



Antibiotic Resistance and Virulence-Associated Gene Profile of *Edwardsiella tarda* Isolated from Cultured Fish in Japan

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Abstract

Edwardsiella tarda is one of the most significant bacterial pathogens to fish aquaculture worldwide. Here, we evaluated antibiotic resistance profiles, virulence genotypes and phylogenetic relationship of 30 *Edwardsiella tarda* isolates derived from cultured fish in Japan. The *E. tarda* isolates were phylogenetically (16S rRNA) clustered into two clads. The isolates of clad 1, which were mainly originated from olive flounder *Paralichthys olivaceus* and Japanese eel *Anguilla japonica*, had all of the known 11 virulence genes (*ank*, *katB*, *LuxS*, *citC*, *astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) for *E. tarda* and mostly one or two of three aminoglycoside resistance genes, i.e. *aac(6')-Ib*, *armA* and *strA-strB*, if exists. In contrast, the isolates of clad 2, which were mainly originated from red seabream *Pagrus major* and black seabream *Acanthopagrus schlegelii*, lacked four (*ank*, *katB*, *LuxS*, *citC*) of the 11 virulence genes and, eight of them harbored mainly a β -lactam resistance gene (*blaCTX-M*). In infection experiments with zebrafish, representative isolates of the clad 1 showed higher virulence than those of the clad 2. These results reveal that the virulence profiles may be applicable for prediction of pathogenicity in fish. Phylogenetic analysis revealed that *E. tarda* isolates derived from olive flounder and red seabream are ancestor differentiated.

Keywords: *Edwardsiella tarda*, edwardsiellosis, antibiotic resistance genes, virulence gene, pathogenicity, phylogenetics, *Paralichthys olivaceus*, *Pagrus major*

Introduction

In recent years, disease outbreaks caused by bacterial pathogens have affected fish farming all over the world. Edwardsiellosis caused by *Edwardsiella tarda* has been reported worldwide in economically important fish species, including Japanese eel *Anguilla japonica*, red sea bream *Pagrus major*, olive flounder *Paralichthys olivaceus*, channel catfish *Ictalurus punctatus*, Asian catfish *Clarias batrachus* and turbot *Scophthalmus maximus* (Alcaide, Herraiz, & Esteve, 2006; Castro et al., 2016; Kim et al., 2014; Xu & Zhang, 2014).

At present, 17 antibiotics (antibacterial drugs) are approved for aquaculture use and from them 5 drugs are used to treat edwardsiellosis in Japan (Ministry of Agriculture, Forestry and Fisheries http://www.maff.go.jp/j/syouan/suisan/suisan_yobo/index.html). However, the massive use of antibiotics in

commercial fish culture increases the selective pressure on microbial flora and promotes the natural increase in antibiotic resistance. Not only resistant bacteria can multiply after an antibiotic has suppressed sensitive bacteria, but also they can transfer the resistance genes bearing plasmids and transposons to other bacteria that have never been exposed to the antibiotics (Aoki, Arai, & Egusa, 1977; Aoki & Kitao, 1981; Aoki, Kitao, & Fukudome, 1989; Stock & Wiedemann, 2001; Wei et al., 2011). Furthermore, previous studies have characterized that antibiotic resistance is more common in pathogenic compared to commensal organisms and is often linked to virulence factors of the pathogen (Beceiro, Tomás, & Bou, 2013; Yu, Cho, Kim, & Kang, 2012).

