34	99.81	99.26	99.24	99.90
gyrB					
E. anguillarum	99.94				
E. hoshinae	84.05	100.00			
E. ictaluri	94.61	84.02	99.73		
E. piscicida	95.88	84.72	94.82	99.78	
E. tarda	85.02	88.86	84.70	85.81	99.47
sodB					
E. anguillarum	99.81				
E. hoshinae	86.86	100.00			
E. ictaluri	92.56	83.95	99.81		
E. piscicida	97.16	86.99	92.39	99.97	
E. tarda	88.54	91.38	86.16	89.12	99.72

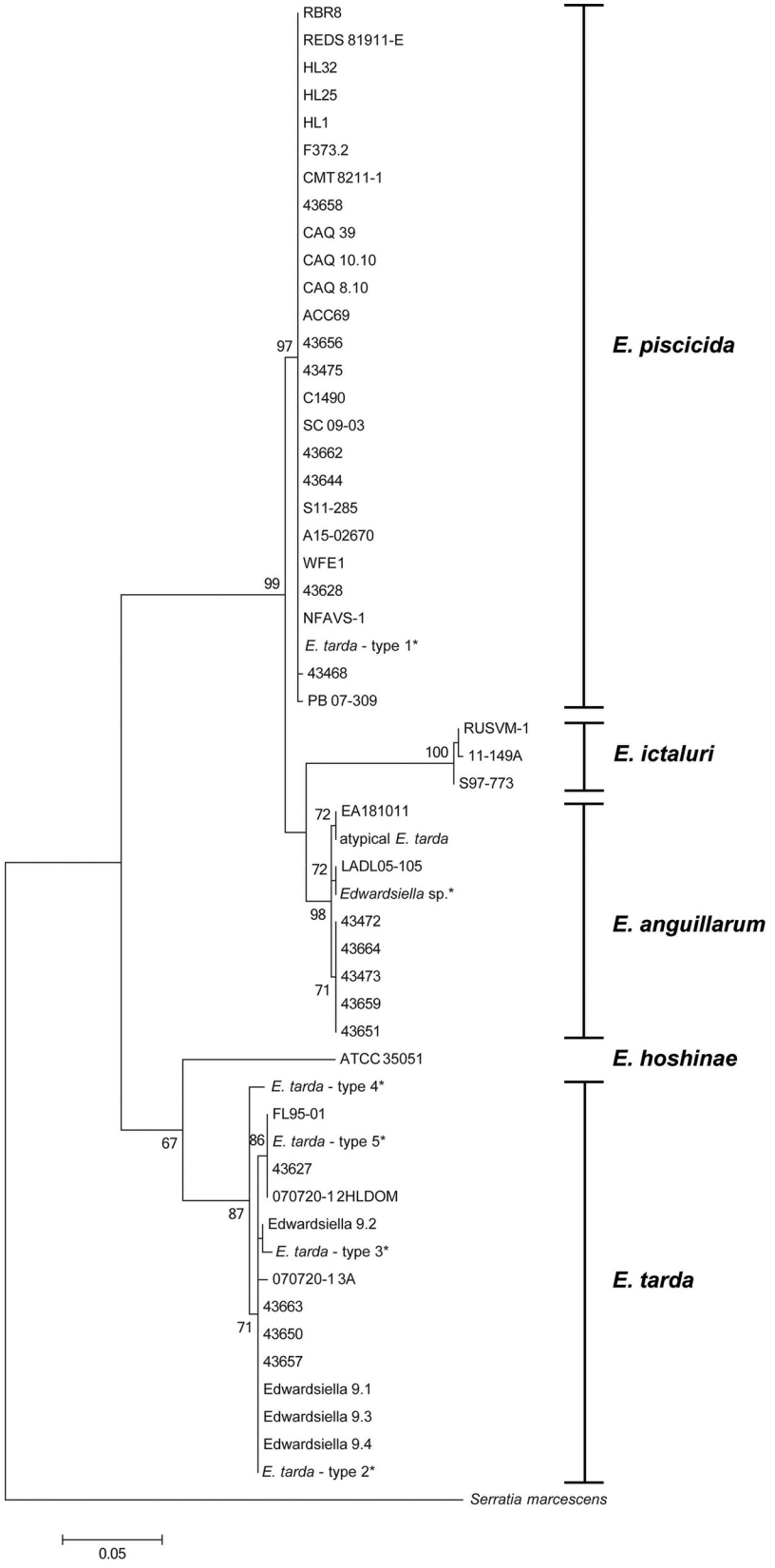

FIG 2 Phylogenetic relationships of *Edwardsiella* spp. based on *sodB* gene sequence. Relatedness was inferred from the maximum likelihood method based and rooted at *Serratia marcescens*. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The isolates described by Yamada and Wakabayashi (33) are marked with asterisks.

TABLE 7 Intragenomic heterogeneity of 16S rRNA for representative *Edwardsiella* genomes

Isolate	No. of 16S copies in genome	No. of differences (bp)	Dissimilarity range (%)
E. anguillarum LADL05-105	9	0–4	0.0–0.3
E. hoshinae ATCC 35051	9	0–3	0.0–0.2
E. ictaluri 93-146	8	0–3	0.0–0.2
E. piscicida S11-285	10	0–6	0.0–0.4
E. tarda FL95-01	9	0–9	0.0–0.6

were calculated (40) from the slope of the log-linear portion of the serial 10-fold dilutions for each *Edwardsiella* species and were within the generally accepted range of 90% to 110% (41).

Plasmid analysis. Twenty-one (45%) of the *Edwardsiella* isolates carried plasmids. Summaries of open reading frames and the putative functions of their predicted

