Vol. 95: 253–258, 2011
doi: 10.3354/dao02363

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 12

NOTE

Intraspecific genetic variability of *Edwardsiella tarda* strains from cultured turbot

N. Castro*, A. E. Toranzo, A. Bastardo, J. L. Barja, B. Magariños

Departamento de Microbiología y Parasitología, Facultad de Biología–CIBUS–Instituto de Acuicultura, Universidad de Santiago de Compostela, Santiago de Compostela, 15782 Spain

ABSTRACT: *Edwardsiella tarda* is an enterobacterial fish pathogen that causes mortality in various fish species worldwide. In this study, we analyzed the intraspecific variability in a collection of *E. tarda* strains isolated from turbot. To do this we employed 4 polymerase chain reaction (PCR)-based methods: (1) random amplified polymorphic DNA (RAPD), (2) enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), (3) repetitive extragenic palindromic-PCR (REP-PCR) and (4) BOX-PCR. *E. tarda* isolates from different hosts were also included for comparison. *E. tarda* strains from turbot showed high molecular homogeneity when RAPD (primers P3 and P6), ERIC-PCR and BOX-PCR were employed. However, with regard to the REP-PCR and RAPD (primers P4 and P5) techniques, different genetic groups could be established within these isolates using either technique. The 2 RAPD types presented an 85% similarity, while those obtained with REP-PCR showed 74% similarity. Based on the results obtained, although a high genetic homogeneity was found in turbot isolates, the RAPD test (with primers P4 and P5) and REP-PCR were capable of discrimination within these strains, and they are therefore considered the most appropriate typing methods for studies of edwardsiellosis in turbot.

KEY WORDS: *Edwardsiella tarda* · Turbot · Genetic variability · Molecular typing

INTRODUCTION

Edwardsiella tarda is a member of the family *Enterobacteriaceae* and the causative agent of edwardsiellosis, a bacterial disease that represents a serious problem for aquaculture, affecting mainly catfish *Ictalurus punctatus*, eel *Anguila japonica* and flounder *Paralichthys olivaceus*. In recent years, it has also caused high economic losses in turbot *Psetta maxima* cultures in Europe (Castro et al. 2006) and is considered one of the most important emergent pathogens. Although this bacterium has been described as biochemically homogeneous (Austin & Austin 1999, Castro et al. 2006), antigenic studies have revealed that all *E. tarda* strains isolated from turbot constitute a homogeneous group different from *E. tarda* isolates from other fish species (Castro et al. 2006). Several studies (Yamada & Wakabayashi, 1998, Nucci et al. 2002, Acharya et al. 2007, Maiti et al. 2008, 2009) have used molecular-based methods for intraspecific characterization of *E. tarda*, although none of them included isolates from turbot.

The determination of randomly amplified polymorphic DNA (RAPD) patterns has been successfully employed for discrimination within bacterial strains in diverse species (Romalde et al. 1999, Magariños et al. 2000, Romalde 2005). In the case of *Edwardsiella tarda*, Nucci et al. (2002) applied this technique to a collection of isolates from fish and humans and found that RAPD could distinguish, with a few exceptions, isolates of human origin from those of fish origin. Moreover, preliminary studies of Castro et al. (2006), employing the RAPD technique on a group of 21 *E. tarda* isolates from turbot, showed that 2 genetic

*Email: nuria.castro@usc.es

groups within the turbot isolates coexisted in the same rearing facility.

Repetitive extragenic palindromic-PCR (REP-PCR) fingerprinting has been shown to be valuable for classifying and typing a variety of Gram-negative and several Gram-positive genera. Three categories of conserved repetitive sequences are used for bacterial typing: (1) the enterobacterial repetitive intergenic consensus sequence (ERIC), (2) the REP and (3) the BOX element. Often, the 3 categories are used in combination in order to achieve better discrimination. ERIC- and REP-PCR have been successfully used to differentiate bacterial strains among diverse species (Bennasar et al. 2002, Bruant et al. 2003, Hahm et al. 2003). Until now, only Maiti et al. (2009) employed ERIC-PCR to differentiate among *Edwardsiella tarda* isolates from pond sediments. BOX-PCR has been applied in numerous taxonomic studies on plant-associated, environmental, medical and food-associated bacteria (Tacão et al. 2005, Marques et al. 2008). In *E. tarda*, Maiti et al. (2008) compared this technique with PCR-ribotyping, employing isolates from freshwater ponds and apparently healthy fish, and finding that the BOX-PCR was the most discriminative.