Pathogenicity of *E. tarda* depends on the number of virulence factors including the ability to resist serum killing (Iida and Wakabayashi, 1983), to invade epithelial cells (Janda et al., 1991; Ling, Wang, Xie, Lim, & Leung, 2000), to resist phagocyte-mediated killing (Srinivasa Rao, Lim, & Leung, 2003), and to produce dermatotoxins, adhesins and hemolysins for disseminating infection (Hirono, Tange, & Aoki, 1997). Virulence of *E. tarda* is believed to be a multifactorial process, but it has not yet been completely understood. The pathogenic strains of *E. tarda* have virulence genes which might be absent in non-pathogenic strains (Castro et al., 2016; Srinivasa Rao et al., 2003). Now, many virulence-related genes in *E. tarda* have been cloned and reported, which include catalase (*katB*), type III secretion system (TTSS) (*ast*), TTSS regulator (*esrB*), type VI secretion system (T6SS) extracellular apparatus (*evp*), putative killing factor (*mukF*), fimbrial operon (*fimA*), glutamate decarboxylase (*gadB*), citrate lyase (*citC*), hemolysins (*hlyA*), ankyrin like proteins (*ank*), and quorum sensing system (*luxS*) (Kim et al., 2014; Okuda, Takeuchi, & Nakai, 2014; Srinivasa Rao et al., 2003). Some studies have been conducted to evaluate the pathogenicity of *E. tarda* using the zebrafish *Danio rerio* as the animal model (Kim et al., 2014; Okuda et al., 2014).

However, the majority of the studies on *E. tarda* have examined either the antibiotic resistance (Lo, Lee, Wang, & Kuo, 2014; Wei et al., 2011) or the pathogenicity (Castro et al., 2016; Okuda et al., 2014; Srinivasa Rao et al., 2003) and thus very little information is currently available concerning both antibiotic resistance and pathogenicity of *E. tarda* strains (Kim et al., 2014; Yu et al., 2012). In this study, we investigated the antibiotic susceptibility, antibiotic resistance and virulence-associated gene profiles, phylogenetic diversity, and pathogenicity to zebrafish on *E. tarda* isolates derived from different cultured fish in Japan.

Materials and Methods

Bacterial Isolates

A total of 30 *E. tarda* isolates, which were derived from four different fish species (15 red seabream *Pagrus major*, 10 olive flounder *Paralichthys olivaceus*, 4 Japanese eel *Anguilla japonica*, 1 black seabream *Acanthopagrus schlegelii*) at commercial farms in Japan between 1980 and 2014, were used for this study (Table 1). *E. tarda* strains were isolated from kidney or liver of diseased (dead or moribund) fish, when mass mortality occurred. Bacteria were routinely grown at 25°C on tryptic soy agar (MB Cell, USA) and in tryptic soy broth (TSB) (MB Cell). Stock cultures were maintained at -80°C as suspensions in supplemented 10% skim milk (MB Cell) containing 25% (vol/vol) glycerol.

(Insert Table 1 here)

16S rRNA Gene Sequencing and Phylogenetic Analysis

All *E. tarda* isolates were grown overnight at 30°C in TSB and genomic DNA was extracted using the Exgene Cell SV kit (GeneAll, Korea) following the manufacturer's protocol. The 16S rRNA gene sequencing was performed using universal primers 12F and 1492R. The amplicons were purified using Exgene PCR SV (GeneAll) purification kit and sequenced at Cosmogenetech Co, Ltd. (Seoul, Korea). Taxonomic identification of the DNA sequences was performed using BLAST option in GenBank database (<http://blast.ncbi.nlm.nih.gov/>).

Based on the 16S rRNA gene sequences, a neighbor-joining phylogenetic tree was obtained with 1000 bootstrap replications. For the analysis, four and two published 16S rRNA sequences data of *E. tarda* and *E. ictaluri*, respectively, and a sequence data of *Aeromonas hydrophila* (Figure 1) were obtained from the GenBank database and MEGA6 sequence analyzing software was used for aligning and construction of the phylogenetic tree.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility of the isolates was examined by the disc-diffusion method with 16 antimicrobial agents: ampicillin (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), tetracycline (15 µg), colistin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), trimethoprim-sulfamethoxazole (25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), streptomycin (10 µg), imipenem (10 µg), gentamicin (10 µg), kanamycin (30 µg), aztreonam (30 µg) and nitrofurantoin (300 µg). Testing was confirmed by duplicating and the resistance profiles (resistant, intermediate, or susceptible) were assigned using criteria described by Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2015). *Escherichia coli* ATCC 25922 was used as the reference strain.

