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Comparative Analysis of *Edwardsiella* Isolates from Fish in the Eastern United States Identifies Two Distinct Genetic Taxa Amongst Organisms Phenotypically Classified as *E. tarda*

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Comparative analysis of *Edwardsiella* isolates from fish in the eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*

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ABSTRACT

Edwardsiella tarda, a Gram-negative member of the family Enterobacteriaceae, has been implicated in significant losses in aquaculture facilities worldwide. Here, we assessed the intra-specific variability of *E. tarda* isolates from 4 different fish species in the eastern United States. Repetitive sequence mediated PCR (rep-PCR) using 4 different primer sets (ERIC I & II, ERIC II, BOX, and GTG_5) and multi-locus sequence analysis of *16S SSU rDNA, groEl, gyrA, gyrB, pho, pgi, pgm, and rpoA* gene fragments identified two distinct genotypes of *E. tarda* (DNA group I; DNA group II). Isolates that fell into DNA group II demonstrated more similarity to *E. ictaluri* than DNA group I, which contained the reference *E. tarda* strain (ATCC #15947). Conventional PCR analysis using published *E. tarda*-specific primer sets yielded variable results, with several primer sets producing no observable amplification of target DNA from some isolates. Fluorometric determination of G + C content demonstrated 56.4% G + C content for DNA group I, 60.2% for DNA group II, and 58.4% for *E. ictaluri*. Surprisingly, these isolates were indistinguishable using conventional biochemical techniques, with all isolates demonstrating phenotypic characteristics consistent with *E. tarda*. Analysis using two commercial test kits identified multiple phenotypes, although no single metabolic characteristic could reliably discriminate between genetic groups. Additionally, anti-microbial susceptibility and fatty acid profiles did not demonstrate remarkable differences between groups. The significant genetic variation (<90% similarity at *gyrA*, *gyrB*, *pho*, *phi* and *pgm*; <40% similarity by rep-PCR) between these groups suggests organisms from DNA group II may represent an unrecognized, genetically distinct taxa of *Edwardsiella* that is phenotypically indistinguishable from *E. tarda*.

1. Introduction

Edwardsiella tarda, a Gram-negative, motile, rod-shaped bacterium, is the causative agent of edwardsiellosis in a wide variety of cultured fish and has been implicated in significant losses in aquaculture worldwide (Mohanty and Sahoo, 2007). First described from humans (Ewing et al.,

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1965), it is the most widespread member of the *Edwardsiella* genera, having been reported from over 20 species of freshwater and marine fish from 25 countries in the Americas, Europe, Asia, Australia, Africa and the Middle East (Hawke and Khoo, 2004). In channel catfish, *E. tarda* is the causative agent of emphesematous putrefactive disease of catfish and was the first member of the genus described as a pathogen in channel catfish (Meyer and Bullock, 1973). Although traditionally considered less important than the closely related *E. ictaluri*, case submissions to the Aquatic Diagnostic Laboratory of the Thad Cochran National Warmwater Aquaculture Center in Stoneville, MS (http://tcnwac.msstate.edu/publications.htm) suggest *E. tarda* is a potential emerging disease in catfish aquaculture in the southeastern United States.

Several studies have demonstrated a wide degree of intraspecific diversity for *E. tarda* from different geographic regions and host species, making the development of broad-spectrum molecular based diagnostic tools difficult (Castro et al., 2006; Panangala et al., 2006, Acharya et al., 2007; Maiti et al., 2008; Maiti et al., 2009; Castro et al., 2011; Wang et al., 2011; Yang et al., 2012). As a result, several researchers have reported the development of *E. tarda*-specific PCR assays with varying levels of success (Chen and Lai, 1998; Sakai et al., 2007; Lan et al., 2008; Sakai et al., 2009). The purpose of this study was to determine if *E. tarda* isolates from fish in the eastern United States demonstrate the same level of intraspecific variability seen in other geographic regions, in turn providing baseline information for the development of more reliable molecular diagnostic tools.

2. Materials and methods

2.1. Isolation and identification of Edwardsiella tarda

A total of 47 *E. tarda* isolates were obtained from the archived collections of the Thad Cochran National Warmwater Aquaculture Center (NWAC), The Aquaculture/Fisheries Center of the University of Arkansas-Pine Bluff, The Department of Biological Sciences at Auburn University, The Aquatic Microbiology Laboratory at Auburn University and the Louisiana Aquatic Diagnostic Laboratory (LADL). All isolates were collected from diseased fish. Briefly, cryostocks were streaked for isolation on Mueller-Hinton agar plates supplemented with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C. Individual colonies were used for identification by the BBL™ Crystal™ Enteric/Nonfermenter ID kit (BD, Franklin Lakes, NJ, USA) following the manufacturer's suggested protocol. Isolates were grouped by biotype based on their identification code and two representatives from each biotype were chosen for further analysis with the exception of biotype 20, which had only 1 representative. An ATCC *E. tarda* isolate (#15947) as well as *Edwardsiella ictaluri* (S94-711; S97-773; S07-698) isolates obtained from diseased channel catfish were included in the analysis.

2.2. Conventional bacterial characterization

For phenotypic analysis, cryostocks were streaked for isolation on Mueller-Hinton agar plates supplemented with 5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C (28 °C for *E. ictaluri*). An individual colony from each isolate was then subcultured onto Trypticase Soy Agar (TSA; Difco, St Louis, MO) for 48 h at 37 °C and each isolate was then characterized by classical microbiological and biochemical tube tests and standardized procedures as described by MacFaddin (1981) and Lennette et al. (1985). All bacteriological media and supplies were prepared and sterilized according to manufacturer's recommendations. Based upon the results from individual tests, bacteria were classified according to referenced flow charts and identification schemes (Lennette et al., 1985; MacFaddin, 1981; Panangala et al., 2006).

2.3. DNA extraction

Individual colonies served to inoculate 5 ml of Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD) overnight at 37 °C (*E. tarda*) or 28 °C (*E. ictaluri*) without shaking and cultures were pelleted by centrifugation. Genomic DNA from all isolates was extracted using the Puregene DNA Isolation Kit (Qiagen, Valencia, CA) following the manufacturer's suggested protocols for gram-negative bacteria and quantified spectrophotometrically (Nanodrop, Wilmington, DE, USA).

2.4. Edwardsiella tarda specific PCR

Genomic DNA from all isolates were analyzed using previously established protocols for PCR amplification of *Edwardsiella* spp., *E. ictaluri*, and *E. tarda* (Chen and Lai, 1998; Sakai et al., 2007; Lan et al., 2008; Sakai et al., 2009; Castro et al., 2011; Griffin et al., 2011) (Table 1). Briefly, the 25-μl PCRs consisted of EconoTaq PLUS GREEN 2X Master Mix (Lucigen Corporation, Middleton, WI, USA); 20 pmol of each primer, 5 ng of DNA template and nuclease-free H_2O to volume. Amplification cycles used for denaturation, primer annealing and extension were carried out according to the respective protocol. Aliquots of each amplification reaction (10 μl) were electrophoresed through a 1.25% (w/v) agarose gel, stained with ethidium bromide and visualized under ultraviolet light for the presence of the appropriate sized bands, determined by direct comparison with concurrently run DNA standards (Hyperladder II, Bioline USA inc., Taunton, MA, USA).

2.5. Repetitive sequence mediated PCR (rep-PCR)

Genetic fingerprinting for each isolate was carried out using modifications to existing protocols (Versalovic et al., 1991, 1994; Castro et al., 2011; Griffin et al., 2011) (Table 2). Three *E. ictaluri* isolates (S94-711; S97-773; S07-698) and an *Escherichia coli* (ATCC# 25952) were included in the analysis. Genomic DNA for these additional isolates was obtained as described above. Briefly, the analysis consisted of 25-μl reactions comprised of 13 μl of IQ Supermix (BioRad, Hercules, CA, USA), 20 (ERIC I and II) or 40 (BOX, ERIC II, GTG5) pmol of primer, 100 ng of DNA template and nuclease-free H_2O to volume. Amplifications were performed on a PTC 200 gradient cycler (MJ Research,

Table 1
Primers used for *Edwardsiella* specific PCR.

Target	Primer	Sequence (5′-3′)	Reference
E. tarda - Hemolysin gene	ChenF ChenR	CCTTATAAATTACTCGCT TTTGTGGAGTAACAGTTT	Chen and Lai, 1998
Edwardsiella tarda - Major fimbrial subunit	EtFimAF EtFimAR	CGGTAAAGTTGAGTTTACGGGTG TGTAACCGTGTTGGCGTAAG	Sakai et al., 2007
E. tarda - Fimbrial subunit	EtFimDF EtFimDR	GGTAACCTGATTTGGCGTTC GGATCACCTGGATCTTATCC	Sakai et al., 2007
E. tarda - DNA gyrase subunit B	LanF LanR	GCATGGAGACCTTCAGCAAT GCGGAGATTTTGCTCTTCTT	Lan et al., 2008
Typical *E. tarda* - Fimbrial gene cluster	EttypF EttypR	TTCCGCAACCATGATCAAAG AGGGCATATATCCACTCACTG	Sakai et al., 2009
Atypical *E. tarda* - Fimbrial gene cluster	EtatypF EtatypR	GAACAGCGCCTCTGTCTG AATTGCTCTATACGCACGC	Sakai et al., 2009
Edwardsiella sp. - Fimbrial gene cluster	GenEDF GenEDR	ACAGCCTGGAAGAGTCCTAC TTGAGAGTCGCTGCTTAC	Sakai et al., 2009
Edwardsiella ictaluri Phosphoserine transaminase	Eict Eict	ACTTATCGCCCTCGCAAC GCCTCTGATAAGTGGTTCTCG	Griffin et al., 2011
16S small-subunit ribosomal DNA	27F 1525R	GAGTTTGATCCTGGCTCAG AGAAAGGAGGTGATCCAGCC	Rainey et al., 1996

Waltham, MA) 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 electrophoresed through a 1.5% (w/v) agarose gel containing ethidium bromide (1 μg/ml) and visualized under ultraviolet light. Band sizes were assigned by direct comparison with concurrently run DNA standards (Hyperladder II, Bioline USA inc., Taunton, MA, USA).