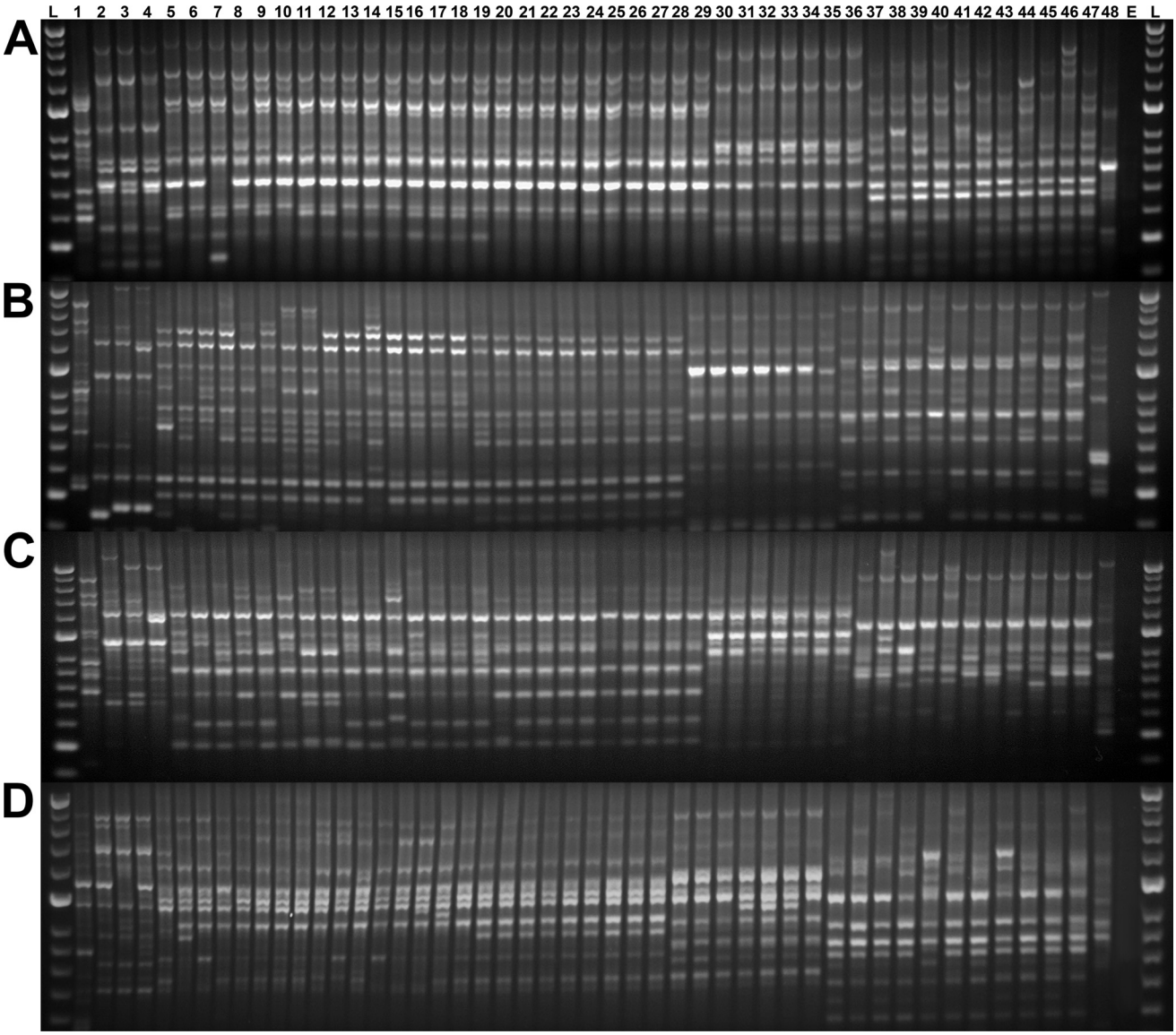

FIG 3 Genetic fingerprints of *Edwardsiella* spp. generated from repetitive extragenic palindromic PCR amplification of gDNA from *Edwardsiella hoshinae* (lane 1), *E. ictaluri* (lanes 2 to 4), *E. piscicida* (lanes 5 to 29), *E. anguillarum* (lanes 30 to 36), and *E. tarda* (lanes 37 to 47) using *E. coli* as an outlier (ATCC 25922, lane 48), a no-template control (lane E), and concurrently run standards (Hyperladder 50 bp, lanes L). Genetic profiles were generated using BOX (A), ERIC I and II (B), ERIC II (C), and GTG_5 (D) primers.

TABLE 8 Specificity of the mPCR assay for each respective target[a]

Species	Mean (SD) C_q				
	gDNA alone from species:				All *Edwardsiella* gDNA mixed together
	E. anguillarum	*E. ictaluri*	*E. piscicida*	*E. tarda*	
E. anguillarum	23.28 (0.07)	—	—	—	23.21 (0.09)
E. ictaluri	—	22.61 (0.10)	—	—	22.49 (0.05)
E. piscicida	—	—	22.63 (0.15)	—	22.50 (0.09)
E. tarda	—	—	—	22.93 (0.13)	22.50 (0.12)

[a]The user-defined fluorescence threshold for C_q determination was set at 50 relative fluorescence units. Dashes indicate no amplification of DNA.

proteins from *E. piscicida* and *E. tarda* plasmids can be found in Tables S1 and S2. Physical maps of isolated plasmids are available in Fig. 5 to 7. Each of the three *E. ictaluri* isolates carried two plasmids, consistent with previous reports for these isolates (42). Nine of the *E. piscicida* isolates (Fr373.2, HL1.1, HL25.1, HL32.1, RBR8.1, ACC69.1, CAQ 8.10, CAQ 10.10, and CAQ 3.9) recovered from turbot between 2005 and 2012 in Europe shared an identical plasmid of 3,782 bp. Four additional *E. piscicida* isolates (PB 07-309, S11-285, SC 09-03, and 43644) from various hosts carried plasmids of different sizes and compositions. The 3,164-bp plasmid identified in isolate S11-285 was in agreement with previous reports (43). Several *E. tarda* isolates carried two plasmids: 070720 3A (2,241 and 6,544 bp), Edwardsiella 9.1 (4,102 and 4,067 bp), Edwardsiella 9.3 (2,328 and 3,189 bp), and Edwardsiella 9.4 (6,920 and 65,317 bp). Additionally, *E. tarda* isolate Edwardsiella 9.2 carried one plasmid of 27,938 bp. No plasmids were detected in any of the *E. anguillarum* isolates or *E. hoshinae* isolate ATCC 35051, consistent with

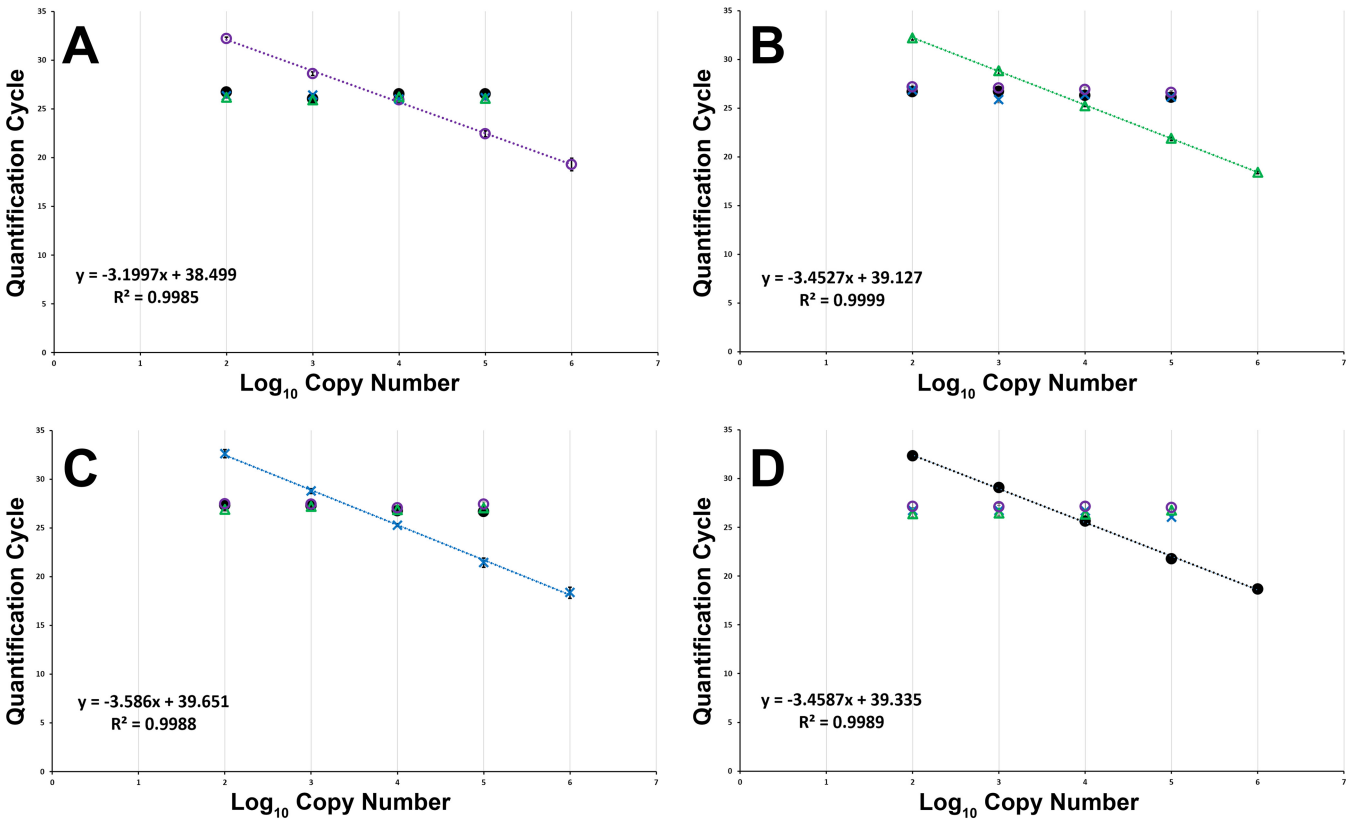

FIG 4 Mean quantification cycles (C_q) for known serial 10-fold dilutions of *E. anguillarum* (○) (A), *E. ictaluri* (△) (B), *E. piscicida* (×) (C), and *E. tarda* (●) (D). A dilution series for each assay was performed in the presence of an equal mixture of ~10,000 copies of each nontarget *Edwardsiella* sp. gDNA. Error bars indicate standard deviations generated from samples run in triplicate on 3 separate plates. The user-defined baseline threshold for C_q determination was set at 50 relative fluorescence units for all runs.

