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Edwardsiella piscicida identified in the southeastern USA by *gyrB* sequence, species-specific and repetitive sequence-mediated PCR

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ABSTRACT: A new *Edwardsiella* taxon was recently described from fishes of Europe and Asia. Phenotypically similar to *E. tarda,* extensive genetic and phenotypic characterization determined this new strain does not belong to any established *Edwardsiella* taxa, leading to the adoption of a new taxon, *E. piscicida*. Concurrent research in the USA also identified 2 genetically distinct taxa within the group of organisms traditionally classified as *E. tarda*. Comparisons of *gyrB* sequences between US isolates and *E. piscicida* from Europe and Asia identified several US isolates with >99.6% similarity to the *gyrB* sequence of the *E. piscicida* type strain (ET883) but <87% similarity to the *E. tarda* type strain (ATCC #15947). A discriminatory PCR was developed for the identification of *E. tarda* and 2 genetic variants of *E. piscicida* (*E. piscicida* and *E. piscicida*-like species). Using these PCR assays, a survey was conducted of 44 archived bacterial specimens from disease case submissions to the Aquatic Research and Diagnostic Laboratory (Stoneville, MS, USA) between 2007 and 2012. All 44 isolates, originally identified phenotypically and biochemically as *E. tarda*, were identified as *E. piscicida* by PCR. Repetitive sequence-mediated PCR (rep-PCR) analysis of these archived specimens suggests they are largely homogenous, similar to what has been observed for *E. ictaluri*. The *gyrB* sequence data, coupled with the *E. piscicida* specific-PCR and rep-PCR data, confirms that *E. piscicida* has been isolated from fish disease cases in the southeastern USA. Moreover, our survey data suggests *E. piscicida* may be more prevalent in catfish aquaculture than *E. tarda*.

KEY WORDS: *Edwardsiella tarda* · *E. piscicida* · *gyrB* · PCR · Repetitive sequence-mediated PCR · Rep-PCR

INTRODUCTION

The genus *Edwardsiella,* a member of the Enterobacteriaceae, was first established following isolation of the genus type species *E. tarda* from humans (Ewing et al. 1965). Until recently, the genus consisted of 3 species: *E. ictaluri, E. hoshinae,* and *E. tarda*. *E. ictaluri* has been traditionally associated with disease outbreaks in cultured channel catfish (Hawke et al. 1981, Hawke & Khoo 2004), although recently it has been implicated in mortality events in cultured tilapia *Oreochromis* sp. and laboratory populations of zebrafish *Danio rerio* (Soto et al. 2012, 2013, Hawke et al. 2013). *E. hoshinae,* first described from birds, reptiles, and aquatic environments (Grimont et al. 1980), also occupies a narrow range of ecological niches, although its role in disease processes is poorly understood (Janda & Abbott 1993).

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By comparison, *E. tarda* is a versatile pathogen with a wide ecological niche and has been isolated from a wide variety of fish, reptilian, avian, and mammalian hosts, including humans (Mohanty & Sahoo 2007).

Recent phenotypic and genetic studies have revealed that *Edwardsiella tarda* isolates from fish fell into different genetic groups, suggesting the existence of multiple distinct taxa within the group of organisms classified as *E. tarda* (Abayneh et al. 2012, Yang et al. 2012). The result of these studies has been the adoption of a fourth member of the *Edwardsiella*, *E. piscicida*, which was characterized from fish in Europe and Asia (Abayneh et al. 2013).

A recent study of *Edwardsiella tarda* isolates from diseased fish in the southeastern United States demonstrated similar findings, suggesting the existence of multiple genetically distinct, yet phenotypically indistinguishable taxa within the group of organism traditionally classified as *E. tarda* (Griffin et al. 2013). The aims of the present study were to determine if *E. piscicida* existed within the group of organisms classified as *E. tarda* in the United States, to develop and validate molecular tools to reliably discriminate between *E. tarda* and *E. piscicida* and to estimate the relative prevalence of *E. piscicida* in catfish aquaculture in the southeastern United States.

MATERIALS AND METHODS

Isolation and identification of *Edwardsiella* isolates from Mississippi farm-raised catfish

A total of 44 *Edwardsiella tarda* isolates were obtained from the archived collections of the Aquatic Research and Diagnostic Laboratory (ARDL) at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS, USA (Table 1). All isolates had been cultured from the brains and/or kidneys of diseased fish on Mueller Hinton agar plates supplemented with 5% sheep blood (Becton Dickinson), according to standard operating procedures of the ARDL. The isolates used in the present study were collected from disease case submissions between 2007 and 2012. All isolates in the present study were identified as *E. tarda* upon initial isolation using the BBL™ Crystal™ Enteric/Nonfermenter ID kit (BD) coupled with indole and oxidase tests (Becton Dickinson) (Table 1). In addition, an American Type Culture Collection (ATCC) *Escherichia coli* isolate (#25942), an ATCC *E. tarda* isolate (#15947), an *Edwardsiella ictaluri* isolate (S97-773), and an isolate identified as atypical *E. tarda* (LADL 05-105) by PCR (Sakai et al. 2009, Griffin et al. 2013) were also included in the analysis.

Table 1. Archived *Edwardsiella* isolates from diseased blue, channel, or hybrid catfish in Mississippi, USA. All isolates were identified as *E. tarda* by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (Becton Dickson) upon initial isolation and represent individual disease case submissions. Isolate identification represents the year and accession number (e.g. S07-262 = Submission #262 for the year 2007) for the individual case submission to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS. All isolates were indole positive and oxidase negative

Isolate	Host	Identification code	Location
S07-262	Channel	2003010103	Leland, MS
S07-275	Channel	2003010103	Belzoni, MS
S07-276	Channel	2003010103	Indianola, MS
S07-346	Channel	2003010103	Hollandale, MS
S07-347	Channel	2003010103	Stoneville, MS
S07-348	Channel	2403010153	Stoneville, MS
S07-356	Channel	2003010103	Belzoni, MS
S07-357	Channel	2003010103	Belzoni, MS
S07-358	Channel	2003010113	Belzoni, MS
S07-534	Channel	2003110113	Itta Bena, MS
S07-907	Channel	2003110113	Tunica, MS
S07-1004	Blue	2002010113	Indianola, MS
S07-1094	Channel	2003110113	Itta Bena, MS
S08-209	Channel	2003010113	Moorhead, MS
S10-279	Channel	2003110113	Stoneville, MS
S10-430	Hybrid	2402010053	Indianola, MS
S10-67	Hybrid	2003010113	Itta Bena, MS
S10-512	Hybrid	2402010113	Glen Allen, MS
S11-62	Channel	2003110143	Itta Bena, MS
S11-159	Channel	2003110113	Itta Bena, MS
S11-222	Channel	2003110113	Belzoni, MS
S11-233	Channel	2003110013	Itta Bena, MS
S11-285	Channel	2403110113	Itta Bena, MS
S11-295	Hybrid	2402010113	Inverness, MS
S11-310	Channel	2003010113	Moorhead, MS
S11-508	Hybrid	2003110113	Indianola, MS
S11-509	Channel	2003110013	Indianola, MS
S11-534	Hybrid	2503110113	Itta Bena, MS
S11-551	Hybrid	2412010113	Clarksdale, MS
S11-552	Channel	2412010113	Clarksdale, MS
S11-553	Channel	2412010113	Clarksdale, MS
S11-616	Hybrid	2003110013	Indianola, MS
S11-632	Channel	2003110113	Itta Bena, MS
S11-680	Channel	2403010113	Itta Bena, MS
S11-688	Channel	2402010113	Doddsville, MS
S12-272	Channel	2403110113	Stoneville, MS
S12-273	Channel	2403110113	Stoneville, MS
S12-281	Hybrid	2403010113	Schlater, MS
S12-307	Channel	2402010013	Indianola, MS
S12-309	Hybrid	2402010013	Indianola, MS
S12-378	Hybrid	2003110113	Itta Bena, MS
S12-408	Channel	2402010113	Itta Bena, MS
S12-419	Hybrid	2402010113	Rolling Fork, MS
S12-420	Hybrid	2402010113	Indianola, MS