Detection of Antibiotic Resistance Genes

All the isolates were tested by PCR assays to detect the genetic determinants associated with resistance to aminoglycosides [*strA-strB*, *aac(3)-IIa*, *armA*, *aphA1-IAB* and *aac(6')-Ib*], β-lactams (*bla_{TEM}*, *bla_{SHV}*, *bla_{OXA}* and *bla_{CTX-M}*), and quinolones (*qnrA*, *qnrB* and *qnrS*) (Chung, Yi, Kim, Kim, & Shin, 2017; Hu et al., 2013). PCR amplifications were conducted in 20 µL volumes consisting of 10 µL of Quick Taq® HS DyeMix (TOYOBO, Japan), 1 µL of 10 pmol/µL each primer, 1 µL of the template and 7 µL of the PCR grade water under standard conditions. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels. Positive controls were implemented with previously characterized enterobacterial strains that harbored the corresponding genes (Chung et al., 2017). The presence of *cr* variant in *aac(6')-Ib* gene was investigated by sequencing and BLAST comparison with GenBank (<http://blast.ncbi.nlm.nih.gov/>).

Detection of Virulence-Associated Genes

The known 11 virulence-associated genes (*ank*, *katB*, *luxS*, *citC*, *astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) of *E. tarda* were detected by PCR amplification using previously reported primers (Kim et al., 2014; Li, Ni, Liu, & Lu, 2011) and under the above-described conditions.

Experimental Infection

Wild type zebrafish (weighed 0.28 ± 0.1 g) were obtained from a commercial aquarium in Seoul. During the acclimatization period, fish were fed with commercial diet for 3 times a day (4% of body weight/day), and maintained in automated water circulation system at $28 \pm 1^\circ\text{C}$ with 12/12 h light/dark cycle. For injection challenge, representative four *E. tarda* isolates were selected randomly from distinct virulence genotypes; two isolates (FH-9104 and E01-21) harboring eleven virulence genes and two isolates (MEE0309 and EY02) harboring seven virulence genes. The inoculum was prepared from the culture in TSB at 28°C for 24 h. Experimental fish ($n=15$) were given an intraperitoneal injection with $10 \mu\text{L}$ of each bacterial suspension containing 1×10^2 to 1×10^6 CFU/fish. Fish ($n=15$) in control group were similarly injected with sterile phosphate-buffered saline (PBS, pH 7.4) instead of bacteria. Mortalities were recorded twice daily for one week. Disease signs of moribund and dead fish were observed, and dead fish were removed from the experimental tanks for bacteriological examination. The mean LD_{50} was determined using the simplified method of Reed and Muench (1938).

Results

Phylogenetic Comparison of 16S rRNA Sequences

Phylogenetic tree derived from the 16S rRNA gene sequences of the present 30 *E. tarda* isolates and the reference strains is illustrated in Figure 1. Neighbor-joining phylogenetic tree indicated two distinct clads. The present *E. tarda* isolates from olive flounder, Japanese eel, one isolate (E01-14) from red seabream and three *E. tarda* reference strains (LTB-4 and HC010907 from turbot *Scophthalmus maximus*, and K20 from Japanese eel *Anguilla japonica*) comprised the clad 1, and the remaining *E. tarda* isolates from red seabream except E01-14 and one isolate (No.1) from black seabream comprised the clad 2. It should be noted that *E. tarda* isolates belonging to clad 2 were closely related to *E. ictaluri* strains (HSS-1 and HSX-2) which were originated from yellow catfish *Pelteobagrus fulvidraco* (Liu, Li, Zhou, Wen, & Ye, 2010).