2.6. Multilocus DNA Sequencing

A panel of 8 different housekeeping genes was selected for multilocus analysis (16S SSU, *gyrA*, *gyrB*, *groEL*, *pgi*, *pgm*, *pho*, *rpoA*). Primers were synthesized commercially (Sigma-Aldrich, The Woodlands, TX, USA) and with the exception of the universal prokaryotic 16S SSU rRNA primers (Rainey et al., 1996), all primers were designed specifically for this study (Table 3). To ensure amplification from all *E. tarda* isolates, targets were selected based on the presence of variable regions flanked by regions conserved between *E. tarda* and *E. ictaluri*. Primers were designed for amplification of ~500–600 bp fragments based on assembled genomes of *E. tarda* (GenBank #CP001135; #CP002154) and *E. ictaluri* (GenBank #CP001600) (Wang et al., 2009; van Soest et al., 2011; Williams et al., 2012).

Table 2
Primers used for repetitive sequence mediated PCR.

Primer	Sequence (5′-3′)	Reference
BOX	CTACGGCAAGGCGACGCTGAC G	Versalovic et al., 1994
ERIC I	ATGTAAGCTCCTGGGGATTCAC	Versalovic et al., 1991
ERIC II	AAGTAAGTGACTGGGGTGAGCG	Versalovic et al., 1991
GTG_5	GTGGTGGTGGTGGTG	Versalovic et al., 1994

Amplification reactions (25 μl) were performed using 13 μl of iProof™ High-fidelity Mastermix (BioRad, Hercules, CA), 20 pmol of each primer, 10 ng of genomic DNA and nuclease-free water to volume using the following thermocycling profile: 3 min denaturation at 98 °C; 45 cycles of 30 s at 98 °C, 30 s at 55 °C, 2 min at 72 °C; and 7 min extension at 72 °C. Amplicons were visualized with UV light after electrophoretic migration through a 1.25% agarose gel containing ethidium bromide (1 μg/ml) and were either gel-excised or purified directly using Qiagen Qiaquick™ columns (Qiagen, Valencia, CA). The purified products were either directly sequenced using the corresponding primers or cloned into a plasmid vector (pCR®4Blunt-TOPO®; Zero Blunt® TOPO® PCR Cloning Kit for sequencing, Invitrogen, San Diego, CA) and sequenced according to the manufacturer's instructions. Purified PCR products or plasmid DNA was cycle-sequenced from both strands using ABI BigDye™ chemistry (Applied Biosystems, Foster City, CA), alcohol-precipitated and run on an ABI Prism 3730™ automated sequencer (Applied Biosystems, Foster City, CA). Reaction components and thermal cycling conditions were the same for all primers sets. Contiguous sequences were assembled using the corresponding chromatograms and the SeqMan™ utility of the Lasergene software package (DNAStar, Madison, WI).

2.7. Phylogenetic analysis

Genetic fingerprints generated by rep-PCR were analyzed using the Quantity One software v. 4.6.5 (Bio-Rad Laboratories Inc., Hercules, CA) to calculate the Dice coefficients and generate a dendrogram based on unweighted pair-group method using arithmetic averages (UPGAMA).

Sequence fragments generated from multilocus sequencing of several housekeeping genes were aligned using the Clustal W application of MEGA version 5.0 (Tamura et al., 2011). For each gene, reference sequences

Table 3
Sequencing primers used for genetic identification. All primer sequences were designed specifically for this study unless otherwise noted*.

Target	Primer	Sequence (5′-3′)
Glucose-6-phosphate isomerase	*PgiF*	TGCCGACCGTTTCTCTAAGT
	PgiR	GACCCAGTCCCAGAACTCAA
Phosphoglucomutase	*PgmF*	TCGCCATTCTGACCCATAAC
	PgmR	GCTGGAGACCAGCGTCTTAC
DNA-directed RNA polymerase α-subunit	*RpoAF*	CGGTACGCTGTACCAGATCA
	RpoAR	CCGAGGTTGAGATTGATGGT
Phosphate transport system regulatory protein	*PhoF*	ATATCCGCACCCAGGTAATG
	PhoR	TGTCAGCAGCTGTTCCAGAT
Chaperonin GroEl	*GroeLF*	GTTCATTCTGCTGGCTGACA
	GroeLR	GCTCTTCGTTGATGCCTTTC
DNA gyrase subunit A	*GyrAF*	AGCGCCTTGTACTCATCCAG
	GyrAR	TGGTGCATGAGATCCCCTAT
DNA gyrase subunit B	*GyrBF*	CCCTGTCTGAAAAGCTGGAG
	GyrBR	CTCGTTCATCAGCGACTCAA
16S small subunit ribosomal DNA	*27F*	GAGTTTGATCCTGGCTCAG[a]
	1525R	AGAAAGGAGGTGATCCAGCC[a]
	16SR2	CTTCTTTTGCAACCCACTCC
	16SR3	CCCAACATTTCACAACACGA
	16SR4	TACGCATTTCACCGCTACAC
	16SR5	GTGCAATATTCCCCACTGCT

[a] Rainey et al. (1996).

from the published genomes of *E. tarda* (GenBank #CP001135; #CP002154) and *E. ictaluri* (GenBank #CP001600) were included in the analysis. Corresponding gene sequences from another member of the Enterobacteriaceae, *Klebsiella pneumoniae* (GenBank #CP000964), were included as outliers. Phylogenetic trees were constructed in MEGA with the neighbor-joining algorithm (Saitou and Nei, 1987), using the Kimura 2-parameter model (Kimura, 1980). Bootstrap support was calculated from 500 repetitions (Felsentein, 1985).

2.8. Estimation of G + C content

The genomic G + C content was estimated by a fluorometric method measuring thermal denaturation of DNA (Gonzalez & Saiz-Jiminez, 2002) and was performed on a CFX96™ Real-time PCR Detection System (BioRad, Hercules, CA) and the accompanying software. Briefly, 50-μl reactions consisted of 1 μl of SsoFast™ EvaGreen® Supermix (BioRad, Hercules, CA), 5 μl of 0.5X saline-sodium citrate buffer (pH 7.0), 5 μg of genomic DNA and nuclease-free water to volume. Thermal conditions consisted of a ramp from 25 °C to 100 °C at 1 °C min $^{-1}$, with fluorescence measurements performed at each step in the ramp. Each isolate was analyzed in triplicate.

2.9. Carbon source utilization

Metabolic fingerprints were determined using Biolog's Microlog Microbial Identification system (GEN II MicroStation System; Biolog, Hayward, CA, USA) according to the manufacturer's suggested protocol. Briefly, after culturing each isolate under the above-mentioned conditions, bacterial suspensions were prepared by removing bacterial colonies from the plate surface with a sterile cotton swab and agitating it in 20 mL of Biolog's GN/GP-IF supplemented with sodium thioglycolate. The bacterial suspension was adjusted to 61% transmittance at 590 nm with a spectrophotometer. A 150 μL aliquot of this suspension was dispensed into each well of a Biolog GN2 microplate. The plates were incubated for 24 h at 35 °C (28 °C for *E. ictaluri*) and analyzed using the MicroLog 3 v 4.20.05 software providing species-level identification (similarity index >0.50).

2.10. Antimicrobial susceptibility

The minimal inhibitory concentration (MIC) of 17 different antimicrobial agents to the 17 *E. tarda* isolates from fish and a quality control (*E. coli*) were tested using the Sensititre 18–24 h MIC and breakpoint susceptibility plates and JustOne strips (Trek Diagnostic System, West Sussex, UK), containing two fold dilutions of florfenicol (0.12–128 μg/ml), and the GN2F–SENSITITRE GRAM NEGATIVE PLATE FORMAT (Trek Diagnostic System, West Sussex, UK), containing two fold dilutions of: Amikacin (8–64 μg/ml), Ampicillin (4–32 μg/ml), Ampicillin/sulbactam 2:1 ratio (4/2–32/16 μg/ml), Aztreonam (8–32 μg/ml), Cefazolin (4–32 μg/ml), Cefepime (4–32 μg/ml), Cefotetan Na (8–32 μg/ml), Ceftriaxone (1–64 μg/ml), Ceftazidime (1–32 μg/ml), Cefoxitin (4–32 μg/ml), Cefuroxime (4–32 μg/ml), Ciprofloxacine (0.5–4 μg/ml), Imipenem (2–16 μg/ml) Gatifloxacin (1–8 μg/ml), Meropenem (1–8 μg/ml), Piperacillin (16–128 μg/ml), Nitrofurantoin (16–128 μg/ml), Piperacillin/tazobactam constant 4 (16/4–128/4 μg/ml), Ticarcillin/clavulanic acid constant 2 (16/2–64/2 μg/ml), Cefpodoxime (2–16 μg/ml), Tobramycin (4–8 μg/ml), Gentamicin (8–0.5 μg/ml), Trimethoprim/sulfamethoxazole (2/38–0.5/9.5 μg/ml), using the manufacturer's suggested protocol. Briefly, the *E. tarda* isolates, along with a reference *E. coli* isolate (ATCC 25922) quality control were plated on Mueller-Hinton agar plates supplemented with 5% sheep