DNA extraction

Cryostocks were streaked for isolation on Mueller-Hinton agar plates supplemented with 5% sheep blood and grown overnight at 37°C (*Edwardsiella tarda* and *Escherichia coli*) or for 48 h at 28°C (*Edwardsiella ictaluri*). Individual colonies served to inoculate 1 ml of Brain Heart Infusion (BHI) broth (Becton Dickinson) and were expanded overnight at 37°C (*E. tarda* and *E. coli*) or 28°C (*E. ictaluri*) without shaking. Cultures were pelleted by centrifugation. Genomic DNA from all isolates was extracted using the Puregene DNA Isolation Kit (Qiagen) following the manufacturer's suggested protocols for Gram-negative bacteria and quantified spectrophotometrically (Nanodrop).

gyrB sequencing for species identification

To determine whether the isolates described by Griffin et al. (2013) and Abayneh et al. (2012, 2013) are conspecific, sequencing of the *gyrB* gene was performed on the 18 *Edwardsiella* isolates genetically characterized by Griffin et al. (2013) (Table 2). As the *gyrB* fragments determined by Abayneh et al. (2012) and Griffin et al. (2013) did not overlap, new sequencing primers were designed to specifically encompass the *gyrB* fragments sequenced in these previous studies so they could be compared (Table 3). Primer locations were based on both complete and incomplete genome sequences available via the National Center for Biotechnology Information's GenBank (Table 4). To ensure amplification from all *Edwardsiella* isolates, targets were selected based on regions conserved between the *E. tarda* and *E. ictaluri* genomes that also flanked the fragments sequenced in previous studies (Abayneh et al. 2012, 2013, Griffin et al. 2013).

Amplification reactions (50 µl) were performed using 25 µl Econotaq Plus Green 2X master mix (Lucigen), 20 pmol of each primer (GyrB620F; GyrB2540R), 10 ng of genomic DNA and nuclease-free water to volume using the following thermocycling profile: 5 min denaturation at 95°C; 45 cycles of 30 s at 95°C, 30 s at 55°C, 2 min at 72°C; and 7 min extension at

Table 2. Results of *gyrB* sequencing and species-specific PCR validation of 18 *Edwardsiella* isolates characterized by Griffin et al. (2013). All archived *Edwardsiella* isolates were identified as *E. tarda* by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (Becton Dickson) accompanied by their corresponding identification code. Primer abbreviations as follows: ET: *E. tarda*; EP: *E. piscicida*; EPL: *E. Piscicida*-like species; ETT: typical *E. tarda* (Sakai et al. 2009); ETA: atypical *E. tarda* (Sakai et al. 2009); ESC: *E. ictaluri* (Bilodeau et al. 2003, Griffin et al. 2011)

Isolate	BBL ID	BBL code	*gyr B* ID	Species-specific PCR (5 ng DNA per reaction)					
				ET	EP	EPL	ETT	ETA	ESC
DNA Group I									
ATCC 15947	2002010013[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
RE-04	2002010013[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
AL 98-87	2003110113[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
LADL 88-209	2003110113[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
FL 95-01	2002010113[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
LADL 99-302	2002010113[a]	*E. tarda*	*E. tarda*	+	–	–	–	–	–
DNA Group II									
MA 97-004	2403110113[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S11-285	2403110113[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
LADL 97-168	2403010113[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
LADL 99-462	2403010113[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-346	2003010103[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-262	2003010103[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-534	2003110103[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-275	2003110103[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-1019	2003010113[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
S07-348	2403010153[a]	*E. tarda*	*E. piscicida*	–	+	–	+	–	–
LADL 05-105	2403014113[a]	*E. tarda*	*E. piscicida*-like	–	–	+	–	+	–
E. ictaluri									
S97-773	2002000113[b]	Unknown	*E. ictaluri*	–	–	–	–	–	+

[a]Indole +; oxidase –
[b]Indole –; oxidase –

Table 3. PCR primers used in the present study. Primer abbreviations as in Table 2. F: forward; R: reverse

Primer	Sequence (5′-3′)	Source
Edwardsiella tarda		Present paper
ETF	CAGTGATAAAAAGGGGTGGA	
ETR	CTACACAGCAACGACAACG	
Edwardsiella piscicida		Present paper
EPF	CTTTGATCATGGTTGCGGAA	
EPR	CGGCGTTTTCTTTTCTCG	
***Edwardsiella piscicida*-like**		Present paper
EPLF	TTTGATCGGGTACGCTGT	
EPLR	AATTGCTCTATACGCACGC	
Typical *Edwardsiella tarda*		Sakai et al. (2009)
ETTF	TTCCGCAACCATGATCAAAG	
ETTR	AGGGCATATATCCACTCACTG	
Atypical *Edwardsiella tarda*		Sakai et al. (2009)
ETAF	GAACAGCGCCTCTGTCTG	
ETAR	AATTGCTCTATACGCACGC	
Edwardsiella ictaluri		Bilodeau et al. (2003)
ESCF2	ACTTATCGCCCTCGCAAC	Griffin et al. (2011)
ESCR2	GCCTCTGATAAGTGGTTCTCG	
***gyrB* sequencing**		Present paper
GyrB630F	GGATAACGCGATTGACGAAG	
GyrB872F	CMCTGTCYGARAAGYTGGAR	
GyrB1245R	ATCRTCYTTCATGGTCGARA	
GyrB1425F	ATGACCCGTACGCTGAACA	
GyrB1949R	GGAGAGCATCTTGTCGAAGC	
GyrB2198F	TAAAGACGATGAGGCGATGG	
GyrB2540R	GCCGTGARCAAARTCRAA	
Repetitive sequence-mediated PCR		Versalovic et al. (1991, 1994)
BOX	CTACGGCAAGGCGACGCTGACG	
ERIC I	ATGTAAGCTCCTGGGGATTCAC	
ERIC II	AAGTAAGTGACTGGGGTGAGCG	

Table 4. Complete and incomplete genome sequences used in primer development

Isolate	Identification	GenBank assembly no.	Source
FL6-60	*Edwardsiella tarda*	ASM14630v1	van Soest et al. (2011)
EIB 202	*Edwardsiella tarda*	ASM2086v1	Wang et al. (2009)
080813	*Edwardsiella tarda*	ASM26476v1	Yang et al. (2012)
ATCC 23685	*Edwardsiella tarda*	ASM16395v1	Yang et al. (2012)
ATCC 15947	*Edwardsiella tarda*	ASM26480v1	Yang et al. (2012)
93-156	*Edwardsiella ictaluri*	ASM2288v2	Williams et al. (2012)
ATCC 33202	*Edwardsiella ictaluri*	ASM26478v1	Yang et al. (2012)

72°C. Amplicons were visualized with UV light after electrophoretic migration through a 1.25% agarose gel containing ethidium bromide (1 µg ml^{-1}) and were purified directly using Qiagen Qiaquick™ columns. The purified products were then directly sequenced using the corresponding external and internal sequencing primers (Table 3). Purified PCR products were cycle-sequenced from both strands using ABI BigDye™ chemistry (Applied Biosystems), alcohol-precipitated, and run on an ABI Prism 3730™ automated sequencer (Applied Biosystems). Contiguous sequences were assembled using the corresponding chromatograms and the SeqMan™ utility of the Lasergene software package (DNAStar).