In this work, RAPD, ERIC-PCR, REP-PCR and BOX-PCR methods have been employed simultaneously to analyze the intraspecific genetic variability of *Edwardsiella tarda* strains causing mortality in cultured turbot, with the aim of evaluating these techniques in studying the epizoology of this bacterial pathogen.

MATERIALS AND METHODS

Bacterial strains. A collection of 38 *Edwardsiella tarda* strains were analyzed. They were isolated from turbot in different outbreaks and years and from 5 diverse rearing facilities in 2 areas of Europe (one in northern Europe and 4 in southern Europe; Table 1). Twelve *E. tarda* strains isolated from other fish hosts were included in this study for comparison.

All strains were characterized by employing classical biochemical tests as well as by using a commercial miniaturized system (API 20E, Biomerieux) and slide agglutination with specific antiserum (Castro et al. 2006). Confirmatory identification of the isolates as *Edwardsiella tarda* was performed by a PCR protocol using the specific primers EtfD (Castro et al. 2010), which amplify a 445 bp sequence of the fimbrial gene. All strains were routinely grown on trypticase soy agar (Pronadisa), supplemented with 1% NaCl (TSA-1), at 25°C for 24 h. Cultures were maintained frozen at –80°C in Criobille tubes (AES Laboratory).

DNA extraction. Chromosomal DNA was extracted using the InstaGene matrix (BioRad), following the manufacturer's recommendations. Extracted DNA was maintained at –30°C until used for PCR reactions. Three DNA extractions were performed from 3 different culture plates for each bacterial strain.

PCR typing analysis. The RAPD reactions were performed using Ready-To-Go RAPD Analysis Beads (GE Healthcare) containing buffer, nucleotides and *Taq* DNA polymerase. Each RAPD bead was resuspended in 19 µl of sterile water and 1 µl of purified bacterial DNA, and 25 pmol of the respective primers (GE Healthcare) were added. The amplification protocol for RAPD analysis started with denaturation (95°C for 1 min) followed by 30 cycles at 95°C for 1 min (denaturation), then at 52°C for 1 min (annealing) and at 72°C for 2 min, with a final step at 72°C for 5 min. ERIC, REP and BOX amplifications were carried out with Ready-To-Go PCR beads (GE Healthcare). These commercial beads, optimized for PCR reactions, contain buffer, nucleotides and *Taq* DNA polymerase. The only reagents added to the reaction, therefore, were template DNA (1 µl), primers (100 pmol each) and sterile distilled water to complete a final volume of 25 µl. The primers employed for REP-PCR, ERIC-PCR and BOX-PCR have been described by Versalovic et al. (1994) and Chowdhury et al. (2004), respectively.

The amplification protocol for ERIC-PCR consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation (92°C for 45 s), annealing (52°C for 1 min) and extension (70°C for 10 min), with a final extension step at 70°C for 20 min. In the case of REP-PCR, an initial denaturation was carried out at 95°C for 7 min. This was followed by 35 cycles of denaturation at 92°C for 45 s, annealing at 40°C for 1 min and elongation at 72°C for 15 min. The final extension step was performed at 72°C for 15 min. For BOX-PCR the program consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min.

All amplifications were performed simultaneously in a T Gradient Thermocycler (Biometra) and a Mastercycler Personal (Eppendorf) instrument. As a negative control, sterile distilled water was included with each set of reactions in place of bacterial DNA.

In all cases, amplification products were analyzed by horizontal 1:5% (w:vol) agarose gels with TAE (0.04 M Tris-acetate, 1 mM EDTA, pH = 8.0) electrophoresis buffer. After staining with ethidium bromide, gels were photographed under UV light. An AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma) and a GeneRuler™ 1 kb DNA Ladder, ready-to-use (250 to 10 000 bp ladder, Fermentas), were employed as molecular-weight markers.