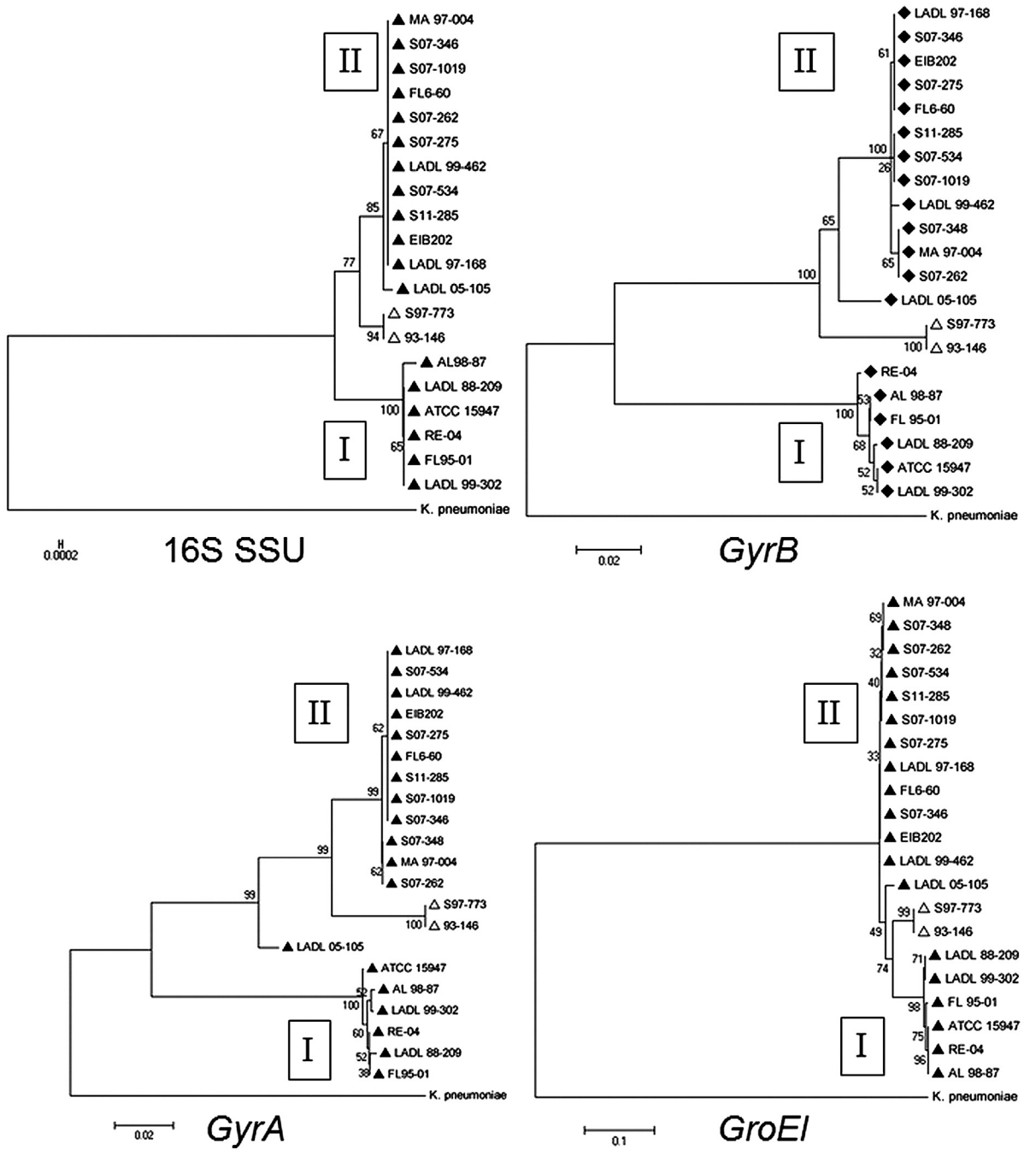

Fig. 1. Phylogenetic analysis of *Edwardsiella tarda* (black triangle) and *Edwardsiella ictaluri* (white triangle) gene sequences identifying two distinct genetic groups (I and II). Reference sequences for *E. tarda* (FL6-60; GenBank #CP002154; EIB202; GenBank #CP001135) and *E. ictaluri* (93-146; GenBank #CP001600) are included. Units are the number of base substitutions per site. *Klebsiella pneumoniae* (GenBank #CP000964) was chosen as an out-group. Bootstrap values are located at the nodes.

serum (Becton, Dickinson and Company, Sparks, MD) and grown overnight at 37 °C and incubated for 24 h, at 27 °C. Inocula were prepared by suspending colonies in 1X PBS to a 0.5 McFarland standard. This suspension was diluted 1000-fold in Mueller-Hinton broth and 50 μl were added to each well of the sensititre plates containing the different antibiotics. For each plate, three wells contained the bacterial inoculum without an antibacterial agent (positive control) and one well contained the bacterial inoculum with an antibacterial agent to prevent bacterial growth (negative control). Tested plates were covered with an adhesive seal, provided by the manufacturer, and incubated for 24 h at 27 °C. Bacterial growth was checked visually after removing the adhesive seal at 24 h post inoculation. The MIC value was defined as the lowest concentration exhibiting no visible growth.

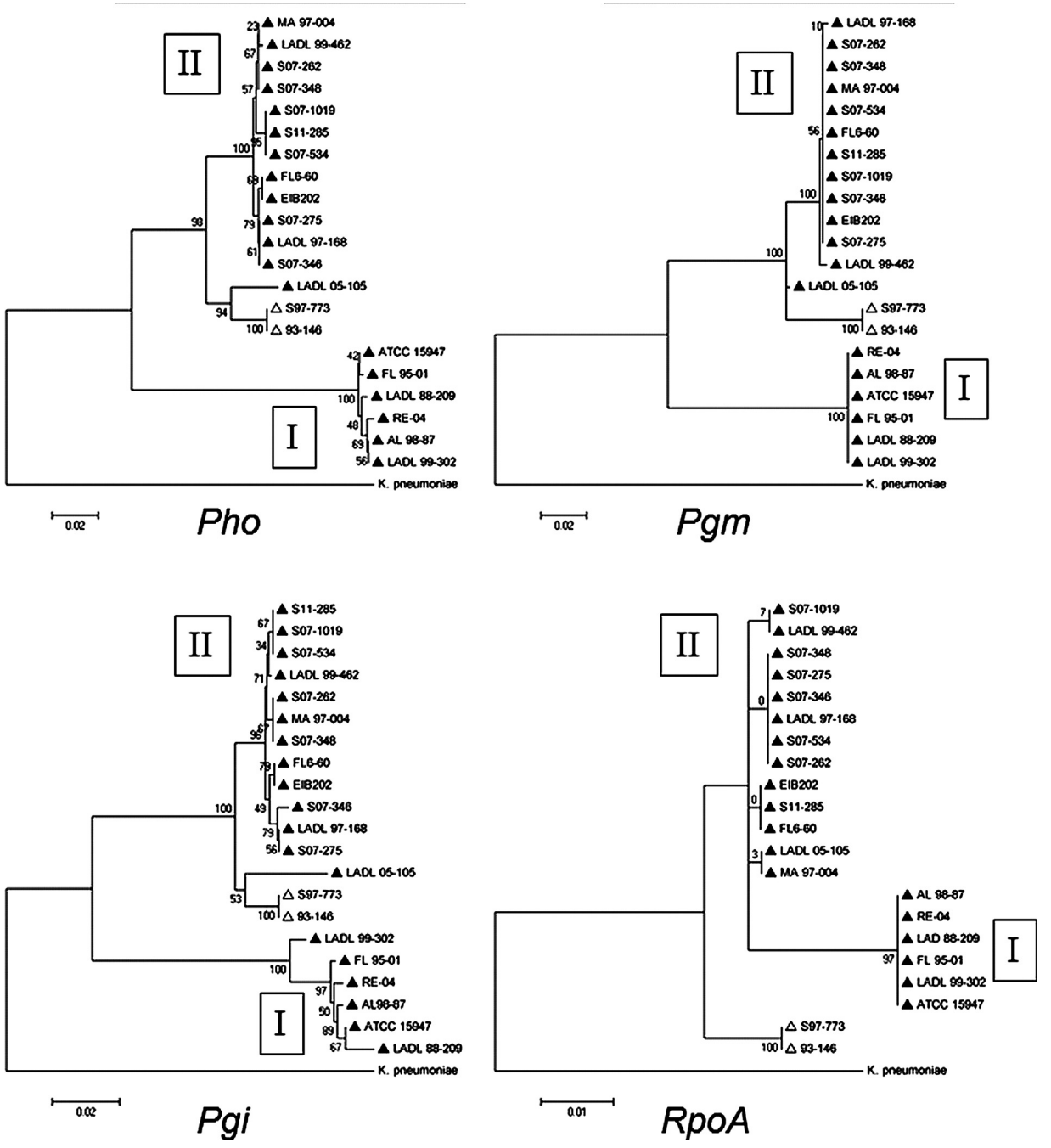

Fig. 2. Phylogenetic analysis of *Edwardsiella tarda* (black triangle) and *Edwardsiella ictaluri* (white triangle) gene sequences identifying 2 distinct genetic groups (I and II). Reference sequences for *E. tarda* (FL6-60; GenBank #CP002154; EIB202; GenBank #CP001135) and *E. ictaluri* (93-146; GenBank #CP001600) are included. Units are the number of base substitutions per site. *Klebsiella pneumoniae* (GenBank #CP000964) was chosen as an out-group. Bootstrap values are located at the nodes.

2.11. Fatty Acid Methyl Ester analysis

Gas chromatographic analysis of cellular fatty acids (GC-FAME) was performed by Microbial Identification, Inc (MIDI, Newark, DE). Species level identification and confidence levels were determined using the Sherlock® Microbial Identification System (Sasser, 1990).

3. Results

3.1. Genetic sequencing and phylogenetic analysis

Multi-locus sequencing identified two distinct genotypes of *E. tarda* (DNA group I; DNA group II). Regardless of gene target, the *E. tarda* isolates formed 2 distinct clusters with high bootstrap support (Figs. 1 and 2). Five of the *E.*

Table 4
Sequence similarity (%) of *Edwardsiella tarda* and *Edwardsiella ictaluri* isolates used in this study to *E. tarda* FL6-60 (GenBank #CP002154) and *E. ictaluri* 93-146 (GenBank #CP001600) assembled genomes.