Edwardsiella ictaluri, *E. piscicida*, *E. piscicida*-like and *E. tarda*-specific PCR

Species-specific PCR primers were developed based on complete and incomplete *Edwardsiella* genomes (Table 3), targeting the fimbrial subunit, which had been used previously to differentiate between what was termed typical and atypical *E. tarda* (Sakai et al. 2009). Genomic DNA from the 18 isolates used for *gyrB* sequencing and characterized initially by Griffin et al. (2013) was analyzed using the newly developed *E. tarda* (ET), *E. piscicida* (EP), and atypical *E. piscicida*-like (EPL) primers as well as previously established *E. ictaluri* (ESC) primers (Bilodeau et al. 2003, Griffin et al. 2011) (Table 3). Briefly, the 20 µl PCRs consisted of 8 µl of EconoTaq PLUS GREEN 2X Master Mix (Lucigen), 20 pmol of each primer, 5 ng of DNA template, and nuclease-free H_2O to volume. Thermal conditions for the ET, EP, EPL, and ESC primers were as follows: 95°C for 5 min followed by 35 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s, with a final extension step of 72°C for 5 min. Aliquots of each amplification reaction (5 µl) were electrophoresed through a 1.5% (wt/vol) agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The presence of the appropriately sized bands (ET: 114 bp; EP: 130 bp; EPL: 128 bp; ESC: 129 bp) was determined by direct comparison with concurrently run DNA standards (Hyperladder II, Bioline USA).

PCR survey of *Edwardsiella* isolates from catfish

Molecular identification for all 44 survey isolates from farm-raised catfish in Mississippi was carried out using the newly developed ET, EP, and EPL primers, the typical (ETT) and atypical *E. tarda* (ETA) primers of Sakai et al. (2009), and the ESC (Bilodeau et al. 2003, Griffin et al. 2011). Reaction components and thermal cycling conditions were carried out as described previously, although the ETT and ETA primers used a slightly different cycling profile of 95°C for 5 min, followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, ad 72°C for 15 s, and a final extension of 72°C for 5 min. Aliquots of each amplification reaction (5 µl) were visualized as described previously, and the presence of the appropriate sized bands (ETT: 268 bp; ETA: 230 bp) was determined by direct comparison with concurrently run DNA standards (Hyperladder II).

Repetitive sequence-mediated PCR (rep-PCR)

Genetic fingerprinting for all 44 survey isolates from farm-raised catfish in Mississippi was performed using published primer sets and modifications to existing protocols (Versalovic et al. 1991, 1994, Griffin et al. 2011, 2013) (Table 3). An *Edwardsiella ictaluri* (S97-773), *E. tarda* (ATCC #15947), atypical *E. tarda* (LADL 05-105), and an *E. coli* (ATCC #25922) were included for phylogenetic analysis. Briefly, the analysis consisted of 25 µl reactions comprising 13 µl of IQ Supermix (BioRad), 20 pmol (ERIC I & II) or 40 pmol (ERIC II; BOX) of primer, 10 ng of DNA template, and nuclease-free H_2O to volume. Amplifications were performed on a PTC-200 gradient cycler (MJ Research) with the following temperature profiles: 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; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 min. Aliquots of each amplification reaction (10 µl each) were visualized as above.

Phylogenetic analysis

Partial sequences (1905 bp) of the *gyrB* gene generated here from the 18 *Edwardsiella* isolates (Table 2) characterized by Griffin et al. (2013) and *gyrB* sequence fragments from *E. piscicida* determined by Abayneh et al. (2012, 2013) (Table 5) were aligned using the Clustal W application of MEGA v. 5.0 (Tamura et al. 2011). Included in the analysis was the *gyrB* sequence from another member of the Enterobacteriaceae, *Klebsiella pneumonia* (GenBank #CP000964), to serve as an outgroup. The GenBank accession numbers for all *gyrB* sequences used in the analysis are listed in Table 5. Phylogenetic trees were constructed in MEGA with the neighbor-joining algorithm (Saitou & Nei 1987), using the Kimura 2-parameter model (Kimura 1980). Bootstrap support was calculated from 1000 repetitions (Felsenstein 1985). Mean base substitutions per site between the newly obtained *gyrB* sequences from isolates categorized by Griffin et al. (2013) and *E. piscicida* (ET883), *E. piscicida*-like species (ET08013), *E. ictaluri* (S97-773), and *E. tarda* (ATCC #15947) were determined using the Kimura 2-parameter model (Kimura 1980) eliminating all positions containing gaps and missing data. Isolates and DNA group assignments determined by Griffin et al. (2013) are listed in Table 2.

Repetitive sequence-mediated PCR analysis of the 44 archived *Edwardsiella tarda* isolates was performed to determine genetic homogeneity. Visibly distinct bands were manually annotated and genetic fingerprints were analyzed using the Quantity One software v. 4.6.5 (Bio-Rad Laboratories) to calculate the Dice coefficients and generate a dendrogram based on the unweighted pair-group method using arithmetic averages (UPGMA).

RESULTS

gyrB sequencing for species identification

Griffin et al. (2013) identified 2 major DNA groups within the 18 isolates they studied, with the majority being classified as DNA Group II. The DNA Group II *gyrB* sequences determined in the present study averaged 99.8% (range 99.6 to 100.0%) homology to the *gyrB* sequence of the *Edwardsiella piscicida* type strain ET883 (Abayneh et al. 2012, 2013) (data not shown). Comparatively, isolates from DNA Group II averaged <87% homology at the *gyrB* locus to the *E. tarda* type strain (ATCC #15947; range 86.5 to 87.0%). Similarly, isolates from DNA Group I demonstrated no greater than 87.1% homology (range 86.8 to 87.1%; mean 86.9%) to ET883, while averaging 99.7% similarity (range 99.6 to 99.9%) to the *E. tarda* type strain (ATCC #15947). One isolate (LADL 05-105), a genetic variant of DNA Group II, was identified as atypical *E. tarda* by PCR (Sakai et al. 2009, Griffin et al. 2013).