(Insert Figure 1)

Antibiotic Susceptibility

Antibiotic resistance profiles of the *E. tarda* isolates to 16 antibiotics are summarized in Table 2. All or most of isolates were susceptible to nitrofurantoin (F), ceftioxin (FOX), tetracycline (T), chloramphenicol (C), nalidixic acid (NAL), ciprofloxacin (CIP) and imipenem (IPM). In contrast, all isolates were resistant to colistin (CL) and erythromycin (E), and six or more isolates were resistant to ampicillin (AMP), cefotaxime (CTX), trimethoprim-sulfamethoxazole (SXT) and aztreonam (ATM), while a few isolates showed resistant/intermediate profiles to streptomycin (S), gentamycin (G) and kanamycin (K).

(Insert Table 2 here)

Distribution of Antibiotic Resistance Genes

The aminoglycoside resistance genes, *aac(6')-Ib*, *armA* and *strA-strB*, were detected in 11, 3 and 1, respectively, from 30 *E. tarda* isolates. The isolates harboring these aminoglycoside resistance genes were mostly olive flounder and Japanese eel, except three isolates (E01-14, MEE0203, E01-40) from red seabream which harbors *aac(6')-Ib* gene. Among the tested β -lactam resistance genes, only the *bla_{CTX-M}* gene was detected in 8 isolates from red seabream. The quinolone resistance gene (*qnrS*) was detected in one isolate (EY01) from red seabream (Table 2).

Distribution of Virulence Genes

Two distinct virulence genotypes were identified in the studied *E. tarda* isolates. Fourteen *E. tarda* isolates, including all the isolates from olive flounder, three isolates (SU44, SU53, SU57) from Japanese eel and one isolate (E01-14) from red seabream, were positive for all 11 tested virulence genes. The rest 16 *E. tarda* isolates, including 14 isolates from red seabream, one isolate (SU28) from Japanese eel and one isolate (No.1) from black seabream, were positive for seven virulence genes (*astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) (Table 2).

LD₅₀ Values

Following the challenge infection with four representative *E. tarda* isolates, disease manifestations appeared in fish between day 1 and 5 post-challenge, showing reduced activity, anorexia, convulsions, and death. Mortalities of fish were different between two different virulence genotypes. Two isolates (FH-9104, E01-21) of the virulence genotype harboring all the tested virulence genes showed LD₅₀ values as 4.8×10^3 and 2.6×10^3 CFU/fish, respectively. Two isolates (MEE0309, EY02) of the virulence genotype harboring seven virulence genes showed LD₅₀ values as 7.6×10^4 and 5.4×10^4 CFU/fish, respectively.

Discussion

In the present study, all of the *E. tarda* isolates derived from cultured fish in Japan were resistant to the colistin and erythromycin. *E. tarda* is considered as intrinsically resistant to colistin and macrolides (Stock & Wiedemann, 2001). However, several studies reported some colistin-sensitive *E. tarda* strains (Michael & Abbott, 1993; Stock & Wiedemann, 2001). The resistant isolates were also found at high frequency for the β -lactams (ampicillin, cefotaxime) and at low frequency for the aminoglycosides (streptomycin, gentamycin, and kanamycin). Previous studies have reported ampicillin-, streptomycin- or kanamycin-resistant *E. tarda* which were originated from cultured fish in Japan (Aoki & Kitao, 1981; Aoki et al., 1989). Wei et al., (2011) reported that 28% and 11% of *E. tarda* isolates from freshwater fish in Malaysia were resistant to ampicillin and kanamycin, respectively. Although only a few isolates were resistant to tetracycline or chloramphenicol in our study, the appearance of *E. tarda* resistant to these antibiotics has been frequently reported in Japanese aquaculture (Aoki et al., 1977; Aoki & Kitao, 1981; Furushita et al., 2003). However, antibiotic resistance and resistant determinants in *E. tarda* strains have not been given enough consideration in Japan. The majority of the studies on this bacterium have examined the pathogenicity while giving a seldom value to the antibiotic resistance (Miyazaki, & Egusa, 1976; Matsuyama, Kamaishi, Ooseko, Kurohara, & Lida, 2005; Okuda et al., 2014). Further studies are needed to explore the prevalence and trend of antimicrobial resistance in *E. tarda* from Japanese aquaculture.