Isolate	*Edwardsiella tarda*								*Edwardsiella ictaluri*							
	16S	*gyrB*	*gyrA*	*groEL*	*pho*	*pgi*	*pgm*	*rpoA*	16S	*gyrB*	*gyrA*	*groEL*	*pho*	*pgi*	*pgm*	*rpoA*
DNA Group I																
ATCC 15947	99.3	85.6	86.4	93.8	88.5	85.9	86.8	97.8	99.3	84.6	85.0	92.5	88.5	85.9	85.5	96.9
RE-04	99.3	86.0	86.2	93.8	88.5	85.5	86.8	97.8	99.3	85.0	85.2	92.5	88.5	85.5	85.5	96.9
AL98-87	99.2	85.8	86.0	93.8	88.5	85.7	86.6	97.8	99.2	85.2	85.4	92.5	88.5	85.7	85.3	96.9
LADL 88-209	99.3	85.6	86.0	94.0	87.7	85.7	86.8	97.8	99.3	84.8	85.0	93.1	87.7	85.4	85.5	96.9
FL95-01	99.3	85.8	86.2	93.8	88.5	85.7	86.6	97.8	99.3	84.6	85.2	92.9	88.5	85.7	85.3	96.9
LADL 99-302	99.3	85.6	86.0	94.0	88.5	85.6	86.6	97.8	99.3	84.6	85.4	93.1	88.5	85.6	85.3	96.9
DNA Group II																
MA 97-004[a]	100	99.6	99.8	99.6	99.4	99.5	100	100	99.7	94.9	95.0	94.8	97.7	95.5	95.5	98.4
S11-285[a]	100	99.8	100	99.8	99.4	98.9	100	100	99.7	94.7	95.2	95.0	97.7	95.3	95.5	98.4
LADL 97-168[a]	100	100	100	100	99.6	99.8	100	100	99.7	94.5	95.2	95.1	97.5	95.5	95.5	98.4
LADL 99-462[a]	100	99.6	100	99.6	99.6	99.3	99.6	100	99.7	94.5	95.2	95.1	97.8	95.3	95.0	98.4
S07-346[a]	100	100	100	100	99.4	99.8	100	100	99.7	94.5	95.2	95.1	97.3	95.5	95.5	98.4
S07-262[a]	100	99.6	99.8	99.6	99.4	99.5	100	100	99.7	94.9	95.0	94.8	97.7	95.5	95.5	98.4
S07-534[a]	100	99.8	100	99.8	99.4	98.9	100	100	99.7	94.7	95.2	95.0	97.7	95.3	95.4	98.4
S07-275[a]	100	100	100	100	99.6	99.6	100	100	99.7	94.5	95.2	95.1	97.5	95.3	95.5	98.4
S07-1019[a]	100	99.8	100	99.8	98.8	98.7	100	100	99.7	94.7	95.2	95.0	97.5	95.1	95.5	98.4
S07-348[a]	100	99.6	99.8	99.6	99.4	99.5	100	100	99.7	94.9	95.0	94.8	97.7	95.5	95.5	98.4
LADL 05-105[b]	99.9	97.0	95.2	97.9	96.1	94.6	98.3	100	99.8	95.4	94.4	95.1	96.7	96.6	96.3	98.4
Edwardsiella ictaluri																
S97-773	99.7	94.5	95.2	95.1	97.5	95.7	95.5	98.4	100	100	100	100	100	100	100	100

[a] Typical *Edwardsiella tarda* as defined by Sakai et al. (2009)
[b] Atypical *Edwardsiella tarda* as defined by Sakai et al. (2009)

tarda isolates (AL 98-87; FL 95-01; LADL 88-209; LADL 99-302; RE-04) clustered with the ATCC reference strain (DNA group I). The remainder of the isolates grouped with the reference *E. tarda* genomes (DNA group II), clustering with *E. ictaluri* to form a clade sister to DNA group I. One genetic variant, LADL 05-105, clustered with DNA group II but diverged from the remainder of the isolates. Regardless of genetic group (I or II), all *E. tarda* isolates demonstrated greater than 98% similarity to *E. tarda* 16S SSU, *GyrB*, *Pgm*, and *Pho* sequences available in GenBank, although each group demonstrated similarities to different entries. For all genes, all isolates from DNA group II were more than 99%

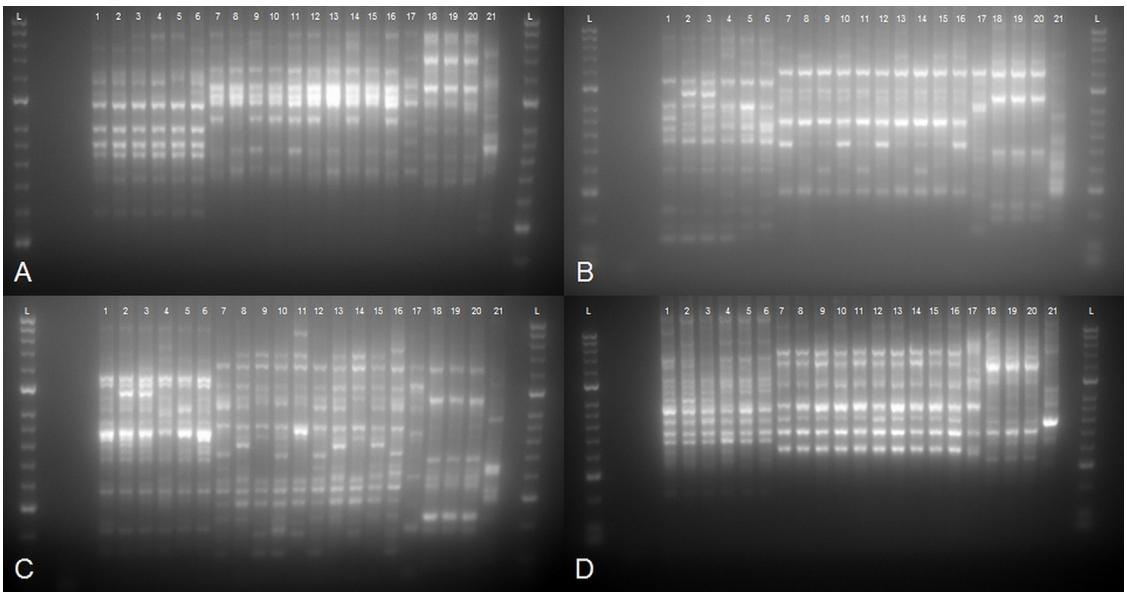

Fig. 3. Repetitive sequence mediated PCR amplification of DNA from *Edwardsiella tarda* (lanes 1–17), *Edwardsiella ictaluri* (Lanes 18–20) and *Escherichia coli* (lane 21). Genetic profiles were generated using GTG_5 (A), ERIC II (B), ERIC I and II (C) and BOX (D) primers. Lane designations are as follows for all gels: L = Hyperladder II; lanes 1 ATCC 15947; 2 = RE-04; 3 = AL98-87; 4 = LADL 88-209; 5 = FL95-01; 6 = LADL 99-302; 7 = MA 97-004; 8 = S11-285; 9 = LADL 97-168; 10 = LADL 99-462; 11 = S07-346; 12 = S07-262; 13 = S07-534; 14 = S07-275; 15 = S07-1019; 16 = S07-348; 17 = LADL 05-105; 18 = S94-711; 19 = S97-773; 20 = S07-698; 21 = ATCC 25922.