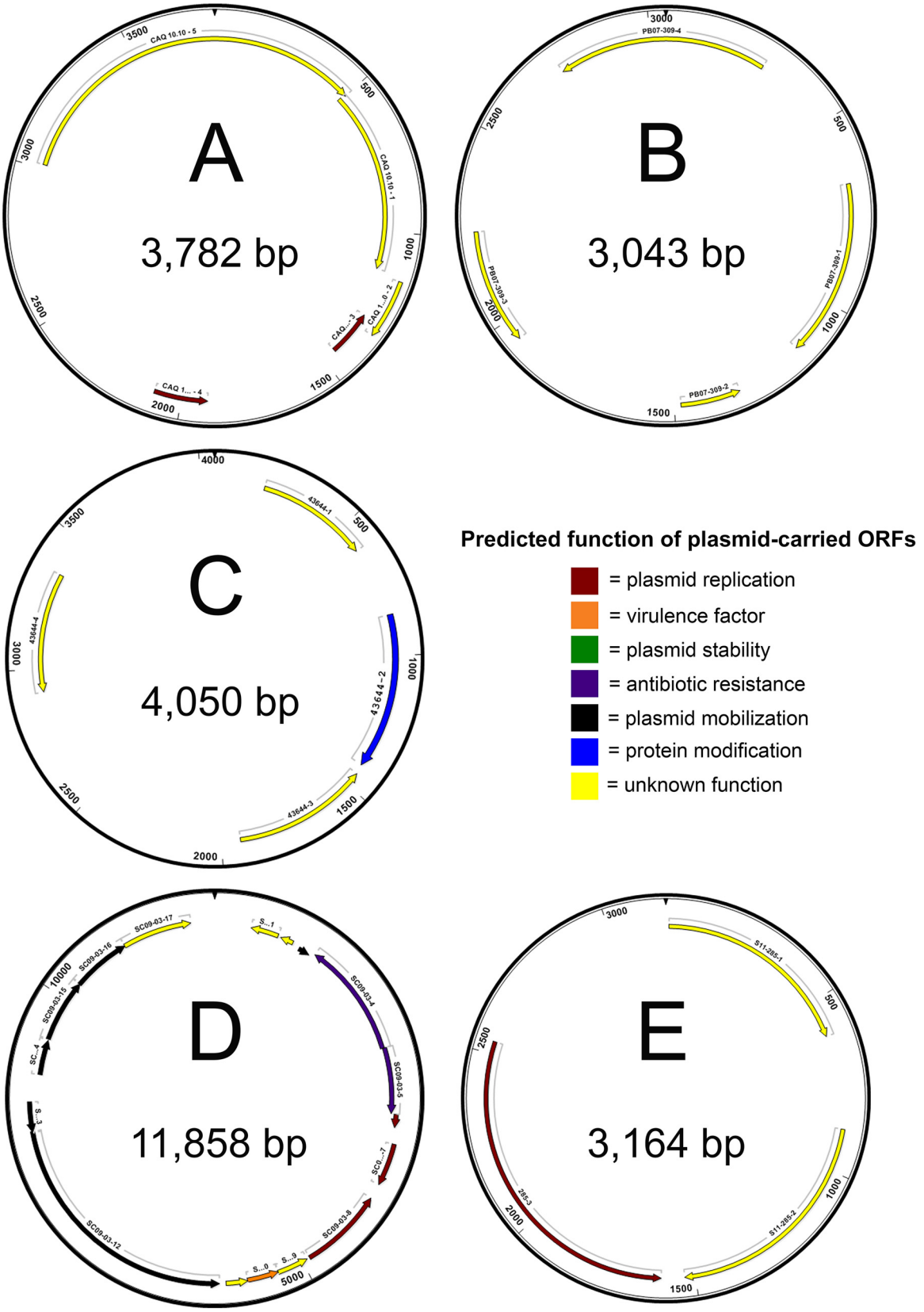

FIG 5 Physical maps of complete nucleotide sequences of plasmids harvested from *E. piscicida* isolates CAQ 10.10, CAQ 3.9, HL1.1, HL25.1, HL32.1, ACC69.1, CAQ 8.10, Fr373.2, and RBR8.1 (A), PB 07-309 (B), 43644 (C), SC 09-03 (D), and S11-285 (E). Maps indicate locations of predicted open reading frames (ORFs), which are color coded according to predicted function. Predicted products and putative functions of ORFs are provided in Table S1.

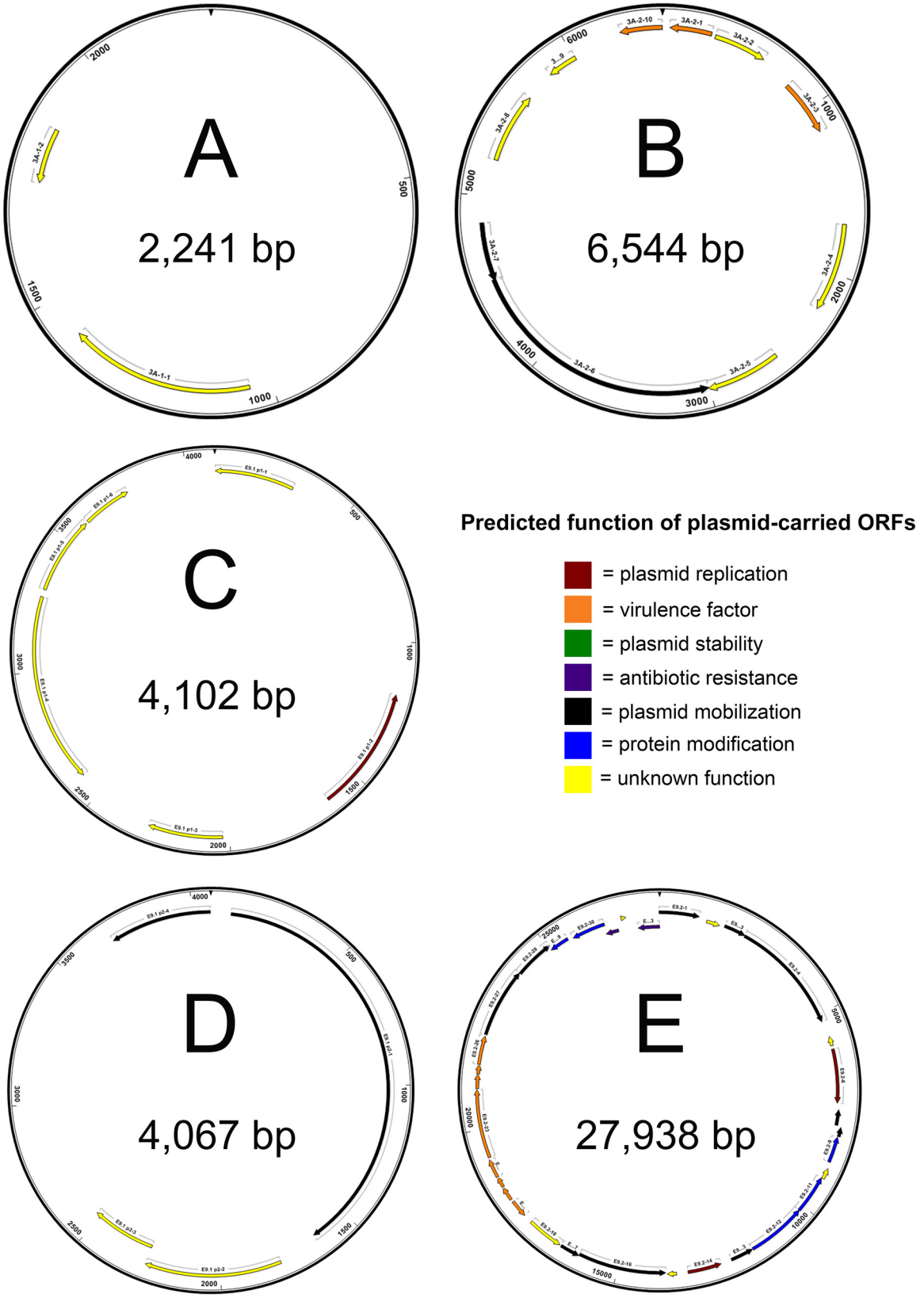

FIG 6 Physical maps of complete nucleotide sequences of plasmids harvested from *E. tarda* isolates 070720-1 3A (A and B), Edwardsiella 9.1 (C and D), and Edwardsiella 9.2 (E). Maps indicate locations of predicted open reading frames (ORFs), which are color coded according to predicted function. Predicted products and putative functions of ORFs are provided in Table S2.