The β -lactams, aminoglycoside and quinolone resistance genes targeted in our study were selected based on the abundance of resistant *E. tarda* strains to the antibiotics. Only one resistance gene (*bla_{CTX-M}*) to the β -lactams was detected in eight isolates from red seabream. All of these isolates were resistant to ampicillin and one isolate (EhiRS10-1) was resistant to cefotaxime (a β -lactam), but other five cefotaxime-resistant isolates (NUF82, NUF251, E01-33, FH-9104, SU53) did not carry the *bla_{CTX-M}* gene. Previous studies have also reported the mismatch phenomenon between antimicrobial resistance phenotypes and genotypes of bacteria (Lou, Liu, Zhang, Pan, & Zhao, 2016; Wimalasena, De Silva, Hossain, Pathirana, & Heo, 2017; Zhang, Zhang, & Fang, 2009). It is interesting that aminoglycoside resistance gene *aac(6')-Ib* was most frequently detected in the present *E. tarda*

isolates and most of the isolates exhibited high multidrug resistance. Since aminoglycoside resistance genes are often located on large plasmids that carry genes encoding resistance to other antimicrobial agents (Huang et al., 2012; Yu et al., 2012), plasmids may play a role to antibiotic resistance of fish-pathogenic bacteria in Japanese aquaculture. According to the available few reports on antibiotic resistance determinants in *E. tarda* from aquaculture, this bacterium consistently harbored tetracycline and chloramphenicol resistance genes but not β -lactams and aminoglycoside resistance genes (Yoo, Huh, Eun, Lee, & Jeong, 2003; Jin et al., 2004).

Recently, studies on pathogenicity mechanism of *E. tarda* have made great progress, and many virulence factors and relevant genes have been identified (Okuda et al., 2014; Srinivasa Rao et al., 2003). Seven of 11 known virulence genes were detected in all the present *E. tarda* isolates. However, the other four virulence genes (*ank*, *katB*, *luxS* and *citC*) were harbored only in 14 *E. tarda* isolates mainly from olive flounder and Japanese eel. The previous study showed that *ank*, *katB* and *citC* genes were present only in virulent strains of *E. tarda* (Nakamura et al., 2013; Srinivasa Rao et al., 2003). Kim et al., (2014) reported less distribution of *ank* and *luxS* genes in *E. tarda* isolated from Japanese eel in Korea, compared with other virulence genes including *fimA*, *evpP*, *esa* and *astA*. Our result, i.e. lack of *ank*, *katB*, *luxS* and *citC* in the majority of the present *E. tarda* isolates from red seabream, suggests the involvement of virulence factors other than these four genes in the pathogenesis to red seabream.

In this study, the adult zebrafish was used as a model organism to evaluate the virulence of *E. tarda* isolated from cultured fish. Previous studies also have used zebrafish as a model organism for pathogenicity test because this species is easy to cultivate and maintain and its whole genome has been sequenced (Okuda et al., 2014; Wang, Yan, Wang, Ding, & Li, 2012). According to the pathogenicity test results, the LD₅₀ values of isolates harboring 11 virulence genes were approximately ten times lower than those of isolates harboring seven virulence genes. This correlation between virulence gene profiles and virulence to zebrafish in *E. tarda* isolates suggests that the virulence gene profiles might be one factor which can be used for the prediction of pathogenicity of *E. tarda*. Similar findings in which the number of virulence genes of *E. tarda* isolates were correlated with the pathogenicity to blue gourami *Trichogaster trichopterus* (Srinivasa Rao et al., 2003) and zebrafish (Wang et al., 2012) were reported. In infection experiments using olive flounder and red seabream, Matsuyama et al. (2005) reported a different host-specificity between *E. tarda* (so-called typical strain) from olive flounder and that (atypical strain) from red seabream. As mentioned above, it seems that the host-specificity of *E. tarda* is associated with difference of virulence genes.