Table 5. *gyrB* sequences of *Edwardsiella piscicida*, *E. piscicida*-like, *E. tarda* and *E. ictaluri* isolates used in phylogenetic analysis. Isolates initially characterized by Griffin et al. (2013) and sequenced in the present study are in **bold**

Isolate	Geographic origin	Source/host species	GenBank accession no.
E. piscicida			
LADL 97-168	Louisiana, USA	Channel catfish *Ictalurus punctatus*	JX866996
LADL 99-462	Louisiana, USA	Channel catfish *I. punctatus*	JX866997
MA97-004	Massachusetts, USA	Tilapia *Oreochromis* sp.	JX866994
S11-285	Mississippi, USA	Channel catfish *I. punctatus*	JX866995
S07-346	Mississippi, USA	Channel catfish *I. punctatus*	JX866998
S07-262	Mississippi, USA	Channel catfish *I. punctatus*	JX866999
S07-534	Mississippi, USA	Channel catfish *I. punctatus*	JX867000
S07-275	Mississippi, USA	Channel catfish *I. punctatus*	JX867001
S07-1019	Mississippi, USA	Blue catfish *I. furcatus*	JX867002
S07-348	Mississippi, USA	Channel catfish *I. punctatus*	JX867003
HL9.1	Northern Europe	Turbot *Scophthalmus maximus*	JN700743
ET883	Norway	European eel *Anguilla anguilla*	JN700725
ET3612	Norway	European eel *A. anguilla*	JN700731
ET2455	Norway	European eel *A. anguilla*	JN700726
ET2493	Norway	European eel *A. anguilla*	JN700727
ET2639	Norway	European eel *A. anguilla*	JN700728
ET2640	Norway	European eel *A. anguilla*	JN700729
ET3381	Norway	European eel *A. anguilla*	JN700730
LTB4	Qingdao, China	Turbot *S. maximus*	JN700740
WY18	Qingdao, China	Turbot *S. maximus*	JN700742
ETA1	Scotland, UK	Turbot *S. maximus*	JN700732
ETA2	Scotland, UK	Turbot *S. maximus*	JN700733
ETA3	Scotland, UK	Turbot *S. maximus*	JN700734
ETB1	Scotland, UK	Turbot *S. maximus*	JN700735
ETB2	Scotland, UK	Turbot *S. maximus*	JN700736
ETB3	Scotland, UK	Turbot *S. maximus*	JN700737
ETK01	South Korea	Korean catfish *Silurus asotus*	JN700738
ETK02	South Korea	Korean catfish *S. asotus*	JN700739
RM298.1	Southern Europe	Turbot *S. maximus*	JN700722
***E. piscicida*-like**			
LADL 05-105	Louisiana, USA	Tilapia *Oreochromis* sp.	JX867004
ET080813	Qingdao, China	Marbled eel *A. marmorata*	JN700723
ET080814	Qingdao, China	Japanese eel *A. japonica*	JN700724
NCIMB 2056	Unknown	Sea bream *Evynnis japonicus*	JN700741
E. tarda			
RE-04	Alabama, USA	Channel catfish *I. punctatus*	JX866989
AL98-87	Alabama, USA	Channel catfish *I. punctatus*	JX866990
FL 95-01	Florida, USA	Channel catfish *I. punctatus*	JX866992
ATCC 15947	Kentucky, USA	Human feces	JX866988
LADL 88-209	Louisiana, USA	Hybrid striped bass *Morone saxitalis* × *M. chrysops*	JX866991
LADL 99-302	Louisiana, USA	Tilapia *Oreochromis* sp.	JX866993
NCIMB 2034	Unknown	Fish unknown species	EU259314
E. ictaluri			
93-146	Mississippi, USA	Channel catfish *I. punctatus*	CP001600
S97-773	Mississippi, USA	Channel catfish *I. punctatus*	JX867005

Table 6. Mean base substitutions per site between *gyrB* sequences from isolates categorized by Griffin et al. (2013) and *Edwardsiella piscicida* (ET883), *E. piscicida*-like species (ET080813), *E. ictaluri* (S97-773), and *E. tarda* (ATCC #15947). Analysis was conducted using the Kimura 2-parameter model (Kimura 1980), eliminating all positions containing gaps and missing data. Isolates and DNA group assignments determined by Griffin et al. (2013) are listed in Table 2

	DNA Group I	DNA Group II	ET883	ET080813	S97-773	ATCC #15947
DNA Group I	0.004	0.149	0.146	0.154	0.149	0.003
DNA Group II	0.149	0.003	0.002	0.042	0.055	0.148
LADL 05-105	0.154	0.042	0.043	0.000	0.065	0.153

This isolate shared 95.9% homology with the *E. piscicida* type strain (ET883), 86.7% similarity to the *E. tarda* type strain (ATCC #15947), 94.9% homology to *E. ictaluri*, and 100% identity with an isolate characterized by Abayneh et al. (2013) as *E. piscicida*-like species (ET080813).

Mean between-group distances for *gyrB* gene sequences determined in the present study demonstrated the DNA Group II isolates characterized by Griffin et al. (2013) to be largely homologous to the type strain of *Edwardsiella piscicida* (ET883) (Table 6). Similarly, the mean between group distances are highly similar between DNA Group I and *E. tarda* (ATCC #15947) as well as between LADL 05-105 and ET080813 from China (Abayneh et al. 2013). Based on *gyrB* sequence comparisons, isolates from DNA Group I are synonymous with *E. tarda* (ATCC #15947), isolates from DNA Group II are conspecific with *E. piscicida* (ET883), and the DNA Group II genetic variant (LADL 05-105) is homologous to *E. piscicida*-like species (ET080813).

Phylogenetic analysis of the *gyrB* sequences (Fig. 1) supported previous findings, demonstrating 2 distinct genetic taxa among organisms previously classified as *Edwardsiella tarda* (Abayneh et al. 2012, Yang et al. 2012, Griffin et al. 2013), supporting the work of Abayneh et al. (2013), who proposed the adoption of *E. piscicida*. The corresponding *gyrB* sequences obtained in the present study were amended to the current *gyrB* sequences for each isolate already in GenBank (GenBank accession numbers JX866988 to JX867004) (Griffin et al. 2013).