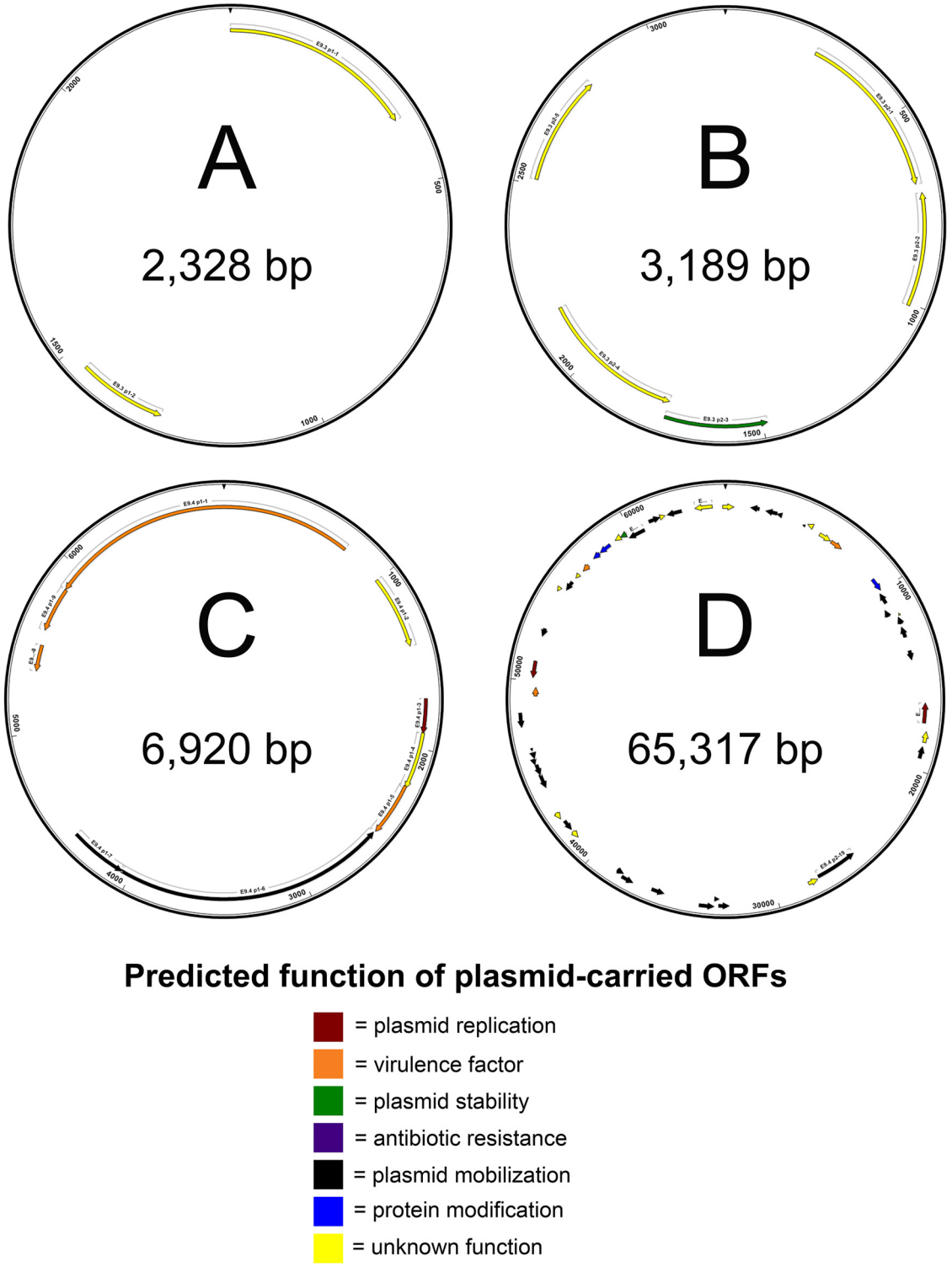

FIG 7 Physical maps of complete nucleotide sequences of plasmids harvested from *E. tarda* isolates Edwardsiella 9.3 (A and B) and Edwardsiella 9.4 (C and D). Maps indicate locations of predicted open reading frames (ORFs), which are color coded according to predicted function. Predicted products and putative functions of ORFs are provided in Table S2.

previous reports (44–46). Alignment of circularized plasmids mapped in this study revealed a variety of locally colinearized blocks (LCBs) shared between some isolates that also matched several *Edwardsiella* plasmid sequences present in GenBank (data not shown). However, plasmids varied widely in content and arrangement and a conserved

LCB or collection of LCBs indicative of a stable plasmid backbone present in all *Edwardsiella* plasmids was not identified.

DISCUSSION

Recent investigations into the phenotypic and genotypic variation of *E. tarda* have led to the recognition that isolates previously classified as *E. tarda* actually represent three distinct taxa: *E. tarda*, *E. piscicida*, and *E. anguillarum*. In light of these findings, the current study was intended to characterize the five *Edwardsiella* species using common phenotypic and genotypic analyses and demonstrate the importance of updating microbial identification systems to reflect contemporary systematics.

Previous work demonstrated variations in biochemical profiles of *Edwardsiella* isolates from different fish hosts and geographic origins (31, 38, 47). The work described here is consistent with these previous studies, with extant intraspecific phenotypic variation within some groups. This is not surprising, given the diversity of fish hosts and the broad geographic distribution and wide temporal range of these isolates. Marked inter- and intraspecific variation was also present in fatty acid content; however, no discriminatory fatty acid was identified, in line with previous findings (31). Similarly, antimicrobial susceptibility profiles were variable within groups and no discriminatory antibiotic agent was identified. Although conventional phenotypic methods are user friendly and relatively inexpensive, certain groups of bacteria are difficult to identify using conventional techniques, specifically rare isolates or isolates with ambiguous profiles (48).

The four microbial identification systems in this study correctly identified the *Edwardsiella* taxa that are recognized and validated for each respective system. However, none of the four databases associated with the systems used here recognize *E. anguillarum* or *E. piscicida*. The increasing use of molecular techniques and the growing number of new bacterial taxa identified using genomics technology pose a problem for phenotype database management, resulting in prokaryote databases that lag behind evolving systematics (49). Moreover, commercial test panel configurations are relatively constant over time, and as new species are defined, more appropriate discriminatory metabolic phenotypic tests may not be present in current test panel arrangements (50). Furthermore, many microbial identification databases still consider 16S rRNA the gold standard for taxon identification (50), the limitations of which are discussed below.

Within the species formerly classified as *E. tarda*, no distinct phenotypic patterns emerged among API 20E and BBL Crystal codes. In addition, no confirmative identifying profile was apparent using the Biolog microbial identification system. It is worth noting, however, that intraspecific variation in phenotypic characters was noted within *Edwardsiella* species. This is consistent with previous work that failed to identify a discriminatory metabolic fingerprint to differentiate among different *E. tarda* phylogroups (31). This suggests that isolates identified phenotypically as *E. tarda*, regardless of the identification system employed, require supplemental confirmation. In light of these findings, and given the rapidly increasing number of representative *Edwardsiella* genomes available, further work establishing a discriminatory metabolic profile for each *Edwardsiella* species is warranted.

Similarly, the role of 16S rRNA sequence for differentiation of the *Edwardsiella* species has recently been called into question (20, 31, 38, 42). The utility of 16S rRNA for bacterial identification has long been a topic of debate, largely due to the high percentage of sequence similarity between closely related species, the lack of a definitive intraspecific dissimilarity value, and the absence of universal guidelines (49–51). Moreover, some organisms possess multiple heterogeneous copies of rRNA, complicating the differentiation between closely related species if intragenomic heterogeneity exceeds interspecific variability (52, 53). As demonstrated in the present research, the intragenomic heterogeneity among *Edwardsiella* 16S rRNA sequences ranges from 0.0 to 0.6%, which exceeds the interspecific variability previously reported for this group (31, 38, 50).

As a result of these limitations, high 16S rRNA sequence identity (>99%) does not

always imply accuracy in microbial identifications, especially in closely related genera (53, 54). This is important to note as many contemporary studies still rely on partial 16S rRNA sequences for molecular confirmation of bacterial identification, often citing 16S rRNA sequences deposited in the National Center for Biotechnology Information's (NCBI's) GenBank and the International Nucleotide Sequence Database (55, 56). These databases are non-peer reviewed and generally accept any listed name and sequence that is submitted. This poses a problem when attempting to identify unknown microorganisms, wherein erroneous identification can occur if archived sequences are inaccurate or misclassified (57). This is further complicated by 16S rRNA searches where inconsistent sequence ends, ambiguous entries, pseudogaps, and insertions can result in misleading sequence matches (54).