Phylogenetic analysis of the 16S rRNA gene sequences showed that the present *E. tarda* isolates were separated into two clads (Figure 1). All the isolates originating from olive flounder and Japanese eel were clustered into clad 1, while all the isolates from red seabream with one exception were clustered into clad 2, indicating source associated phylogenetic clustering in *E. tarda* isolates from Japanese cultured fish. This suggests that *E. tarda* isolates derived from olive flounder and red seabream are differentiated from a common ancestor in response to niche adaption (Yang et al., 2012). Furthermore, it is interesting that the clad 2 isolates were clustered with *E. ictaluri* (HSX-2 reference strain originated from yellow catfish in China). A comparative phylogenomic study revealed that *E. tarda* could be classified into two genotypes (EdwGI, EdwGII) and EdwGI is more closely related to *E. ictaluri* than to *E. tarda* (Yang et al., 2012). Further pathological and genetic studies are required to explain the host-specificity of fish-pathogenic *E. tarda* and *E. ictaluri*.

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Table 1. *Edwardsiella tarda* isolates used in this study

Bacterial strain	Host fish	Year	Location (Prefecture)	Reference
NE8003	Olive flounder	1980	Nagasaki	Okuda et al. (2007)
NUF82	Olive flounder	1984	Nagasaki	Okuda et al. (2007)
NUF251	Olive flounder	1986	Nagasaki	Okuda et al. (2007)
PE113	Olive flounder	2001	Ehime	Okuda et al. (2007)
PE210	Olive flounder	2001	Ehime	-
E01-21	Olive flounder	2001	Ehime	-
E01-33	Olive flounder	2001	Ehime	Okuda et al. (2007)
E01-37	Olive flounder	2001	Ehime	Okuda et al. (2007)
FH-9104	Olive flounder	1991	Hiroshima	-
FK1051	Olive flounder	2001	Hiroshima	-



REF001	Red seabream	1996	Mie	Okuda et al. (2007)
REF146	Red seabream	1996	Mie	Okuda et al. (2007)
REF179	Red seabream	2001	Mie	Okuda et al. (2007)
MEE0203	Red seabream	2002	Mie	-
MEE0301	Red seabream	2003	Mie	-
MEE0309	Red seabream	2003	Mie	-
E01-14	Red seabream	2001	Ehime	Okuda, Kiriya, Yamanoi, and Nakai (2008)
E01-17	Red seabream	2001	Ehime	Okuda et al., (2009)
E01-40	Red seabream	2001	Ehime	-
EhiRS10-1	Red seabream	2011	Ehime	-
EhiRS12-1	Red seabream	2011	Ehime	-
EhiRS12-2	Red seabream	2011	Ehime	-
EY01	Red seabream	2014	Ehime	-
EY02	Red seabream	2014	Ehime	-
Yama-S	Red seabream	-	Ehime	-
SU28	Japanese eel	1980	Shizuoka	-
SU44	Japanese eel	1980	Shizuoka	-
SU53	Japanese eel	1980	Shizuoka	-
SU57	Japanese eel	1980	Shizuoka	-
No. 1	Black porgy	2003	Hiroshima	-



^a Antibiotic resistance was designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI, 2015). Antibiotics; FOX= ceftiofur (30 µg), T= tetracycline (15 µg), C= chloramphenicol (30 µg), NAL= nalidixic acid (30 µg), CL= colistin (10 µg), E= erythromycin (15 µg), AMP= ampicillin (10 µg), CTX= cefotaxime (30 µg), SXT= trimethoprim-sulfamethoxazole (25 µg), ATM= aztreonam (30 µg), S= streptomycin (10 µg), G= gentamicin (10 µg) and K= kanamycin (30 µg).