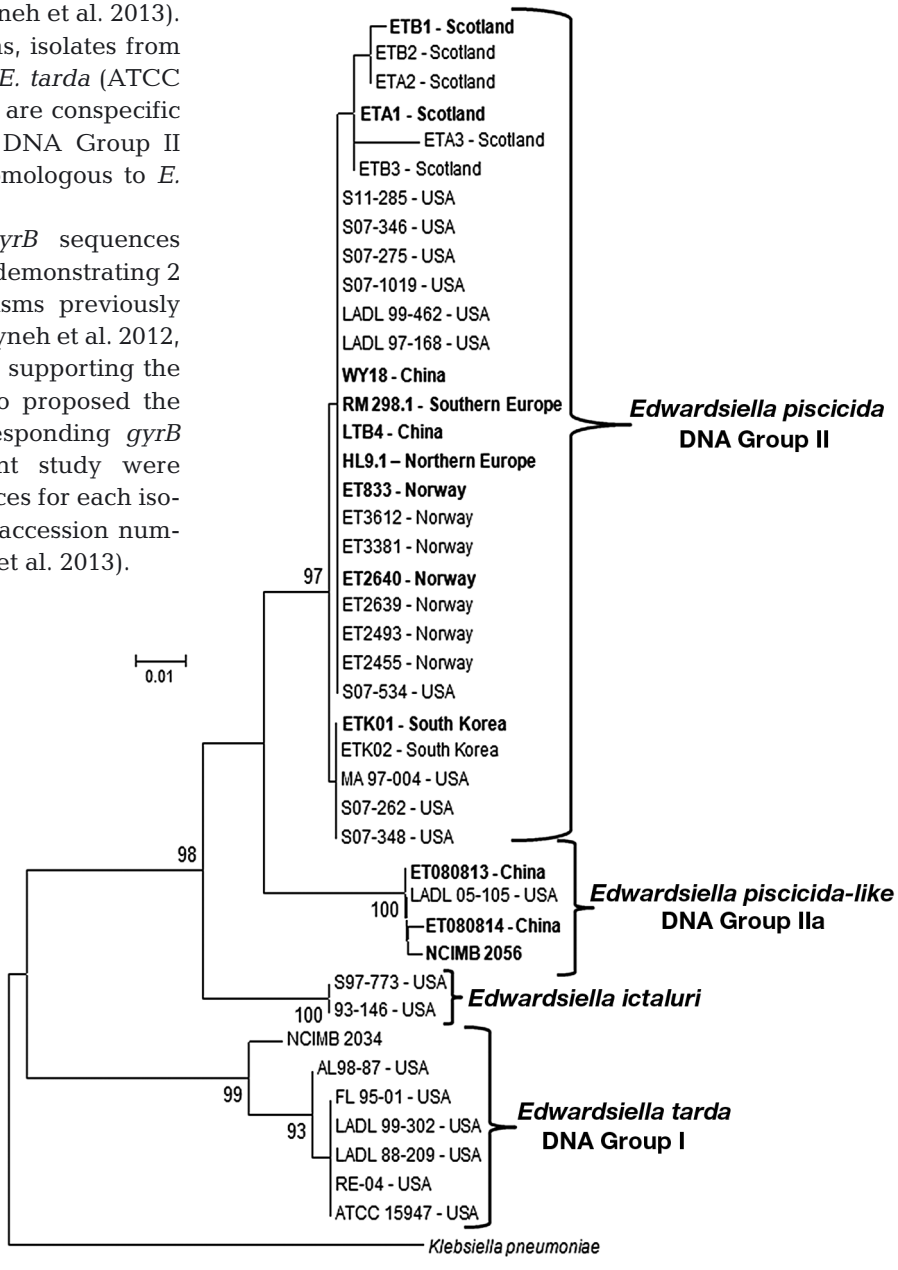

Fig. 1. Phylogenetic relationships of *Edwardsiella* isolates from fish inferred from the neighbor-joining method based on *gyrB* gene sequence and rooted at *Klebsiella pneumoniae* (GenBank #CP003200). The *E. tarda* group includes the ATCC type strain (#15947). *E. piscicida* isolates (ET833, ET2640, LTB4, WY18, ETK01, ETA1, ETB1, RM 298.1, and HL9.1) and *E. piscicida*-like isolates (ET080813, ET080814, and NCIMB 2056) characterized by Abayneh et al. (2013) are in **bold**. DNA Groups I, II, and IIa determined by Griffin et al. (2013) are identified. The percentage of replicate trees in which the associated taxa clustered together in the boot-strap test (1000 replicates) is shown next to the branches. GenBank accession numbers for sequences used in this analysis are listed in Table 5

Edwardsiella ictaluri, *E. piscicida*, *E. piscicida*-like and *E. tarda*-specific PCR

The newly developed PCR primers were specific to their respective groups. There was robust amplification using the ET primers from all 6 isolates identified as *Edwardsiella tarda* by *gyrB* sequence, with no amplification observed from isolates identified as *E. piscicida, E. piscicida*-like, or *E. ictaluri.* Similar results were found for the EP and EPL primers, with strong amplification from their respective isolates and no false positives from non-target organisms (Table 2, Fig. 2). The EP and EPL primers were in

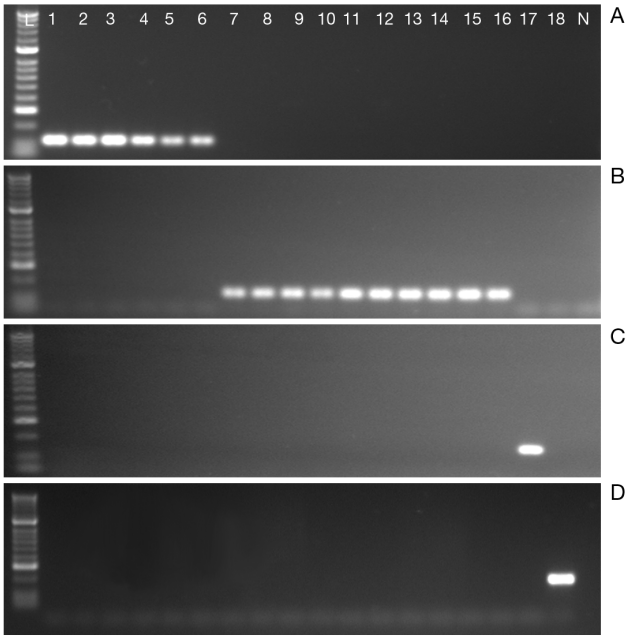

Fig. 2. Validation of (A) *Edwardsiella tarda*, (B) *E. piscicida*, (C) *E. piscicida*-like, and (D) *E. ictaluri* primer sets for discrimination between different *Edwardsiella* species. Lane L: molecular weight marker; Lanes 1–6: DNA Group I isolates; Lanes 7–16: DNA Group II isolates; Lane 17: DNA Group IIa; Lane 18: *E. ictaluri*. Lane N: no-template control

agreement with the primers originally designed by Sakai et al. (2009), suggesting that what they had classified as typical and atypical *E. tarda* and what Abayneh et al. (2013) classified as *E. piscicida* and *E. piscicida*-like species are synonymous.

Survey results of the 44 isolates from diseased catfish identified every archived specimen as *Edwardsiella piscicida*. Similarly, target amplification was observed from all 44 isolates using the ETT primer set designed by Sakai et al. (2009), suggesting that what Sakai referred to as typical *E. tarda* and what Abayneh et al. (2013) described as *E. piscicida* are the same. None of the 44 archival isolates were PCR-positive for *E. ictaluri, E. piscicida*-like species, or *E. tarda* (Fig. 3).

Repetitive sequence-mediated PCR (rep-PCR)

Genomic fingerprinting was in agreement with the *gyrB* phylogeny, demonstrating a high degree of genetic homogeneity between the 44 archival isolates identified as *Edwardsiella piscicida* by PCR. There were varying degrees of resolution among primer sets, with the lowest degree of variability demonstrated by the BOX primer set, followed by ERIC II and lastly the ERIC I and II primers (Fig. 4). However, regardless of primer set, all the archived *E. piscicida* isolates collected from diseased catfish demonstrated greater similarity to other *E. piscicida* isolates than to *E. piscicida*-like species (LADL 05-105), *E. ictaluri* (S97-773), the *E. tarda* type strain (ATCC #15947), or the *Escherichia coli* outlier (ATCC #25922). For all primer sets, the UPGMA tree clustered the catfish isolates together as a single, monophyletic group, separate from the other *Edwardsiella* spp. included in the analysis and the *E. coli* outgroup (Figs. 5–7).

DISCUSSION

The genetic diversity of *Edwardsiella tarda* has been well documented, and several studies have demonstrated the existence of 2 distinct genetic groupings of *E. tarda* (Castro et al. 2006, 2011, Panangala et al. 2006, Acharya et al. 2007, Maiti et al. 2008, 2009, Wang et al. 2011). Based on these historical findings as well as their own data, Abayneh et al. (2012) suggested fish-pathogenic *E. tarda* isolates have been previously misclassified and likely represented >1 genetically distinct, yet unrecognized taxa within the genus *Edwardsiella*. This was supported by multi-locus sequence analysis of several housekeeping genes. Their work demonstrated that *E. tarda* from humans (ATCC #15947), as well as a reference *E. tarda* from fish (NCIMB #2034), forms a distinct clade, sister to a clade that includes other *E. tarda* (ET883-like) from fish and *E. ictaluri*.