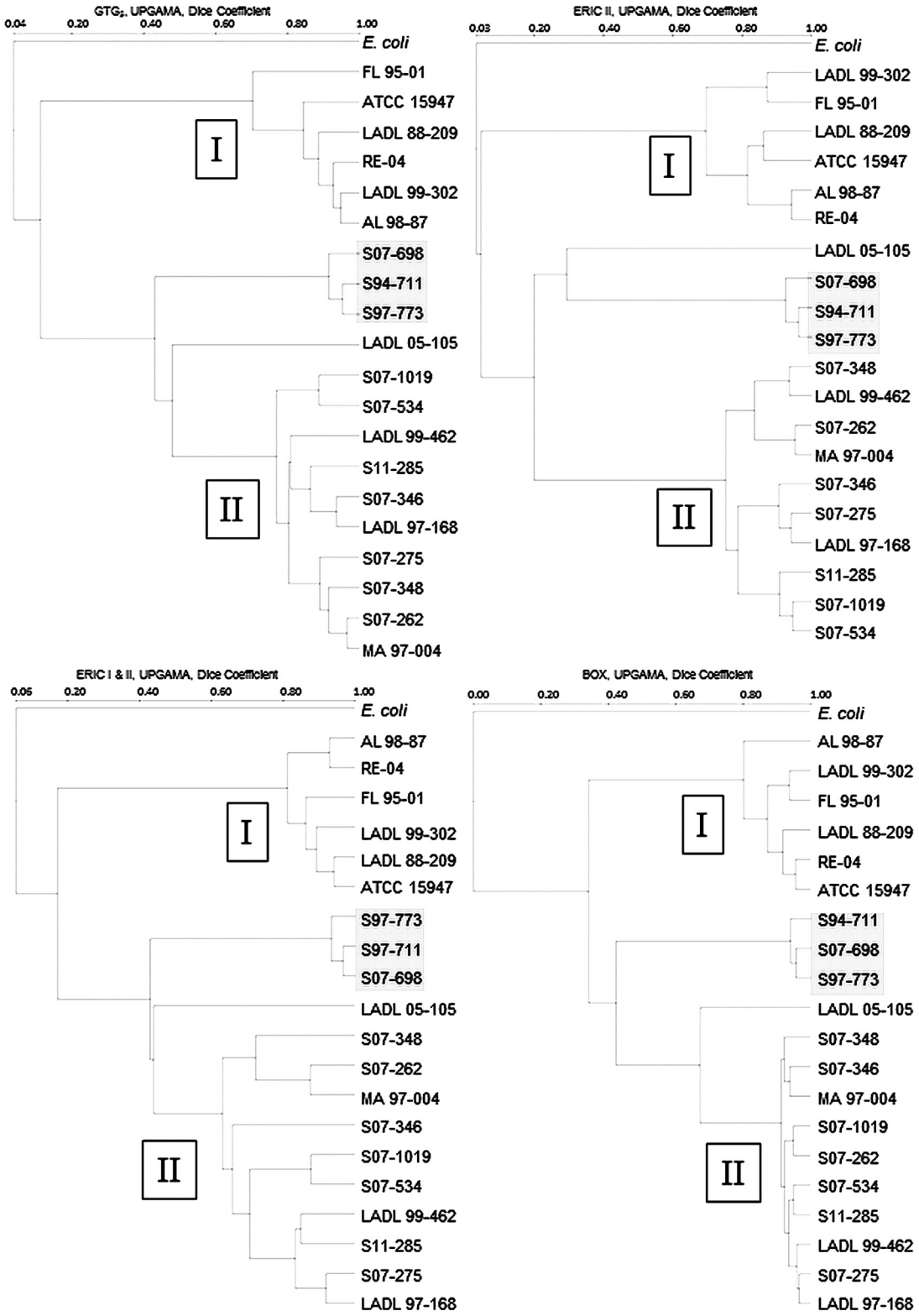

Fig. 4. UPGAMA dendograms based on Dice coefficient matrices generated from PCR amplification of *Edwardsiella tarda* DNA using four different primer sets. The isolates consistently formed two distinct clusters (I and II). Three *E. ictaluri* (S94-711; S97-773; S07-696; gray highlight) isolates were also included in the analysis.

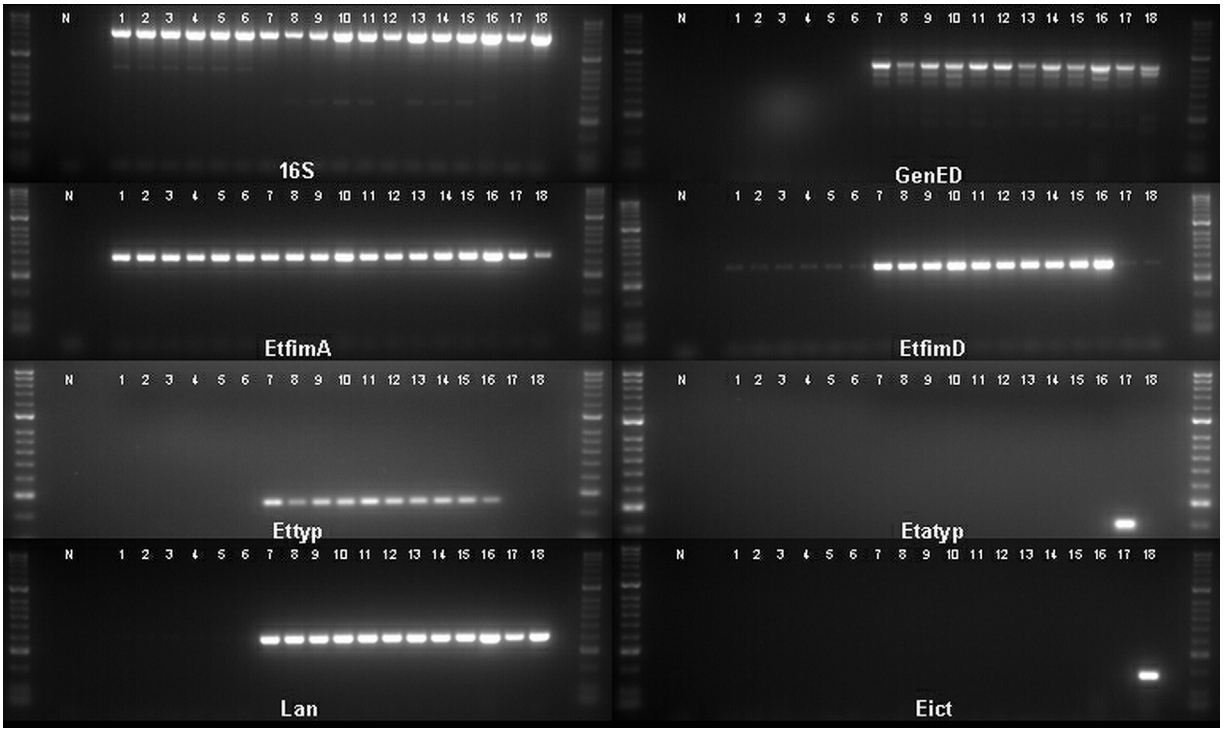

Fig. 5. Polymerase chain reaction amplification of bacterial isolates identified biochemically and by 16S sequencing as *Edwardsiella tarda* (lanes 1–17) or *Edwardsiella ictaluri* (lane 18). Primer sequences and corresponding publications can be found in Table 1. Lane designations are as follows for all gels: N = no template control; 1 = ATCC 15947; 2 = RE-04; 3 = AL98-87; 4 = LADL 88-209; 5 = FL95-01; 6 = LADL 99-302; 7 = MA 97-004; 8 = S11-285; 9 = LADL 97-168; 10 = LADL 99-462; 11 = S07-346; 12 = S07-262; 13 = S07-534; 14 = S07-275; 15 = S07-1019; 16 = S07-348; 17 = LADL 05-105; 18 = S97-773 (*Edwardsiella ictaluri*).

similar to the published *E. tarda* genomes, with the exception of LADL 05-105, which demonstrated slightly more variability (94–100%). Alternatively, DNA group I, which includes the ATCC reference strain, demonstrated remarkable differences from these genomes for all targets (Table 4). For all gene targets, DNA group II, including LADL 05-105, demonstrated mofre similarity to *E. ictaluri* than to DNA group I. Sequences for all targets for all isolates have been deposited in GenBank (#JX866952–JX867095).

3.2. Repetitive sequence mediated PCR

Genetic profiles for the isolates used in this study were consistent with the multi-locus sequencing data, regardless of primer set. The isolates formed two distinct clusters (Figs. 3 and 4), with LADL 05-105 again falling sister to DNA group II. The two predominant *E. tarda* clusters demonstrated no greater than 40% similarity between them, regardless of primer set. All isolates, consistently fell into the same clusters, with 5 of the isolates (AL 98-87; FL 95-01; LADL 88-209; LADL 99-302; RE-04) grouping with the ATCC reference strain, and the remaining isolates grouping more closely to *E. ictaluri* (Fig. 4).

3.3. Edwarsiella tarda-specific PCR

Results for *Edwardsiella*-specific PCRs from 5 ng of template DNA were variable (Fig. 5). All isolates were positive by 16S rDNA PCR. Positive reactions were also observed for DNA group I from the *E. tarda* fimbrial subunit primers (EtfimD; Sakai et al., 2007), with faint bands observed for the *E. tarda* major fimbrial subunit primers (EtfimA; Sakai et al., 2007). There was no amplification from DNA group I isolates with any other primer sets. In contrast, strong positive reactions were observed for isolates from DNA group II for the *Edwardsiella* fimbrial gene cluster (GenEd; Sakai et al., 2009), the *E. tarda* fimbrial subunit (EtfimD), and *E. tarda* DNA gyrase subunit B (EtgyrB; Lan et al., 2008). All group II isolates were positive for the *E. tarda* major fimbrial subunit (EtfimA) and the typical *E. tarda* fimbrial gene cluster (Ettyp; Sakai et al., 2009), with the exception of LADL 05-105, which had only a faint positive for the *E. tarda* major fimbrial subunit (EtfimA) and was negative for the typical *E. tarda* fimbrial gene cluster (Ettyp). However, LADL 05-105 was positive for the atypical *E. tarda* fimbrial gene cluster (Etatyp; Sakai et al., 2009). By comparison, positive reactions were observed for *E. ictaluri* isolate S97-773 from the *Edwardsiella* fimbrial gene cluster (GenEd), the *Edwardsiella* fimbrial subunit, *E. tarda* DNA gyrase subunit B and *E. ictaluri* specific primers, with a faint positive for the *E. tarda* major fimbrial subunit. No positive reactions were observed for any *E. tarda* isolates from *E. ictaluri* specific primers or primers amplifying the *E. tarda* hemolysin gene (Chen and Lai, 1998).

Table 5
Fluorometric estimation of G + C content (%) based on genomic DNA melting temperatures(T_m). G + C content (%) was estimated by the mean T_m (°C) from triplicate reactions.

Isolate	G + C content (%)	T_m(±S.D.)
DNA Group I		
ATCC 15947	56.0	82.3 (±0.2)
RE-04	55.7	82.1 (±0.3)
AL98-87	57.2	82.9 (±0.2)
LADL 88-209	56.6	82.6 (±0.0)
FL95-01	56.5	82.5 (±0.1)
LADL 99-302	56.1	82.3 (±0.1)
Group mean	56.4	82.5 (±0.3)
DNA Group II		
MA 97-004	59.9	84.3 (±0.1)
S11-285	60.5	84.5 (±0.4)
LADL 97-168	58.9	83.7 (±0.1)
LADL 99-462	60.3	84.5 (±0.2)
S07-346	60.6	84.6 (±0.0)
S07-262	60.7	84.7 (±0.1)
S07-534	60.3	84.5 (±0.4)
S07-275	60.6	84.6 (±0.4)
S07-1019	60.5	84.5 (±0.1)
S07-348	60.5	84.5 (±0.1)
LADL 05-105	60.3	84.5 (±0.1)
Group mean	60.3	84.5 (±0.3)
Edwardsiella ictaluri		
S97-773	58.4	83.5 (±0.2)

3.4. Estimation of G + C content

All isolates from DNA group I, which includes the ATCC type strain (#15947) demonstrated lower melting temperatures than DNA group II and *E. ictaluri*. The average melting temperature for DNA group I was 82.3 °C compared to 84.5 °C for DNA group II. Isolate LADL 05-105, a genetic variant of DNA group II, demonstrated a melting temperature of 84.5 °C, while S97-773, the *E. ictaluri* isolate melted at 83.5 °C. Using the relationship between fluorometric estimations of genomic melting temperatures and G + C content (Gonzalez and Saiz-Jimenez, 2002), the mean G + C content for DNA group I was 56.4%, 60.2% for DNA group II, and 58.4% for *E. ictaluri*. Results of this analysis are tabulated in Table 5.