Although 16S sequencing is useful in identifying unknown isolates to genus level, the discriminatory power significantly diminishes at the species level, especially in closely related species (48). In these instances, alternative reference genes should be considered. The single-copy *gyrB* gene, encoding the ATPase domain of DNA gyrase, is essential for DNA replication and is present in all prokaryotes. It contains conserved motifs that facilitate the development of genus-specific or family-specific primers (52). The *gyrB* gene has been used to explore the diversity of a wide range of bacteria and is more resolute than 16S rRNA in differentiating closely related members of the *Enterobacteriaceae*, including the *Edwardsiella* spp. (31, 38, 58). The utility of *gyrB* in *Edwardsiella* classification and identification has been demonstrated elsewhere (31, 38, 42), and the work reported here further supports the use of *gyrB* as an appropriate marker for discrimination of *Edwardsiella* species.

Similar to *gyrB*, the iron-cofactored superoxide dismutase gene (*sodB*) has high discriminatory power among *Edwardsiella* spp. Prior to the segregation of *E. tarda* and the identification of *E. piscicida* and *E. anguillarum* as discrete taxa, an internal fragment of *sodB* was used to distinguish between fish-pathogenic and fish-nonpathogenic *E. tarda* (33). This work raised questions whether fish-pathogenic *E. tarda* and the fish-nonpathogenic *E. tarda* type strain from humans (ATCC 15947) were truly monophyletic. The present analysis of *sodB* sequences found similar groupings and allowed for correlation between these historical analyses and contemporary nomenclature.

The current work confirms that what was defined as typical motile fish-pathogenic *E. tarda* is synonymous with *E. piscicida*. *E. piscicida* isolates in the current study share 99.8% to 100% similarity at *sodB* to typical motile fish-pathogenic *E. tarda* isolates described previously (33) (GenBank AB009853). Similarly, *sodB* sequence analysis showed that atypical nonmotile fish-pathogenic *E. tarda* is conspecific with *E. anguillarum*, while isolates identified here as *E. tarda* were found to be synonymous with fish-nonpathogenic *E. tarda* (including the *E. tarda* type strain from humans, ATCC 15947). This agrees with previous genomic assessments demonstrating high genome sequence homology (>97%) between the typical motile (NUF806) and atypical nonmotile (FPC503) *E. tarda* strains characterized by Matsuyama et al. (34) and the new species *E. piscicida* and *E. anguillarum*, respectively (32, 37).

Repetitive extragenic palindromic PCR (rep-PCR) fingerprinting is another common molecular technique used to estimate the relative degrees of similarity between bacterial isolates (59, 60). The rep-PCR analysis in this study produced distinctive banding patterns for each member of the *Edwardsiella* genus, with some intraspecific variation. This variation was anticipated and congruent with previous research (31, 38, 42), demonstrating the ability of rep-PCR to distinguish among *Edwardsiella* spp.

Plasmid analysis revealed that slightly less than half of the bacterial isolates in this study carry at least one native plasmid. Plasmid content included several predicted genes associated with replication, antibiotic resistance, and virulence, although this content varied by group and by isolate. The plasmids harvested from *E. ictaluri* isolates in this study supported previous characterization (42). While these *E. ictaluri* plasmids are similar in size, they differ in composition and arrangement, which is likely a function of being isolated from different fish hosts in different geographic locales.

Nine of the *E. piscicida* isolates (Fr373.2, HL1.1, HL25.1, HL32.1, RBR8.1, ACC69.1, CAQ

8.10, CAQ 10.10, and CAQ 3.9) all carried an identical plasmid. This can be expected, however, as these isolates were recovered from a single fish host (turbot) in Europe during a short temporal range (2005 to 2012). This plasmid encodes a replication initiation factor and RNA polymerase, along with several hypothetical proteins.

Plasmids from remaining isolates all vary in size, composition, and arrangement. This is attributed to the diversity of fish hosts, geographic origins, and years of isolation. Of note, 6 of the 13 (46%) remaining plasmids harvested encode mobilization proteins, nucleases, and transposases and carry various resistance genes. *Edwardsiella piscicida* isolate SC 09-03, recovered from a smallmouth bass in South Carolina, carries an 11,858-bp plasmid with several ORFs containing genes related to tetracycline resistance. During MIC analysis, SC 09-03 demonstrated resistance to the highest concentrations of tetracycline, oxytetracycline, and minocycline analyzed in the current study.

The plasmid harvested from Edwardsiella 9.1 (*E. tarda*), the original isolate from the description of emphysematous putrefactive disease in channel catfish (61), encoded a DNA polymerase, a mobilization protein, and several hypothetical proteins. Plasmids from *E. tarda* isolates Edwardsiella 9.2 and Edwardsiella 9.4, recovered from channel catfish in the United States, contain ORFs encoding transposases and conjugal transfer proteins. In addition, the 27,938-bp plasmid from Edwardsiella 9.2 contains ORFs encoding mercury resistance.

It is important to note that the methods employed here may be limited in their ability to isolate very large plasmids or plasmids with low copy numbers. For example, multidrug resistance plasmids belonging to the IncA/C family are widely distributed among enterobacterial isolates (62) and have been reported from some *E. ictaluri* isolates from farm-raised and research channel catfish in the southeastern United States as well as *Yersinia ruckeri* and *Aeromonas salmonicida* isolates from salmonids (63–66). The IncA/C plasmids are usually very large and typically present in low copy numbers. While an IncA/C-type plasmid was not observed in any of these isolates, future studies employing more robust techniques suitable for the harvest of very large and/or low-copy-number plasmids are warranted.

The MAUVE program identified several shared LCBs representing regions of homologous sequence shared between different plasmids, although analysis failed to identify a single core region shared across all *Edwardsiella* plasmids. As plasmids rarely carry fundamental genes required for bacterial growth and replication, but rather an assortment of genes that may be advantageous for survival in specific environmental niches or in response to particular selective pressures, it is unsurprising that a temporally diverse collection of congeneric isolates from an assortment of host and geographic origins would carry plasmids demonstrating a diverse array of organization and function. While not particularly useful in providing confirmatory diagnosis or differentiating between the *Edwardsiella* species, characterization of plasmid profiles has utility in diagnostics from an epidemiological standpoint. Given the potential dispersal of *Edwardsiella*-associated plasmids with imported and exported aquaculture products and the capacity of plasmids to be spread across multiple bacterial taxa, the identification and characterization of a "core" set of genes associated with *Edwardsiella* plasmids warrant further study.

Real-time quantitative PCR (qPCR) assays are becoming more common in fish disease research and diagnostics. Assays are currently available for a variety of bacterial, viral, and parasitic fish pathogens (67). Previous research validated qPCR assays for the detection and quantification of *E. anguillarum*, *E. piscicida*, and *E. tarda* in broth culture, pond water, and catfish tissue (68). The real-time multiplex PCR validated here demonstrated appropriate specificity, sensitivity, reproducibility, and repeatability to reliably discriminate among *E. anguillarum*, *E. ictaluri*, *E. piscicida*, and *E. tarda* (40). In addition, the presence of large quantities of nontarget DNA had no measurable effect on PCR efficiency, suggesting that this assay could also have application as a research tool for environmental DNA (eDNA) assessments in aquaculture systems, similar to other qPCR assays (69–71), and warrants further study. Still, because no distinguishing phenotypic character has been identified for *E. anguillarum*, *E. piscicida*, and *E. tarda*,

this assay is a valuable diagnostic tool, providing a rapid method of confirmatory identification for all *Edwardsiella* species infecting fish.

Last, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) is an emerging technology for microbial identification. MALDI-TOF generates protonated ions and uses time of flight to generate a peptide mass fingerprint for each sample (72). It can be used for rapid microbial identification from a pure culture, dramatically improving time to identification (73). The use of MALDI-TOF mass spectrometry (MS) for species and subspecies identification has been reported in several different bacteria (74–76).