The work of Abayneh et al. (2012) is supported by Yang et al. (2012), who performed comparative phylogenomics on several *Edwardsiella tarda* and *E. ictaluri* isolates. They also identified 2 distinct groups of *E. tarda*, termed EdwGI and EdwGII. The group EdwGI contained isolates EIB202 isolated from diseased turbot *Scophthalmus maximus* in Shandong, China (Wang et al. 2009), isolate 080813 from a Japanese eel *Anguilla japonica* in Fugian, China (Wang et al. 2011), and isolate FL6-60 isolated from a striped bass *Morone saxatilis* in Maryland, USA (Baya et al. 1997). Comparatively, EdwGII contained the type strains from humans (ATCC #15947 and ATCC #23685) as well as isolate DT from oscar *Astronotus ocellatus* in Guangzhou, China (Castro et al. 2011). Yang et al. (2012) employed the average nucleotide identity (ANI) method (Konstantinidis & Tiedje 2005) to determine genomic relatedness. An ANI value of 94% corresponds to a DNA-DNA

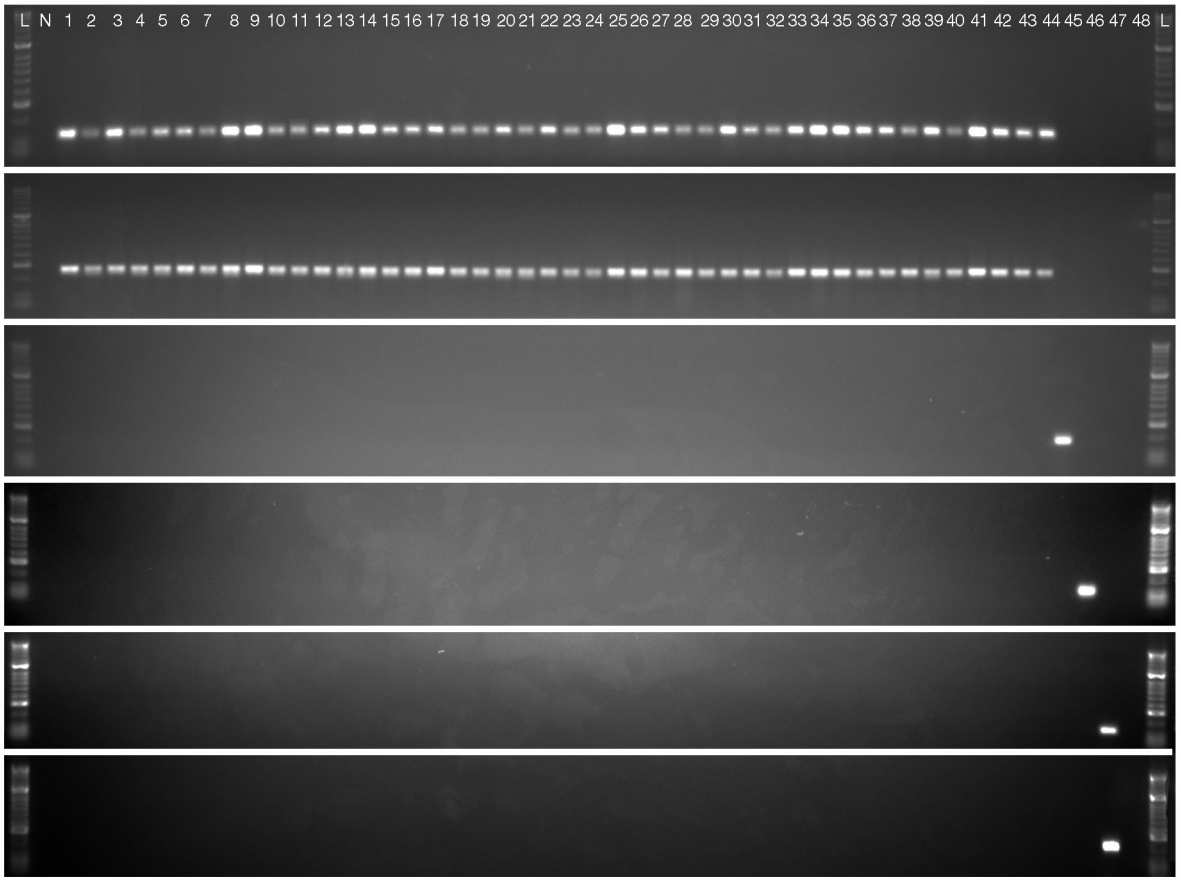

Fig. 3. PCR analysis of archived *Edwardsiella* isolates identified as *E. tarda* by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (Becton Dickson). Analysis was performed using (A) *E. piscicida* (EP), (B) typical *E. tarda* (ETT; Sakai et al. 2009), (C) *E. ictaluri* (ESC; Bilodeau et al. 2003, Griffin et al. 2011), (D) *E. tarda* (ET), (E) *E. piscicida*-like (EPL), and (F) atypical *E. tarda* (ETA; Sakai et al. 2009) primer sets. Lane designations as follows: L, Hyperladder II molecular weight marker (Bioline USA); N, no-template control; 1–44, *Edwardsiella* species from diseased channel catfish (Table 1); 45, *E. ictaluri* (S97-773); 46, *E. tarda* (ATCC #15947); 47, atypical *E. tarda*/*E. piscicida*-like sp. (LADL 05-105); 48, *Escherichia coli* (ATCC #25922)

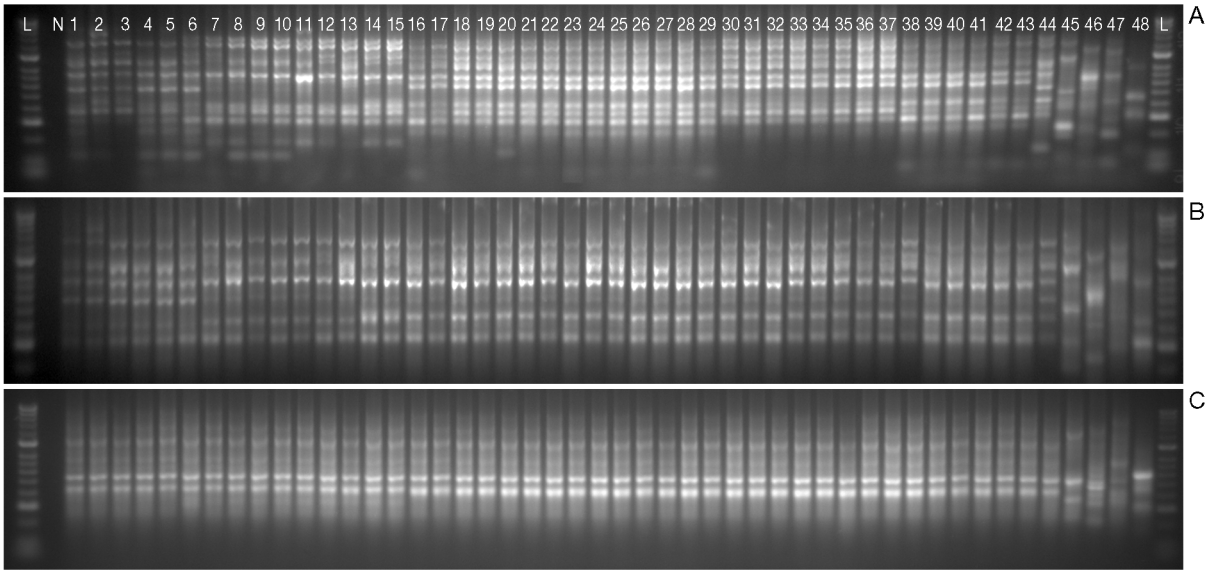

Fig. 4. Repetitive sequence-mediated PCR analysis of archived *Edwardsiella* isolates identified as *E. tarda* by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (Becton Dickson). Analysis was performed using (A) ERIC I and II, (B) ERIC II, and (C) BOX primer sets. Lane designations as in Fig. 3