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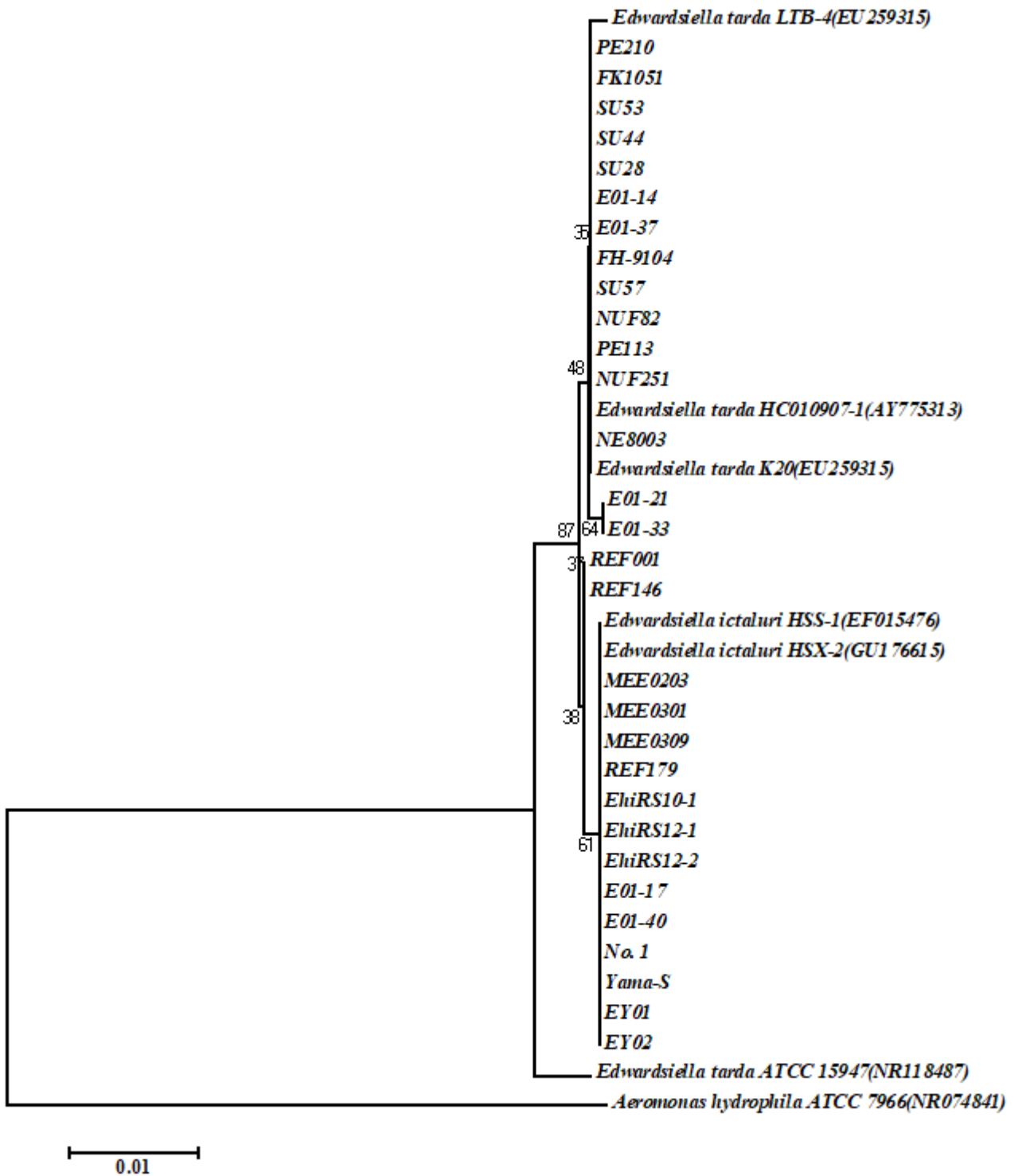


Figure 1. Phylogenetic analysis of the 16S rRNA genes from *Edwardsiella tarda* isolates. The tree was generated based on nucleotide sequences using the maximum-likelihood model in MEGA 6 software. The numbers below the branches indicate the 1000 bootstrap values.