Initially, MALDI-TOF classified all *E. anguillarum* and *E. piscicida* isolates as *E. tarda*. This was expected, as *E. anguillarum* and *E. piscicida* are not currently recognized by the microbial peptide mass spectrum database v5.0.0.0 (Bruker Daltonics). However, observation of individual spectral profiles revealed that discriminatory peaks were present for each *Edwardsiella* species. Thus, in spite of deficiencies in the current microbial database, MALDI-TOF can discriminate among the five current *Edwardsiella* taxa, including the three species formerly classified as *E. tarda*.

Molecular typing methods described here were all in agreement with taxonomic assignments for all isolates. Despite the lack of a discriminatory metabolic or phenotypic character, MALDI-TOF correlated with multiplex PCR, *gyrB*, *sodB*, and rep-PCR identifications and classifications. While molecular confirmation of suspect *Edwardsiella* isolates is ideal in terms of generating archival data for comparison in future studies, MALDI-TOF offers a dependable, cost-effective alternative for clinical laboratories that require rapid, reliable identification.

Another significant finding resulting from the current research is the confirmation that Edwardsiella isolate 9.1, recovered from the original description of emphysematous putrefactive disease in catfish aquaculture in the 1970s, as well as other suspected *E. tarda* isolates from catfish aquaculture in the early 1980s, is factually *E. tarda*. Recent molecular surveys suggest that *E. piscicida* is far more common in U.S. catfish aquaculture than *E. tarda*, and it was suspected that these original isolates, in addition to other reports of *E. tarda* in fish prior to the adoption of *E. piscicida*, may have been unintentionally misclassified (38). Although this may be the case in some instances, it does not hold true for all historical isolates. In light of recent developments in regard to *Edwardsiella* systematics, *E. piscicida* and *E. anguillarum* appear more commonly associated with disease outbreaks in fish than *E. tarda*, although comparisons of archived *sodB* sequences from previous reports to data generated here suggest that *E. tarda* (as it is currently defined) still occasionally causes disease in fish (20, 31, 33, 38, 68, 77, 97).

Proper identification of bacterial isolates is the foundation on which clinical diagnostics and infectious disease research are built. Consistent taxonomic assignment of bacteria facilitates the definition of host-microbe relationships and the development of therapeutic and preventative strategies, and it is the cornerstone of epidemiological investigations (50). This is especially true for *Edwardsiella*, as different members of the genus demonstrate various degrees of pathogenicity to different hosts (6, 20, 34, 68, 77). The methodologies described here provide reliable methods of identification of the *Edwardsiella* species and are consistent with current taxonomic schemes. Moreover, the zoonotic potential of *E. tarda* and the variable pathogenicity of *E. anguillarum*, *E. piscicida*, and *E. tarda* in different hosts make proper identification of isolates recovered from fish and aquaculture systems extremely important.

Edwardsiella tarda plays an important role in zoonotic infections and is one of the principal pathogens acquired from fish and shellfish, including ornamental pet fish (78–80). The clinical disease that manifests in humans infected with *E. tarda* may be associated with necrotic skin lesions, gastroenteritis, and, in severe cases, a septicemia leading to osteomyelitis, meningitis, or cholecystitis (81). At present, the zoonotic potential of *E. anguillarum* and *E. piscicida* is unknown, and it is unclear if these previous reports are in reference to *E. tarda* as it is currently defined or to one of the newly recognized species. Consistent methods of identification in line with contemporary

TABLE 9 *Edwardsiella* isolates analyzed in the current study

Isolate	Species	Host	Geographic origin	Yr of isolation
EA181011	*E. anguillarum*	White grouper	Israel	2011
LADL05-105	*E. anguillarum*	Tilapia	Louisiana, USA	2005
43472	*E. anguillarum*	Blue striped grunt	Maryland, USA	2003
43664	*E. anguillarum*	Striped bass	Maryland, USA	1994
43473	*E. anguillarum*	Tilapia	Maryland, USA	1997
43659	*E. anguillarum*	Tilapia	Maryland, USA	1998
43651	*E. anguillarum*	Tilapia	Maryland, USA	1999
ATCC 35051	*E. hoshinae*	Monitor	Chad	1978
11-149A	*E. ictaluri*	Zebrafish	Florida, USA	2011
S97-773	*E. ictaluri*	Channel catfish	Mississippi, USA	1997
RUSVM-1	*E. ictaluri*	Tilapia	Western Hemisphere	2012
PB 07-309	*E. piscicida*	Smallmouth bass	Arkansas, USA	2007
NFAVS-1	*E. piscicida*	Largemouth bass	Florida, USA	2014
Fr373.2	*E. piscicida*	Turbot	France	2012
HL1.1	*E. piscicida*	Turbot	Holland	2006
HL25.1	*E. piscicida*	Turbot	Holland	2006
HL32.1	*E. piscicida*	Turbot	Holland	2006
WFE1	*E. piscicida*	Flounder	Japan	2002
S11-285	*E. piscicida*	Channel catfish	Mississippi, USA	2011
C1490	*E. piscicida*	Largemouth bass	New York, USA	2014
CMT 8211-1	*E. piscicida*	Rainbow trout	North Carolina, USA	2014
REDS 81911-E	*E. piscicida*	Rainbow trout	North Carolina, USA	2014
RBR8.1	*E. piscicida*	Turbot	Portugal	2008
SC 09-03	*E. piscicida*	Smallmouth bass	South Carolina, USA	2009
ACC69.1	*E. piscicida*	Turbot	Southern Europe	2005
CAQ 8.10	*E. piscicida*	Turbot	Spain	2009
CAQ 10.10	*E. piscicida*	Turbot	Spain	2009
CAQ 3.9	*E. piscicida*	Turbot	Spain	2009
A15-02670	*E. piscicida*	Blotched fantail stingray	Georgia, USA	2015
43628	*E. piscicida*	Koi	Maryland, USA	2000
43662	*E. piscicida*	Seatrout	Maryland, USA	1988
43644	*E. piscicida*	Striped bass	Maryland, USA	1994
43475	*E. piscicida*	Striped bass	Pennsylvania, USA	1996
43658	*E. piscicida*	Striped bass	Pennsylvania, USA	1996
43468	*E. piscicida*	Striped bass	Maryland, USA	1999
43656	*E. piscicida*	Striped bass	Maryland, USA	2000
Edwardsiella 9.1	*E. tarda*	Channel catfish	Arkansas, USA	1969
Edwardsiella 9.2	*E. tarda*	Channel catfish	West Virginia, USA	1977
Edwardsiella 9.3	*E. tarda*	Flounder	Virginia, USA	1984
Edwardsiella 9.4	*E. tarda*	Channel catfish	Georgia, USA	1979
FL95-01	*E. tarda*	Channel catfish	Florida, USA	1995
070720-1 3A	*E. tarda*	Tilapia	Michigan, USA	2007
070720-1 2HLDOM	*E. tarda*	Tilapia	Michigan, USA	2007
43657	*E. tarda*	Bottlenose dolphin	Maryland, USA	2000
43650	*E. tarda*	Hooded seal	Maryland, USA	2004
43627	*E. tarda*	Tilapia	Pennsylvania, USA	2000
43663	*E. tarda*	Toadfish	Maryland, USA	1988

systematic nomenclature will limit ambiguity in such reports moving forward. Therefore, it is imperative that nomenclature consistency is applied across different laboratories and throughout different countries. The limitations of databases such as GenBank and the unverified taxon classifications associated with submissions further emphasize the importance for researchers and diagnosticians of remaining attentive to the current literature.

MATERIALS AND METHODS

Bacterial isolates. Isolates of *E. anguillarum*, *E. hoshinae*, *E. ictaluri*, *E. piscicida*, and *E. tarda* were obtained from collaborators and biological collections and expanded in porcine brain heart infusion broth (BHIb) (Bacto; Becton, Dickinson and Company) at optimal growth temperatures for each species (37°C for *E. anguillarum*, *E. hoshinae*, *E. piscicida*, and *E. tarda* and 28°C for *E. ictaluri*). Aliquots of broth cultures were stored cryogenically (−80°C) with 15% (vol/vol) glycerol. A collection of 47 representative isolates from 10 countries and 19 host species, isolated over a 47-year period, was chosen for analyses (Table 9). Of note, isolate Edwardsiella 9.1 was recovered during the original description of emphysematous putrefactive disease in channel catfish *Ictalurus punctatus* in the 1970s (61). For all phenotypic

analyses, cryostocks of archived isolates were revived by isolation streaking on Mueller-Hinton II agar (BBL, Becton, Dickinson and Company) supplemented with 5% defibrinated sheep blood (Hemostat Laboratories) and grown for 24 h (*E. anguillarum*, *E. hoshinae*, *E. piscicida*, and *E. tarda*) or 48 h (*E. ictaluri*) at temperatures optimal for each respective isolate.