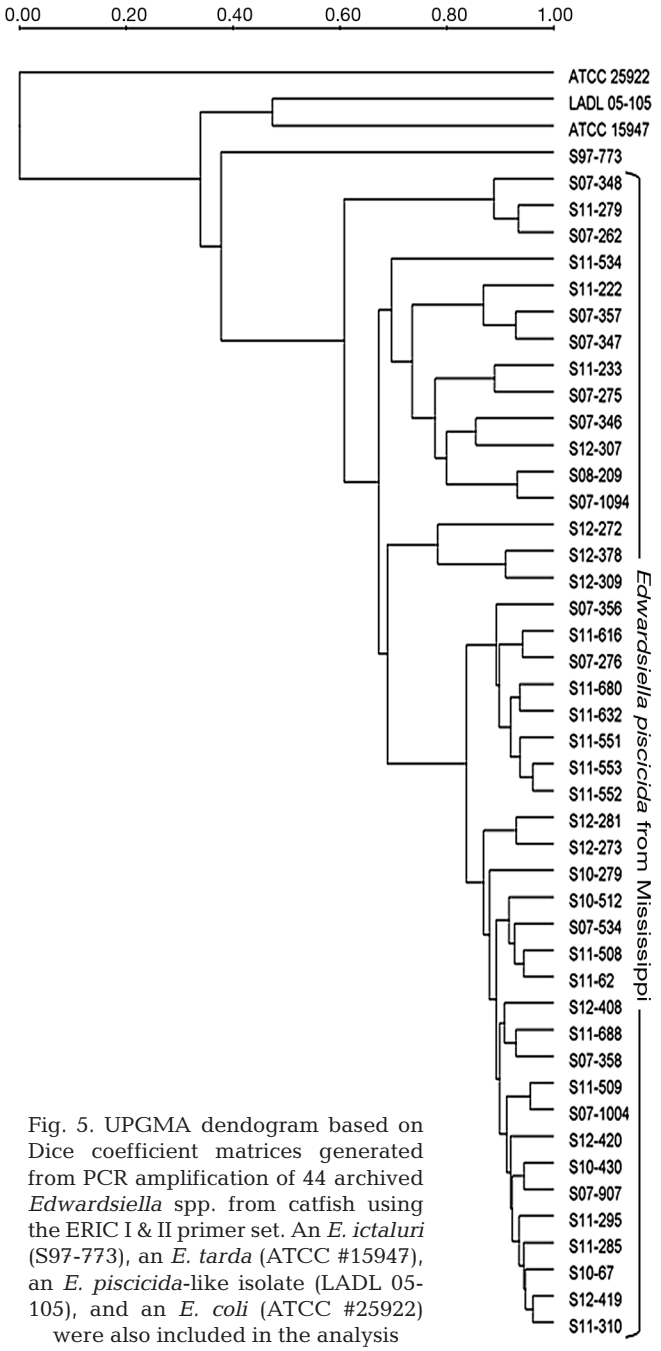

Fig. 5. UPGMA dendogram based on Dice coefficient matrices generated from PCR amplification of 44 archived *Edwardsiella* spp. from catfish using the ERIC I & II primer set. An *E. ictaluri* (S97-773), an *E. tarda* (ATCC #15947), an *E. piscicida*-like isolate (LADL 05-105), and an *E. coli* (ATCC #25922) were also included in the analysis

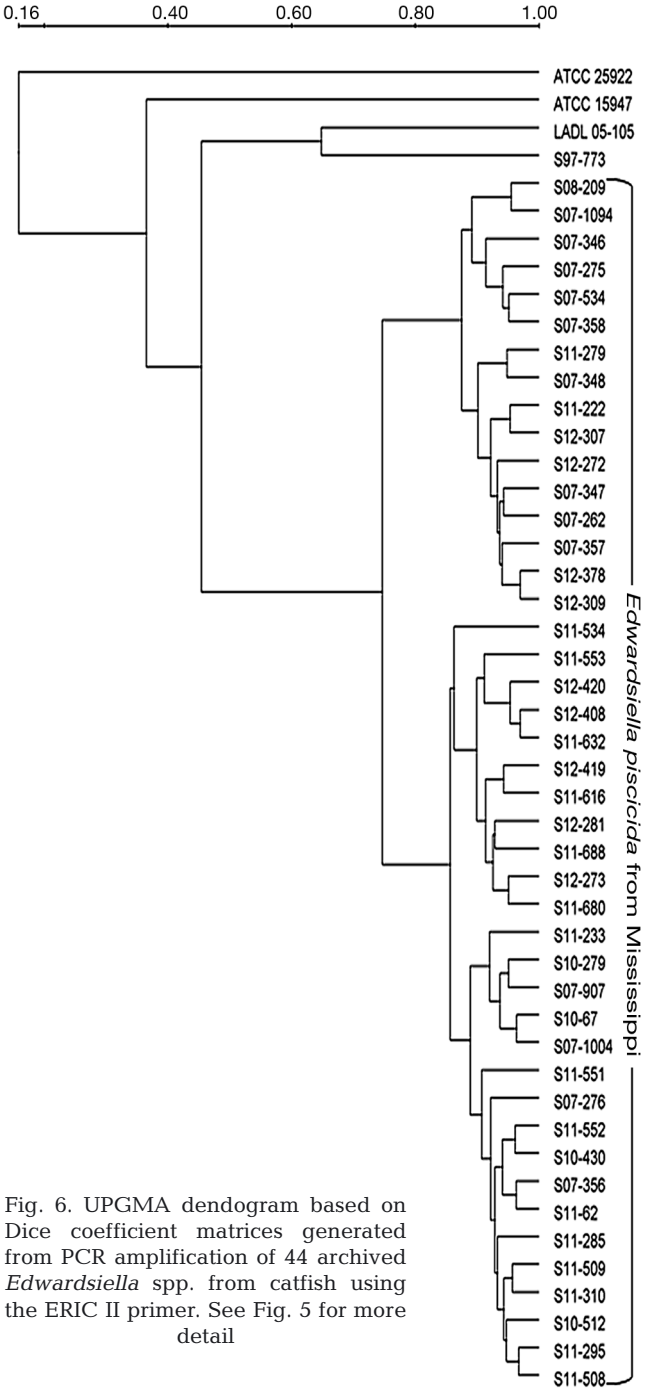

Fig. 6. UPGMA dendogram based on Dice coefficient matrices generated from PCR amplification of 44 archived *Edwardsiella* spp. from catfish using the ERIC II primer. See Fig. 5 for more detail

hybridization value of 70%, traditionally used as the cutoff for species designation (Wayne et al. 1987, Goris et al. 2007). Yang et al. (2012) demonstrated an ANI of ≥94% within the EdwGI but <83% between EdwGI and EdwGII, suggesting that EdwGI and EdwGII represent 2 different, genetically distinct bacterial species.