Table 6
Biochemical identification of *Edwardsiella* isolates used in this study; CL = confidence level; Sim = similarity index.

Isolate	Host (State)	BBL Crystal ID	BBL Crystal Code[a,b]	Biolog ID; (CL; Sim)
DNA Group I				
ATCC 15947	Human (KY)	*Edwardsiella tarda*	2002010013[a]	*E. tarda* (100; 0.884)
RE-04	Channel catfish (AL)	*E. tarda*	2002010013[a]	*E. tarda* (N/A; 0.448)
AL98-87	Channel catfish (AL)	*E. tarda*	2003110113[a]	*E. tarda* (98; 0.571)
LADL 88-209	Hybrid Striped Bass (LA)	*E. tarda*	2003110113[a]	*E. tarda* (100; 0.715)
FL95-01	Channel catfish (FL)	*E. tarda*	2002010113[a]	*E. tarda* (100; 0.733)
LADL 99-302	Tilapia (LA)	*E. tarda*	2002010113[a]	*E. tarda* (100; 0.634)
DNA group II				
MA 97-004	Tilapia (MA)	*E. tarda*	2403110113[a]	*E. tarda* (100; 0.814)
S11-285	Channel catfish (MS)	*E. tarda*	2403110113[a]	*E. tarda* (93; 0.653)
LADL 97-168	Channel catfish (LA)	*E. tarda*	2403010113[a]	*E. tarda* (92; 0.658)
LADL 99-462	Channel catfish (LA)	*E. tarda*	2403010113[a]	*E. tarda* (86; 0.660)
S07-346	Channel catfish (MS)	*E. tarda*	2003010103[a]	*E. tarda* (99; 0.916)
S07-262	Channel catfish (MS)	*E. tarda*	2003010103[a]	*E. tarda* (78; 0.751)
S07-534	Channel catfish (MS)	*E. tarda*	2003110103[a]	*E. tarda* (86; 0.518)
S07-275	Channel catfish (MS)	*E. tarda*	2003110103[a]	*E. tarda* (98; 0.712)
S07-1019	Blue catfish (MS)	*E. tarda*	2003010113[a]	*E. tarda* (100; 0.901)
S07-348	Channel catfish (MS)	*E. tarda*	2403010153[a]	*E. tarda* (N/A; 0.492)
LADL 05-105	Tilapia (LA)	*E. tarda*	2403014113[a]	*E. tarda* (N/A; 0.468)
Edwardsiella ictaluri				
S97-773	Channel catfish (MS)	No ID	2002000113[b]	*E. ictaluri* (90; 0.538)
S94-711	Channel catfish (MS)	No ID	2002000113[b]	N/A
S07-698	Channel catfish (MS)	No ID	2002000113[b]	N/A

[a] Indole + Oxidase -
[b] Indole -; Oxidase -

Table 7
Comparison of characteristics of *Edwardsiella tarda* isolates from this study. Tests listed in bold differed from the classical description of *Edwardsiella tarda* (Mohanty and Sahoo, 2007); CyOx = Cytochrome oxidase; VP = Vogues-Proskaueer; ONPG = β-galactosidase activity.

	DNA Group				DNA Group		
	I	II[a]	LADL 05-105		I	II[a]	LADL 05-105
Gram Stain	–	–	–	Acid from			
Morphology	R	R	R	Glucose	+	+	+
CyOx	–	–	–	**Arabinose**	–	–	+
TSI	K/A	K/A	K/A	Adonitol	–	–	–
H_2S on TSI	+	+	+	Cellobiose	–	–	–
OF glucose	Fg	Fg	Fg	Dulcitol	–	–	–
Motility at 37 °C	+	+	+	Erythrytol	–	–	–
Motility at 25 °C	+	+	+	Galactose	+	+	+
Gelatin	–	–	–	Inositole	–	–	–
Indole	+	+	+	Lactose	–	–	–
Bile esculine	–	–	–	**Maltose**	+	+	+
Urease	–	–	–	Melibiose	–	–	–
Simmon's citrate	–	–	–	Mannitol	–	–	–
Phenylalanine	–	–	–	**Mannose**	+	+	+
Nitrate reduction	+	+	+	**Rhamnose**	–	–	–
Methyl Red	+	+	+	Salicin	–	–	–
VP	–	–	–	Sorbitol	–	–	–
ONPG	–	–	–	Sorbose	–	–	–
Malonate	–	–	–	Sucrose	–	–	–
Catalase	+	+	+	**Trehalose**	–	–	–
Arginine	–	–	–	Xylose	–	–	–
Lysine	+	+	+				
Ornithine	+	+	+	Growth in TSB			
RBC-hemolysis	β	β	β	0% NaCl	+	+	+
				0.5% NaCl	+	+	+
Growth at				1.0% NaCl	+	+	+
20 °C	+	+	+	1.5% NaCl	+	+	+
25 °C	+	+	+	2.0% NaCl	+	+	+
30 °C	+	+	+	2.5% NaCl	+	+	+
35 °C	+	+	+	3.0% NaCl	+	+	+
40 °C	+	+	+				

[a] Isolate LADL 05-105 was excluded from DNA group II.

3.5. Biochemical identification, substrate metabolism, FAME analysis and antimicrobial susceptibilty

All isolates were identified as *E. tarda* by the BBL™ Crystal™ Enteric/Nonfermenter ID kit, representing 9 different phenotypes based on identification code (Table 6). There is no identification code available for *E. ictaluri*, however, the identification code is consistent with codes seen for *E. ictaluri* isolates from diseased channel catfish (Griffin, unpublished data). Traditional tests demonstrated the same characteristics for all *E. tarda* isolates, with the exception of one (LADL 05-105), which produced acid from arabinose (Table 7).

The Biolog system provided different carbon utilization patterns for all 17 *E. tarda* isolates, with *E. tarda* being the closest match for all 17 isolates. The isolates metabolized 29 different substrates; of which no single substrate discriminated between DNA groups (Table 8). In addition, the *E. tarda* isolates used in this study were relatively homogeneous in regards to cellular fatty acid composition (Table 9) and antimicrobial susceptibility patterns to the chemotherapeutic agents evaluated (data not shown).

4. Discussion

With the high degree of phenotypic diversity within bacterial species and the promiscuous nature genetic material is shared between environmental microbes, the accuracy of a biochemical identification can be limited if a large number of isolates have not been tested, especially from phenotypically heterogenous or cryptic genera. As such, current descriptions of bacterial species require both phenotypic and genetic characteristics, although this does little to deconvolute species described prior to the advent of molecular techniques.

The BBL crystal kit provided multiple identification codes for the isolates used in this study; however, the kit offers a multitude of codes for *E. tarda*, suggesting the existence of numerous phenotypes for this species. Similarly, the Biolog system generated multiple phenotypic profiles for the isolates tested, identifying all 17 isolates as *E. tarda*, although several isolates received low (<0.50) similarity index scores (RE-04; S07-348, LADL 05-105). Regardless, metabolic profiles were unable to differentiate between groups. Likewise, biochemical characterization using conventional bacteriology techniques and cellular fatty acid compositions were inconclusive in discriminating between the two genetic groups. Lastly, there were no observable differences between the two groups in terms of antimicrobial susceptibility.

Contradictory to the phenotypic data, genetic fingerprinting by rep-PCR identified two distinct genetic groups within the *E. tarda* isolates tested. All four primer sets were in agreement with the grouping of isolates, demonstrating

Table 8
Substrate metabolism for *Edwardsiella tarda* (ET) isolates; + = positive reaction; − = negative reaction; +/− = borderline positive.