DNA isolation. Cryostocks were revived as described above, and individual colonies were expanded for 24 to 48 h in static BHIb at appropriate temperatures for each isolate. Cultures were pelleted by centrifugation, and genomic DNA (gDNA) was isolated using a commercial DNA isolation kit according to the manufacturer's suggested protocols for Gram-negative bacteria (Gentra Puregene DNA isolation kit; Qiagen). Isolated gDNA was resuspended in 100 μl of DNA hydration solution (DHS; Gentra Puregene DNA isolation kit; Qiagen), quantified spectrophotometrically (NanoDrop 2000; Thermo Fisher Scientific), diluted with DHS to a final concentration of 10 ng/μl, and cryogenically stored (-80°C) until further use.

Motility and TSI. Individual colonies of *Edwardsiella* isolates were stabbed into motility medium (Difco) and evaluated for dispersal after 48 h at 37°C (*E. anguillarum*, *E. hoshinae*, *E. piscicida*, and *E. tarda*) or 28°C (*E. ictaluri*). Glucose, sucrose, and/or lactose fermentations, in addition to hydrogen gas and/or hydrogen sulfide production in triple sugar iron medium (TSI; Oxoid Ltd.), were determined using similar incubation conditions.

Microbial identification systems. The commercial API 20E system (bioMérieux) was used for all bacterial species in accordance with the manufacturer's instructions. Briefly, API 20E strips were inoculated and incubated for 24 h at 37°C for *E. anguillarum*, *E. hoshinae*, *E. piscicida*, and *E. tarda* and 48 h at 28°C for *E. ictaluri*. All reagents were added, a seven-digit profile number was generated, and profile numbers were submitted to bioMérieux for microbial identification. Additionally, isolates were analyzed using the Biolog microbial identification system (Biolog) according to the manufacturer's instructions. In short, isolates were streaked for isolation from archived cryostocks on Biolog Universal Growth (BUG; Biolog) agar with 5% sheep blood. After 24 h at 28°C, colonies were picked and added to the inoculating fluid A (IF-A; Biolog) to reach 92% to 98% transmittance (%*T*). Gen III microplates were inoculated and incubated at 28°C for 24 h, after which reactions were read and identification was performed using OmniLog data collection software (Biolog).

Last, bacterial isolates were subjected to the BBL Crystal Enteric/Nonfermentor identification kit (Becton, Dickinson and Company). Cryostocks were streaked for isolation on Mueller-Hinton II agar (BBL; Becton, Dickinson and Company) supplemented with 5% defibrinated sheep blood (Hemostat Laboratories). Individual colonies were picked using a sterile toothpick and resuspended in inoculating fluid to achieve a 0.5-McFarland-standard turbidity before addition to the assay panel. Panels were incubated at 28°C for 24 to 48 h, and reactions were visualized and recorded.

A commercial matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Bruker MALDI-TOF Biotyper LT) was used for bacterial identification and generation of peptide mass spectral profiles. Bacterial colonies were applied to a spot on the MALDI-TOF target plate and overlaid with freshly made 70% formic acid and α-cyano-4-hydroxycinnamic acid matrix solution according to the manufacturer's recommended protocol. The spectra were captured in positive linear mode in a mass range of 2 to 20 kDa with a laser frequency of 60 Hz (IS1, 20 kV; IS2, 18 kV; lens, 6 kV; extraction delay time, 100 ns). Spectra were acquired in automatic mode by accumulating a maximum of 240 profiles (6 $\times$ 40 laser shots from different positions of the target spot). Bacterial identification was performed using the default settings of the software provided with the Bruker MALDI-TOF system. A score of >2 indicated highly probable bacterial genus and species identification. The peptide spectra were collected and analyzed using FlexAnalysis software (Bruker).

FAME analysis. The 47 *Edwardsiella* isolates were streaked onto sheep blood agar (SBA; Remel) and incubated for 24 h at 28°C. Following incubation, an average of 35 mg (wet weight) of bacteria was harvested from each plate, placed into individual Pyrex glass tubes, and centrifuged for 1 min at 5,250 $\times$ *g*. Bacteria were saponified by adding 1.0 ml of saponification reagent (150 ml of deionized distilled water combined with 150 ml of high-performance liquid chromatography [HPLC]-grade methanol and 45 g of sodium hydroxide). Sequentially, each tube was vortexed for 5 to 10 s, boiled for 5 min in water at 100°C, vortexed for 10 s, and then boiled for an additional 25 min at 100°C. Samples were methylated by the addition of 2.0 ml methylation reagent (162.5 ml of 6.0 N hydrochloric acid with 137.5 ml of HPLC-grade methanol), vortexed for 10 s, and immediately cooled on ice for 10 min.

Following methylation, FAMEs were extracted by the addition of 1.25 ml extraction solvent (200 ml of HPLC-grade hexane combined with 200 ml of HPLC-grade methyl-tert-butyl ether). Samples were loaded into a circular rotator and centrifuged for 10 min at 3,000 $\times$ *g* to ensure adequate combination of sample and extraction solvent. Tubes were then centrifuged for 1 min at 5,250 $\times$ *g* to separate extraction waste. The bottom phase was removed and discarded using a long-tip Pasteur pipette. Three milliliters of base wash (5.4 g of sodium hydroxide diluted in 45 ml of distilled water) was added to the top phase of each sample and centrifuged for 5 min at 3,000 $\times$ *g*. Samples were held upright at room temperature to complete the separation between the bottom and top phases. The top phase (100 μl) from each sample was removed and transferred into a glass vial (National Scientific Target Vials; C4011-1) fitted with a 100-μl glass insert with polymer feet and lid.

Samples (1 μl) were analyzed on HP-Ultra-2 analytical capillary columns (25 by 0.200 mm, 0.33-μm film thickness; Agilent Technologies, Inc.) using an Agilent Technologies 6850 network gas chromatography system (Agilent Technologies, Inc.). Nitrogen was the carrier gas at a constant flow rate of 0.3 ml/min. The oven was programmed at an initial temperature of 170°C and then heated to 288°C at a ramp rate of 28°C/min. The split ratio was 40:1, and the total run was 6.23 min. Data were analyzed using the Sherlock Microbial Identification System (MIS) RCLIN6 6.2 library (MIDI 2012).

TABLE 10 Primers and probes used in the current study

Purpose and primer	Sequence (5′–3′)	Reference(s)
16S sequencing		95
27F	GAGTTTGATCCTGGCTCAG	
1525R	AGAAAGGAGGTGATCCAGCC	
gyrB sequencing		37
GyrB630F	GGATAACGCGATTGACGAAG	
GyrB1245R	ATCRTCYTTCATGGTCGARA	
GyrB2198F	TAAAGACGATGAGGCGATGG	
GyrB2540R	GCCGTGARCAAARTCRAA	
sodB sequencing		32
E1F	ATGTCRTTCGAATTACCTGC	
497R	TCGATGTARTARGCGTGTTCCCA	
Repetitive sequence-mediated PCR		
BOX	CTACGGCAAGGCGACGCTGACG	87
ERIC I	ATGTAAGCTCCTGGGGATTCAC	87
ERIC II	AAGTAAGTGACTGGGGTGAGCG	59
GTG_5	GTGGTGGTGGTGGTG	59
Multiplex real-time PCR		
E. tarda		89
ET3518F	CAGTGATAAAAAGGGGTGGA	
ET3632R	CTACACAGCAACGACAACG	
ET3559P	AGACAACAGAGGACGGATGTGGC	
E. piscicida		89
EP14529F	CTTTGATCATGGTTGCGGAA	
EP14659R	CGGCGTTTTCTTTTCTCG	
EP14615P	CCGACTCCGCGCAGATAACG	
E. anguillarum		89
EA1583F	GATCGGGTACGCTGTCAT	
EA1708R	AATTGCTCTATACGCACGC	
EA1611P	CCCGTGGCTAAATAGGACGCG	
E. ictaluri		70, 96
EI481F	ACTTATCGCCCTCGCAACTC	
EI658R	CCTCTGATAAGTGGTTCTCG	
EI561P	CCTCACATATTGCTTCAGCGTCGAC	

Antimicrobial susceptibility profiles. MICs of 39 different antimicrobial agents were determined for all 47 *Edwardsiella* isolates to identify susceptibility patterns that could be exploited to differentiate among the *Edwardsiella* spp. MICs were evaluated using the Sensititre GN4F and Avian1F plate formats (Trek Diagnostic Systems) using the manufacturer's suggested protocol. *Escherichia coli* ATCC 25922 was used as the quality control strain. Each inoculum was prepared by suspending individual colonies in sterile distilled water to a 0.5-McFarland-standard turbidity; 30 μl of the suspension was added to 11 ml of cation-adjusted Mueller-Hinton broth (Sigma-Aldrich), and 50 μl of the inoculum was added to each well. Plates were covered with an adhesive seal (provided by the manufacturer) and incubated (24 h at 37°C for *E. anguillarum*, *E. hoshinae*, *E. piscicida*, and *E. tarda* and 48 h at 28°C for *E. ictaluri*). Following incubation, plates were checked visually, and MIC values were defined as the lowest drug concentration exhibiting no visible growth.