Similar work performed by Griffin et al. (2013) supports the findings of Yang et al. (2012) and Abayneh et al. (2012), demonstrating 2 distinct genetic groups of *Edwardsiella tarda* from fishes in the United States. Griffin et al. (2013) supported the claims of Abayneh et al. (2012), suggesting the existence of 2 genetically distinct, yet phenotypically indistinguishable taxa of *Edwardsiella* within the organisms traditionally classified as *E. tarda*. Similar to the work of Yang et al. (2012), Griffin et al. (2013) identified 2 distinct genetic groups, one (DNA Group I) that contained the *E. tarda* type strain from humans (ATCC #15947) and another that consisted solely of isolates

Fig. 7. UPGMA dendogram based on Dice coefficient matrices generated from PCR amplification of 44 *Edwardsiella* spp. from catfish using the BOX primer set. See Fig. 5 for more detail

collected from diseased fish (DNA Group II). Multilocus sequence analysis of several housekeeping genes (*gyrB*, *gyrA*, *pho*, *pgi*, and *pgm*) demonstrated the DNA Group II isolates to be ≥99% similar to the EIB-like isolates described by Yang et al. (2012). Comparatively, the isolates identified as DNA Group I, which included the ATCC type strain from humans (ATCC #15947), demonstrate <87% similarity at the same loci. Moreover, DNA Group II showed ≥94% similarity to *E. ictaluri* at these same gene targets.

In their description of *Edwardsiella piscicida*, Abayneh et al. (2013) performed DNA-DNA hybridization (DDH) between isolates they identified as *E. piscicida* and the *E. tarda* type strain (ATCC #15947). The DDH re-association values between *E. piscida* isolates and *E. tarda* were low (15 to 43%), well below the 70% DDH threshold required for inclusion within the same species (Wayne et al. 1987). Based on these and other findings, Abayneh et al. (2013) proposed that *Edwardsiella* isolates from fish, previously classified as *E. tarda* (ET883-like strains), did not belong to *E. tarda* or any of the previously established taxa within the genus *Edwardsiella* and proposed the adoption of *E. piscicida* sp. nov., with their isolate ET883 as the type strain.

The *gyrB* gene has been demonstrated as a suitable phylogenetic marker for identification and classification of bacteria. A single-copy gene that is present in all bacteria, *gyrB* has been demonstrated to be more reliable for determining relationships between closely related bacterial species than 16S rDNA (Dauga 2002). The *gyrB* gene sequences obtained here from the DNA Group II isolates described by Griffin et al. (2013) demonstrated high homology (99.6 to 100%) to the *gyrB* sequences of the *Edwardsiella piscicida* type strain (ET883) and to similar isolates described by Abayneh et al. (2012, 2013), suggesting they are conspecific. In addition, based on *gyrB* sequence homology and selected gene fragments from previous studies, *E. tarda* genomes FL-60 (GenBank Assembly# ASM14630v1) (van Soest et al. 2011) and EIB 202 (GenBank Assembly# ASM2086v1) (Wang et al. 2009) are also homologous to *E. piscicida* (Abayneh et al. 2012, Griffin et al. 2013).

The PCR primers developed here are specific to their respective isolates and allow for rapid molecular discrimination between phenotypically similar organisms in pure culture and potentially in field samples, though further validation would be required for the latter. Moreover, the present work indicates that the ETT and ETA primers by Sakai et al. (2009) are valid for their respective taxa and specific to isolates homologous to *E. piscicida* and *E. piscicida*-like species (Abayneh et al. 2013). More importantly, 100% of the archived *E. tarda* isolates collected from diagnostic case submissions of diseased fish in Mississippi were identified as *E. piscicida* by PCR. Genomic fingerprinting of isolates identified as *E. piscicida* demonstrated a high degree of genetic similarity between the isolates, suggesting *E. piscicida* isolates from diseased catfish represent a genetically homogenous group, similar to what has been demonstrated for *E. ictaluri* (Griffin et al. 2011).

These data suggest, as with *E. ictaluri*, that culture species, management practices, and geographic proximities associated with farm-raised catfish have resulted in selective pressures and epidemiological links favoring a relatively homogenous genetic group. Although noteworthy, the biological significance of these findings is unclear.

Previous work has suggested that some strains of *Edwardsiella tarda* were pathogenic to fish, while others were not (Sakai et al. 2009). Although we do not have challenge data to support this hypothesis, all the archived isolates in the present study were obtained from disease case submissions, and all of them were identified as *E. piscicida* by PCR. This would suggest *E. piscicida* may be more commonly associated with disease outbreaks in catfish than *E. tarda.* However, several isolates characterized by Griffin et al. (2013) and identified here as *E. tarda* by *gyrB* sequencing and species-specific PCR (DNA Group I) were isolated from diseased fish, suggesting that although not as common as *E. piscicida*, *E. tarda* can still be associated with fish kills. Still, based on our findings, it is possible that many *E. tarda* strains historically identified as fish-pathogenic *E. tarda* may be misclassified *E. piscicida*.

From 2001 to 2006, there were 5 cases of *Edwardsiella tarda* diagnosed from case submissions to the ARDL in Stoneville, MS, representing <0.1% of total case submissions. From 2007 to 2012, there were 116 cases, representing ~3% of total case submissions (http://tcnwac.msstate.edu/publications.htm). In all cases, the bacteria isolated from diseased fish were identified biochemically as *E. tarda.* Of the 116 cases diagnosed as *E. tarda* from 2007 to 2012, 44 isolates (38%) were chosen for the present study, all of which were molecularly confirmed as *E. piscicida.* Although *E. ictaluri* and *Flavobacterium columnare* are still the most commonly diagnosed bacterial infections at the ARDL, recent findings suggest *E. piscicida* may be an emerging pathogen in catfish aquaculture in the southeastern United States.

Future research will focus on the identification of a discriminatory phenotypic characteristic to differentiate between these *Edwardsiella tarda* and *E. piscicida*. Furthermore, additional work is required to elucidate how widespread *E. piscicida* is in cultured fishes in the United States, most notably, how often isolates originally identified as *E. tarda* have been misclassified. Lastly, although both taxa have been identified from diseased catfish, investigations into the variable pathogenicity and virulence associated with these 2 organisms must be undertaken before any firm claims regarding the pathogenicity of *E. tarda* and *E. piscicida* to catfish can be made.

Results of the present study confirm previous findings, suggesting the existence of 2 genetically distinct, yet phenotypically indistinguishable taxa within the group of organisms traditionally classified as *Edwardsiella tarda* (Abayneh et al. 2012, Griffin et al. 2013). The PCR assays described here were demonstrated specific to their respective targets and identified all 44 survey isolates, classified as *E. tarda* upon initial isolation, to be genetically homogenous to *E. piscicida*. In addition, the present research supports the work of Abayneh et al. (2013) and the adoption of *E. piscicida* as a fourth member of the *Edwardsiella*.

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