Substrate	DNA Group I						DNA Group II										
	ATCC 15947	RE-04	AL 98-87	LADL 88-209	FL 95-01	LADL 99-302	MA 97-004	S11-285	LADL 97-168	LADL 99-462	S11-346	S07-262	S07-534	S07-275	S07-1019	S07-348	LADL 05-105
Dextrin	−	−	+	+/−	−	+	−	+	+	+/−	+/−	+/−	+	+	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+/−	+	+	+	+/−	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Fucose	−	−	−	+/−	−	+	−	−	−	−	−	−	−	−	−	−	−
D-Galactose	+	+	+	+	+	+	+	+	+/−	+	+	+	+	+	+	+/−	+/−
a-D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+/−	+	+	+	+	+
D-Mannose	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
D-Psicose	+	+	+	+	+	+	−	+	+	−	+/−	−	+	+	+/−	+/−	+
Pyruvic Acid Methyl Ester	+/−	−	+	−	+	+	−	+/−	−	+/−	+/−	+/−	−	−	−	−	−
Succinic Acid Mono-Methyl Ester	−	+/−	+	−	−	+	−	+	−	−	−	−	−	+	−	+/−	+
Citric Acid	+/−	+	−	−	+/−	+	−	+	+/−	−	−	−	+	+/−	−	−	+/−
D-Gluconic Acid	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	+/−	+/−
D-Clucuronic Acid	+/−	−	−	−	−	−	−	+/−	−	−	−	−	−	−	−	−	+
a-Ketoglutaric acid	−	−	+	+	+	+	−	−	−	−	−	−	−	+/−	+/−	−	−
D,L-Lactic Acid	+	+	+	+	+	+	+	+	−	−	−	−	−	+/−	+	+	+
Succinic Acid	−	+/−	+	−	+/−	+	−	+/−	−	+/−	−	−	−	+	−	+	−
Bromosuccinic Acid	−	+	+	+	+	+	−	+	+	−	−	−	−	+	+	+	+/−
L-Asparagine	+	+	+	+	+	+	+	+	+	+	+	+/−	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+	+	−	+	+	+	+	+/−	+	+	+	+	+
Glycyl-L-Aspartic Acid	+	+	+	+	+	+	+	+	+/−	+	+	+/−	−	+	+	+	+
Glycyl-L-Glutamic Acid	−	−	−	−	+/−	−	−	+	−	−	+/−	−	−	+	−	+/−	+/−
D-Serine	+	+	+	+	+	+	+	+	+	−	+	−	−	+	+	−	−
Inosine	+/−	+/−	−	−	+/−	+	−	+/−	−	−	+/−	−	−	+/−	−	+	−
Uridine	+	−	+	+	+	+	−	+	+	−	+/−	+/−	+	+	+/−	+	−
Thymidine	+	+	+	+	+	+	−	+	+	−	+/−	+/−	+	+	−	+/−	−
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
D,L,a-glycerol phosphate	+	+	+	+/−	+	+	+	+	−	+	+	+	−	+	+/−	+	+/−
a-d-glucose-1-phosphate	+	+	+	+	+	+	+	+	+	+	+	+/−	+	+	+	+	+
D-glucose-6-phosphate	+	+	+/−	+/−	+	+	−	+	+/−	+	+/−	+/−	+	+	+/−	+/−	−
ID	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET	ET
Prob	100	−	98	100	100	100	100	93	92	86	99	78	86	98	100	−	−
Sim	.88	.448	.57	.72	.73	.63	.81	.65	.66	.66	.92	.75	.52	.71	.90	0.49	0.47

Table 9
Comparison of fatty acid composition (%) from *Edwardsiella tarda* strains. LADL 05-105 was excluded from DNA Group II.

	DNA Group I							DNA Group II											
	ATCC 15947	RE-04	AL 98-87	LADL 88-209	FL 95-01	LADL 99-302	Mean	MA 97-004	S11-285	LADL 97-168	LADL 99-462	S11-346	S07-262	S07-534	S07-275	S07-1019	S07-348	Mean	LADL 05-105
Fatty Acid (%)																			
12:0	1.9	1.6	1.8	1.9	1.9	1.7	1.8	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.8
13:0	0.6	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.7	0.4	0.5	0.5	0.5	0.5	0.6	0.4	0.5	0.5	–
12:0 3OH	–	–	–	0.09	–	–	–	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	–
14:0	12.6	12.7	11.1	13.3	12.4	11.7	12.3	13.4	14.8	12.6	14.4	15.8	14.2	14.4	14.2	14.5	14.1	14.2	16.6
15:1 w8c	0.1	0.1	–	–	–	–	–	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–
15:0 3OH	–	–	–	–	–	–	–	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	–
16:1 w5c	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
16:0	33.6	34.0	34.5	36.3	35.7	35.7	35.0	34.0	34.5	32.7	33.5	33.7	34.9	34.9	34.0	34.7	34.8	34.1	37.4
17:0 anteiso	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.04	0.05	–	–
17:0 iso	–	–	0.2	–	–	–	–	–	0.1	0.1	–	0.1	–	0.1	0.1	0.1	0.1	–	–
17:1 w8c	0.4	0.3	0.3	–	–	0.3	–	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.4	0.3	0.4	0.4	–
17:0 cyclo	16.3	16.8	8.2	19.3	17.5	10.8	14.8	13.2	14.9	17.2	14.2	15.7	16.0	17.1	16.0	16.0	16.2	15.7	11.2
17:0	1.23	0.7	0.7	0.6	0.8	0.6	0.8	0.9	0.9	1.3	1.0	0.9	0.7	0.6	1.0	0.6	0.7	0.9	0.4
18:1 w6c	0.3	0.2	–	–	0.2	–	–	0.2	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	–
18:1 w9c	1.6	2.0	1.9	1.7	1.6	2.2	1.9	1.5	1.9	2.0	1.6	2.0	1.8	1.8	2.0	1.7	1.7	1.8	2.0
18:0	1.5	1.7	1.5	1.6	1.4	1.7	1.8	1.3	1.4	1.5	1.2	1.4	1.5	1.5	1.4	1.3	1.4	1.4	1.4
18:1 w7c	3.8	3.7	6.9	2.8	3.5	6.4	4.5	6.0	4.4	5.8	5.1	4.2	4.5	4.4	4.9	4.7	4.5	4.8	2.8
19:0 cyclo w8c	2.6	2.4	0.5	3.3	3.4	0.9	2.2	1.1	1.2	2.0	1.1	1.3	1.2	1.4	1.4	1.2	1.3	1.3	1.3
19:0	0.4	0.3	0.2	0.4	0.4	0.2	0.3	0.2	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.2	0.2	0.3	–
20:4 w6,9,12,15c	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.5	0.3	0.3	0.4	0.4	0.3
Summed features																			
2	9.4	9.0	8.8	8.9	9.2	8.1	8.9	8.5	8.5	8.8	8.6	8.8	8.4	8.4	8.2	8.3	8.6	8.5	9.0
3	11.0	1.3	20.8	6.8	8.3	16.9	10.9	16.2	12.4	10.9	14.7	11.3	12.3	10.8	11.9	12.5	12.0	12.5	14.1
5	2.0	2.0	1.8	2.1	2.0	1.8	1.9	1.6	1.8	2.1	1.7	2.0	1.7	1.7	1.9	1.7	1.7	1.8	2.0
8	4.0	3.9	6.9	2.8	3.6	6.4	4.6	6.1	4.6	6.1	5.2	4.4	4.7	4.5	5.1	4.8	4.7	5.0	2.8
Sim Index to *E. tarda*	0.77	0.77	0.87	0.89	0.86	0.68	0.81	0.57	0.64	0.7	0.48	0.70	0.66	0.75	0.68	0.64	0.68	0.65	<0.3

remarkable differences (<40% similarity) between DNA groups I and II. Although banding patterns for respective primer sets varied in complexity, all primer sets demonstrated substantial differences between the two main groups. Isolate LADL 05-105, likely a genetic variant, consistently grouped outside of DNA group II, although this isolate shared more similarities with DNA group II and *E. ictaluri* then DNA group I.

Similarly, fluorometric estimation of G + C content identified marked differences between DNA group I and DNA group II, with all isolates from DNA group I demonstrating melting temperatures 1–2 degrees lower than DNA group II. This difference in melting temperatures represents an approximate difference in G + C content of nearly 4%, with both groups of *E. tarda* demonstrating more similar G + C ratios to *E. ictaluri* than to each other.

This study also demonstrates the limitations of using 16S SSU sequencing for bacterial identification, which has been well reviewed (Fox et al., 1992; Janda and Abbott, 2002; Clarridge, 2004). In general, a range of about 0.5–1% difference (99–99.5% similarity) is often used as a cutoff for species discrimination, although there are several instances where even a 1% difference is overly conservative (Clarridge, 2004). Because the 16S SSU gene is so highly conserved, multilocus sequence typing has been proposed as an alternative method of genetic characterization (Cooper and Feil, 2004). Although the *E. tarda* groups in this study demonstrate greater than 99% similarity between them (~1300 bp) at the 16S SSU locus, DNA group II is more similar to *E. ictaluri* than DNA group I at every other target used in the analysis. In addition, DNA groups I and II share less than 90% similarity at several targets (*GyrA*, *GyrB*, *Pho*, *Pgi*, *Pgm*).

Phylogenetic analysis consistently separated the two *E. tarda* groups, placing members of DNA group I in their own distinct cluster with high bootstrap support (range 97–100%). Regardless of gene target, phylogram topographies repeatedly placed *E. ictaluri* sister to DNA group II as part of a larger cluster sister to DNA group I. This data suggests a monophyletic origin for the three species of bacteria (DNA group I, DNA group II and *E. ictaluri*), which likely arose from a single common ancestor, with DNA group II and *E. ictaluri* diverging in a later event. The significant genetic separation between DNA group I and DNA group II, and the fact DNA group II shares greater similarity to *E. ictaluri* than to DNA group I, suggests these two DNA groups are not conspecific. This supports similar findings in China, where comparative phylogenomics and multilocus sequencing identified two distinct genetic groups of *E. tarda* (Yang et al., 2012) and is in agreement with other studies (Castro et al., 2006; Panangala et al., 2006; Acharya et al., 2007; Maiti et al., 2008; Maiti et al., 2009; Castro et al., 2011; Wang et al., 2011).

The data presented here also clarifies why a definitive diagnostic PCR for *E. tarda* has yet to be developed. There have been several attempts to develop a PCR assay for the detection of *E. tarda* from a wide variety of substrates, with mixed results. Chen and Lai (1998) initially developed a PCR for the detection of the Hemolysin gene (GenBank L43071) of *E. tarda* from infected fish and environmental samples. Their PCR amplified the appropriate sized amplicon from 40 different *E. tarda* isolates. However, none of the isolates used in the current study were positive by this PCR, suggesting this primer set is specific to a different *E. tarda* variant.

Similarly, Sakai et al. (2007) developed primers specific to subunits of the type 1 fimbrial genes *E. tarda* isolates from different species of fish. They found primer sets EtfimA and EtfimD produced PCR products of the appropriate size from all 17 pathogenic isolates examined; however, there was inconsistent amplification from non-pathogenic isolates and the EtfimD produced false positives from *E. ictaluri*. A follow up study developed two new primer sets that separated *E. tarda* isolates into two groups, typical (Ettyp) and atypical (Etatyp) (Sakai et al., 2009); however, data presented here suggests typical and atypical isolates both fall into DNA group II.