Phylogenetic analysis. Three different gene targets were chosen for amplification and sequencing to link historical *E. tarda* isolates to contemporary phylogenomic assignments. Primers used for amplification and sequencing of the 16S rRNA, *gyrB*, and *sodB* genes are listed in Table 10. Amplification reactions (50 μl) were performed using 43 μl of Platinum High-Fidelity PCR SuperMix (Invitrogen), 20 pmol of each primer, ~50 ng of gDNA, and nuclease-free water to volume. Amplifications were performed using a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.). For 16S rRNA and *gyrB*, the following cycling conditions were used: 3 min of denaturation at 94°C; 45 cycles of 30 s at 94°C, 30 s at 52°C, and 2 min at 68°C; and 7 min of extension at 68°C. For *sodB*, the following cycling conditions were used: 2 min of denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C; and 7 min of extension at 72°C. Amplicons were visualized with UV light after electrophoretic passage through a 0.8% agarose gel containing ethidium bromide (0.5 μg ml^{-1}), excised, and purified using QIAquick columns (Qiagen). Purified PCR products were sequenced commercially using the same primers employed to generate the amplicons (Eurofins Genomics, Louisville, KY). Contiguous sequences were assembled, and ambiguous base calls were manually annotated from corresponding chromatograms using Geneious v10.0.7 (Biomatters, Ltd.) (82).

Sequences from 16S rRNA, *gyrB*, and *sodB* were trimmed and aligned using the MUSCLE (83) application of MEGA v6 (84), and pairwise sequence similarities were determined. Moreover, *sodB*

sequences of *Edwardsiella* spp. were compared to *sodB* sequences of typical motile fish-pathogenic *E. tarda* (GenBank accession no. AB009853), atypical nonmotile fish-pathogenic *E. tarda* (GenBank accession no. AB009584), and fish-nonpathogenic *E. tarda* (GenBank accession no. AB009850) (33). The Bayesian inference criterion identified the Kimura 2-parameter model with gamma distribution (16S rRNA), the Tamura-Nei model with gamma distribution (*gyrB*), and the Tamura 3-parameter model with gamma distribution (*sodB*) as the best-fit nucleotide substitution model for maximum likelihood analysis (85). All positions containing gaps and missing data were eliminated. The final trees were constructed from 1,000 bootstrap replicates. Additionally, intragenomic heterogeneity of the 16S rRNA was evaluated by BLASTN searches of 16S rRNA sequences against the complete genomes of isolates *E. anguillarum* LADL05-105, *E. hoshinae* ATCC 35051, *E. piscicida* S11-285, and *E. tarda* FL95-01, which were closed ancillary to the current project (43–45, 86), as well as *E. ictaluri* 93-146 (87).

Genetic fingerprinting. Repetitive extragenic palindromic PCR (rep-PCR) fingerprinting was performed on isolates using previously published primer sets (Table 10) and modifications to existing protocols (31, 38, 59, 60, 88). Briefly, 50-μl reaction mixtures comprised 25 μl of IQ Supermix (Bio-Rad; Hercules, CA), 20 pmol (ERIC I and II) or 40 pmol (ERIC II, BOX, and GTG_5) of primer, 10 ng of DNA template, and nuclease-free water to volume. Amplifications were performed on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Inc.) with the following temperature profiles: BOX, ERIC II, and ERIC I and II, 1 cycle at 95°C for 10 min; 5 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 5 min; and 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 min; GTG_5, 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 1 min, 46°C for 1 min, and 72°C for 3 min with a final extension at 72°C for 10 min. Aliquots of each amplification reaction mixture (10 μl each) and a molecular weight standard (Hyperladder 50 bp; Bioline) were electrophoresed through a 1.5% (wt/vol) agarose gel with ethidium bromide (0.5 μg ml^{-1}) and visualized under UV light. Genetic fingerprints generated by the BOX primer were analyzed using Quantity One software v.4.6.9 (Bio-Rad Laboratories, Inc.). Band sizes were estimated by comparison with concurrently run standards, and distinct bands were manually annotated to calculate Dice coefficients and generate a dendrogram based on the unweighted pair group method using arithmetic averages (UPGMA).

Multiplex real-time PCR. A real-time multiplex PCR (mPCR) specific to *E. anguillarum*, *E. ictaluri*, *E. piscicida*, and *E. tarda* was developed based on previously published primers, probes, and protocols (38, 68, 89). Primers and probes (Table 10) were synthesized commercially (Eurofins MWG; Louisville, KY); each probe was labeled with a fluorescent reporter dye (*E. anguillarum*, Texas Red; *E. ictaluri*, hexachlorofluorescein [HEX]; *E. piscicida*, 6-carobxyfluorescein [6-FAM]; *E. tarda*, Cy5) on the 5′ end and appropriate quencher dye (black hole quencher 1 for HEX and 6-FAM; black hole quencher 2 for Texas Red and Cy5) on the 3′ end. The 25-μl reaction mixture contained 12 μl of PCR master mix (TaqMan Environmental Mastermix 2.0; Applied Biosystems), 5 pmol of each primer, 0.5 pmol of each probe, DNA template, and nuclease-free water to volume. Amplifications were performed on a CFX96 thermal cycler (Bio-Rad Laboratories, Inc.) with the following temperature profile: 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data collection occurred following the 60°C annealing/extension step at the end of each cycle.

The specificity of the mPCR assay was tested against both target and nontarget *Edwardsiella* gDNA. Additionally, the specificity and sensitivity of the assay were tested using serial 10-fold dilutions of target gDNA, supplementing each reaction mixture with an equal mixture of ~10,000 copies of each nontarget *Edwardsiella* sp. gDNA to ensure that large quantities of nontarget DNA did not impair reaction efficiency. Samples, as well as no-template controls, were run in triplicate under the reaction conditions described above. Each plate was run in triplicate on three separate occasions to assess the repeatability and reproducibility of the assay. Quantification cycles (C_q) for each reaction were based on a user-defined baseline threshold of 50 relative fluorescence units (RFU).

Plasmid analysis. For all isolates, plasmid DNA was harvested from 3 ml of expanded BHIb cultures using the QIAprep Spin miniprep kit (Qiagen). Plasmids were identified by separation on a 0.8% agarose gel. When present, plasmid sizes were approximated with concurrently run standards (Supercoiled DNA Ladder; New England BioLabs). Harvested plasmids were submitted to the complete plasmid sequencing service of the DNA Core Facility of the Center for Computational and Integrative Biology at Massachusetts General Hospital (Boston, MA, USA) for sequencing. Open reading frames (ORFs) were predicted using GeneMark.hmm prokaryotic v3.25 (90, 91) and Glimmer v3.02 (92, 93). Putative functions of plasmid ORFs were predicted using a BLASTX search of the NCBI nonredundant protein database using the *Bacteria* and *Archaea* code with E values of $\geq$1e−2 considered insignificant. Last, circularized plasmids were aligned using the progressiveMauve algorithm utility in Geneious, with seed weights and minimum locally colinear block (LCB) scores calculated automatically (94).

Accession number(s). 16S, *sodB*, and *gyrB* sequences have been submitted to GenBank under the accession numbers MG225458 to MG225535 and MG230270 to MG230308. Annotated plasmid sequences have been submitted to GenBank under the accession numbers MG212496 to MG212499 and MG225254 to MG228262.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00970-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

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