Similarly, Lan et al. (2008) attempted to amplify the *GyrB* gene from a non-motile *E. tarda* isolated from turbot in Japan. The assay produced the desired product in 20 of 68 isolates, all of which were identified as *E. tarda* biochemically and by 16S SSU sequence. In the development of this PCR the *gyrB* genes of 3 separate isolates were sequenced and deposited into GenBank (EU259315, EU259316, and EU259317), all of which fall into DNA group II. Unfortunately, the *gyrB* sequences of the isolates that were PCR negative were not sequenced.

More recently, the above mentioned primer sets were comparatively evaluated for their ability to detect *E. tarda* in turbot (Castro et al., 2010). They evaluated 53 different isolates of *E. tarda*, confirmed biochemically and serologically, as well as 18 isolates from other bacterial strains isolated from an array of fish hosts. Contradictory to our findings, 11 of the 53 *E. tarda* isolates and 1 *E. ictaluri* isolate were positive for the hemolysin gene targeted by the primers of Chen and Lai (1998), whereas none of the isolates were positive for the *gyrB* primers of Lan et al. (2008). The EtfimA primers and EtfimD primers of Sakai et al. (2007) were the most consistent, with 49 of the 53 tarda isolates positive using the EtfimA primers, although these primers also amplified target DNA from *E. ictaluri*, *E. hoshinae*, *E. coli*, *Enterobacter aerogenes*, *Aeromonas salmonicida* ssp. *Salmonicida* and two isolates of *Yersinia ruckeri*. Conversely, all 53 isolates of *E. tarda* were amplified by the EtfimD primers, with no amplification of other bacterial strains.

It should be noted that all of the above mentioned PCR assays are valid for their respective isolates; however, sequence data is unavailable for most isolates used in the validation of these assays. While the best efforts were made to mimic the original conditions of the PCR, many factors could explain the variable results demonstrated here and in other studies, most notably the intraspecific variability of *E. tarda*. Further compounding the problem of a species-specific PCR is that one group of *E. tarda* (DNA group II) shares a greater degree of genetic similarity with *E. ictaluri* than other *E. tarda* strains. As such, the development of a PCR specific to all *E. tarda* but discriminatory against *E. ictaluri* will be unlikely. Though identified biochemically as *E. tarda*, it is evident the isolates used in these studies do not share the same genetic composition and possibly represent two genetically

distinct taxa. Although our current research identifies only two distinct genotypes, it is possible more exist. The data generated here will be utilized in the development of more accurate molecular diagnostics to better understand the role these genetically distinct *E. tarda* groups play in fish health.

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References

Acharya, M., Maiti, N.K., Mohanty, S., Mishra, P., Samanta, M., 2007. Genotyping of *Edwardsiella tarda* isolated from freshwater culture system. Comp. Immunol. Microbiol. Infect. Dis. 30, 33–40.

Castro, N., Toranzo, A.E., Barja, J.L., Núñez, S., Magariños, B., 2006. Characterization of *Edwardsiella tarda* strains isolated from turbot, *Psetta maxima* (L.). J. Fish Dis. 29, 541–547.

Castro, N., Toranzo, A.E., Núñez, S., Osorio, C.R., Magariños, B., 2010. Evaluation of four polymerase chain reaction primer pairs for the detection of *Edwardsiella tarda* in turbot. Dis. Aquat. Organ. 18, 55–61.

Castro, N., Toranzo, A.E., Bastardo, A., Barja, J.L., Magariños, B., 2011. Intraspecific genetic variability of *Edwardsiella tarda* strains from cultured turbot. Dis. Aquat. Organ. 95, 253–258.

Chen, J.D., Lai, S.Y., 1998. PCR for direct detection of *Edwardsiella tarda* from infected fish and environmental water by application of the hemolysin gene. Zool. Stud. 37, 169–176.

Clarridge, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17, 840–862.

Cooper, J.E., Feil, E.J., 2004. Multilocus sequence typing—what is resolved? Trends Microbiol. 12, 373–377.

Ewing, W.H., Mcwhorter, A.C., Escobar, M.R., Lubin, A.H., 1965. Edwardsiella, a new genus of Enterobacteriaceae based on a new species, *E. tarda*. Int. J. Syst. Evol. Microbiol. 15, 33–38.

Felsentein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fox, G.E., Wisotzkey, J.D., P. Jurtshuk, P.J.R., 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.

Gonzalez, J.M., Saiz-Jimenez, C., 2002. A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. Environ. Microbiol. 4, 770–773.

Griffin, M.J., Mauel, M.J., Greenway, T.E., Khoo, L.H., Wise, D.J., 2011. A real-time polymerase chain reaction assay for quantification of *Edwardsiella ictaluri* in catfish pond water and genetic homogeneity of diagnostic case isolates from Mississippi. J. Aquat. Anim. Health. 23, 178–188.

Hawke, J.P., Khoo, L.H., 2004. Infectious diseases. In: Tucker, C.S., Hargreaves, J.A. (Eds.), Biology and Culture of the Channel Catfish. Elsevier B.V., Amsterdam, The Netherlands, pp. 387–443.

Janda, J.M., Abbott, S.L., 2002. Bacterial identification for publication: when is enough enough? J. Clin. Microbiol. 40, 1887–1891.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Lan, J., Zhang, X.H., Wang, Y., Chen, J., Han, Y., 2008. Isolation of an unusual strain of *Edwardsiella tarda* from turbot and establish a PCR detection technique with the *gyrB* gene. J. Appl. Microbiol. 105, 644–651.

Lennette, E.H., Ballows, A., Hauser Jr., W.J., Shadomy, H.J., 1985. Manual of Clinical Microbiology, 4th edition. American Society for Microbiology, Washington, DC 1149 pp.

MacFaddin, J.F., 1981. Biochemical Tests for Identification of Medical Bacteria, 2nd edition. Williams and Wilkins, Baltimore/London 527 pp.

Maiti, N.K., Mandal, A., Mohanty, S., Samanta, M., 2008. Comparative analysis of genome of *Edwardsiella tarda* by BOX-PCR and PCR-ribotyping. Aquaculture 280, 60–63.

Maiti, N.K., Mandal, A., Mohanty, S., Mandal, R.N., 2009. Phenotypic and genetic characterization of *Edwardsiella tarda* isolated from pond sediments. Comp. Immunol. Microb. 32, 1–8.

Meyer, F.P., Bullock, G.L., 1973. *Edwardsiella tarda*, a new pathogen of channel catfish (*Ictalurus punctatus*). Appl. Microbiol. 25, 155–156.

Mohanty, B.R., Sahoo, P.K., 2007. Edwardsiellosis in fish: a brief review. J. Biosci. 32, 1331–1344.

Panangala, V.S., Shoemaker, C.A., McNulty, S.T., Arias, C.R., Klesius, P.H., 2006. Intra- and interspecific phenotypic characteristics of fish-pathogenic *Edwardsiella ictaluri* and *E. tarda*. Aquacult. Res. 37, 49–60.

Rainey, F.A., Ward-Rainey, N.L., Janssen, P.H., Hippe, H., Stackebrandt, E., 1996. *Clostridium paradoxum* DSM 7308^T contains multiple 16S rRNA genes with heterogenous intervening sequences. Microbiology 142, 2087–2095.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sakai, T., Iida, T., Osatomi, K., Kanai, K., 2007. Detection of Type 1 fimbrial genes in fish pathogenic and non-pathogenic *Edwardsiella tarda* strains by PCR. Fish Pathol. 42, 115–117.

Sakai, T., Yuasa, K., Sano, M., Iida, T., 2009. Identification of *Edwardsiella ictaluri* and *E. tarda* by species-specific polymerase chain reaction targeted to the upstream region of the fimbrial gene. J. Aquat. Anim. Health. 21, 124–132.

Sasser, M., 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101. MIDI Inc., Newark, Delaware.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

van Soest, J., Stockhammer, O.W., Ordas, A., Bloemberg, G.V., Spaink, H.P., Meijer, A.H., 2011. Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen *Edwardsiella tarda*. BMC Immunobiol. 12, 58, http://dx.doi.org/10.1186/1471-2172-12-58.

Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19, 6823–6831.

Versalovic, J., Schneider, M., de Bruijn, F.J., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Method Mol. Cell. Biol. 5, 25–40.

Wang, Q., Yang, M., Xiao, J., Wu, H., Wang, X., Lv, Y., Xu, L., Zheng, H., Wang, S., Zhao, G., Liu, Q., Zhang, Y., 2009. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. PLoS ONE 4, e7646, http://dx.doi.org/10.1371/journal.pone.0007646.

Wang, Y.M., Wang, Q.Y., Xiao, J.F., Liu, Q., Wu, H.Z., Zhang, Y.X., 2011. Genetic relationships of *Edwardsiella* strains isolated in China aquaculture revealed by rep-PCR genomic fingerprinting and investigation of *Edwardsiella* virulence genes. J. Appl. Microbiol. 111, 1337–1348.

Williams, M.L., Gillaspy, A.F., Dyer, D.W., Thune, R.L., Waldbieser, G.C., Schuster, S.C., Gipson, J., Zaitshik, J., Landry, C., Banes, M.M., Lawrence, M.L., 2012. Genome sequence of *Edwardsiella ictaluri* 93-146, a strain associated with a natural channel catfish outbreak of enteric septicemia of catfish. J. Bacteriol. 194, 740–741.

Yang, M., Lv, Y., Xiao, J., Wu, H., Zheng, H., Liu, Q., Zhang, Y., Wang, Q., 2012. Edwardsiella comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms. PLoS ONE 7 (5) e36897.