Table 1. *Edwardsiella tarda* in *Psetta maxima*. Summary of differentiation among *E. tarda* isolates from turbot and reference strains using molecular typing methods. nd: no data

Strain	Year	Origin[a]	Molecular groups						
			RAPD P3	RAPD P4	RAPD P5	RAPD P6	ERIC	REP	BOX
Turbot strains									
ACC35.1	2005	SA1	I	I	I	I	I	II	I
ACC36.1	2005	SA1	I	I	I	I	I	I	I
ACC51.1	2006	SA2	I	I	I	I	I	I	I
ACC52.1	2006	SA2	I	I	I	I	I	I	I
ACC53.1	2006	SA2	I	I	I	I	I	I	I
ACC69.1	2006	SA3	I	I	I	I	I	II	I
ACC70.1	2006	SA3	I	I	I	I	I	I	I
ACC121.1	2009	SA4	I	II	II	I	I	I	I
RM288.1	2006	SB1	I	I	I	I	I	I	I
RM289.1	2006	SB1	I	I	I	I	I	II	I
RM290.1	2006	SB1	I	I	I	I	I	I	I
RM293.1	2006	SB1	I	I	I	I	I	I	I
RM294.1	2006	SB1	I	I	I	I	I	I	I
HL1.1	2004	NC1	I	I	I	I	I	I	I
HL8.1	2005	NC2	I	I	I	I	I	I	I
HL9.1	2005	NC2	I	I	I	I	I	I	I
HL10.1	2005	NC2	I	I	I	I	I	I	I
HL12.1	2005	NC3	I	I	I	I	I	II	I
HL14.1	2005	NC3	I	I	I	I	I	II	I
HL21.2	2006	NC4	I	II	II	I	I	II	I
HL21.3	2006	NC4	I	II	II	I	I	I	I
HL23.1	2006	NC5	I	II	II	I	I	I	I
HL24.1	2006	NC5	I	II	II	I	I	I	I
HL25.1	2006	NC5	I	II	II	I	I	I	I
HL30.1	2006	NC5	I	II	II	I	I	I	I
HL31.1	2006	NC5	I	II	II	I	I	I	I
HL37.1	2006	NC6	I	II	II	I	I	I	I
HL38.1	2006	NC6	I	II	II	I	I	I	I
HL42.1	2006	NC7	I	II	II	I	I	I	I
HL43.1	2006	NC7	I	II	II	I	I	I	I
RBR7.1	2008	SD1	I	II	II	I	I	I	I
RBR8.1	2008	SD1	I	II	II	I	I	I	I
RBR13.1	2008	SD2	I	II	II	I	I	I	I
ACR326.1	2009	SE1	I	II	II	I	I	I	I
ACR327.1	2009	SE1	I	II	II	I	I	I	I
ACR328.1	2009	SE1	I	II	II	I	I	I	I
ACR1.1-10	2010	SE2	I	II	II	I	I	I	I
ACR2.1-10	2010	SE2	I	II	II	I	I	I	I
Reference strains from various hosts									
E.11-2[b]	nd	Japan	I	V	III	II	II	VII	II
EDK1[b]	nd	Japan	I	III	III	II	II	VI	II
9.8[c]	1986	USA	I	VII	III	IV	III	VIII	II
FL4-534K[c]	1997	USA	I	VI	III	II	II	V	II
WFE1[d]	2002	Japan	I	VIII	III	VIII	II	VIII	II
WFE10[d]	2002	Japan	I	IX	III	VIII	II	VIII	II
ET001[e]	2002	Japan	III	X	V	VII	IV	IX	III
ET009[e]	2002	Japan	IV	XI	V	VII	IV	IX	III
KGE7901[f]	nd	Japan	I	IV	III	II	III	IV	II
81.48[g]	nd	USA	II	XIV	VI	V	V	III	IV
FL3-22[h]	nd	USA	II	XII	VI	V	V	III	IV
NCIMB2034[i]	1977	NCIMB collection	II	XIII	IV	III	VI	III	V

[a]S: Southern Europe; N: Northern Europe; A to E: farm identifier. Number indicates different outbreaks. Hosts: [b]Japanese eel *Anguilla japonica*; [c]striped bass *Morone saxatilis*; [d]Japanese flounder *Paralichthys olivaceus*; [e]red sea bream *Pagellus bogaraveo*; [f]tilapia *Tilapia nilotica*; [g]catfish *Ictalurus punctatus*; [h]angel fish *Pterophyllum* sp.; [i]unknown fish sp.

Computer-assisted analysis of genomic fingerprints. All gels were scanned and images captured by a Gel Doc-2000 gel documentation system (Bio-Rad). Data analysis was performed using Diversity Database software (Bio-Rad). The computed similarities among isolates were estimated by means of the Dice coefficient (Dice 1945). Dendograms were produced using the unweighted pair group method average (UPGMA). Moreover, a composite tree was generated using a combined data set of all genotypic data and BioNumerics version 4.0 (Applied Maths).

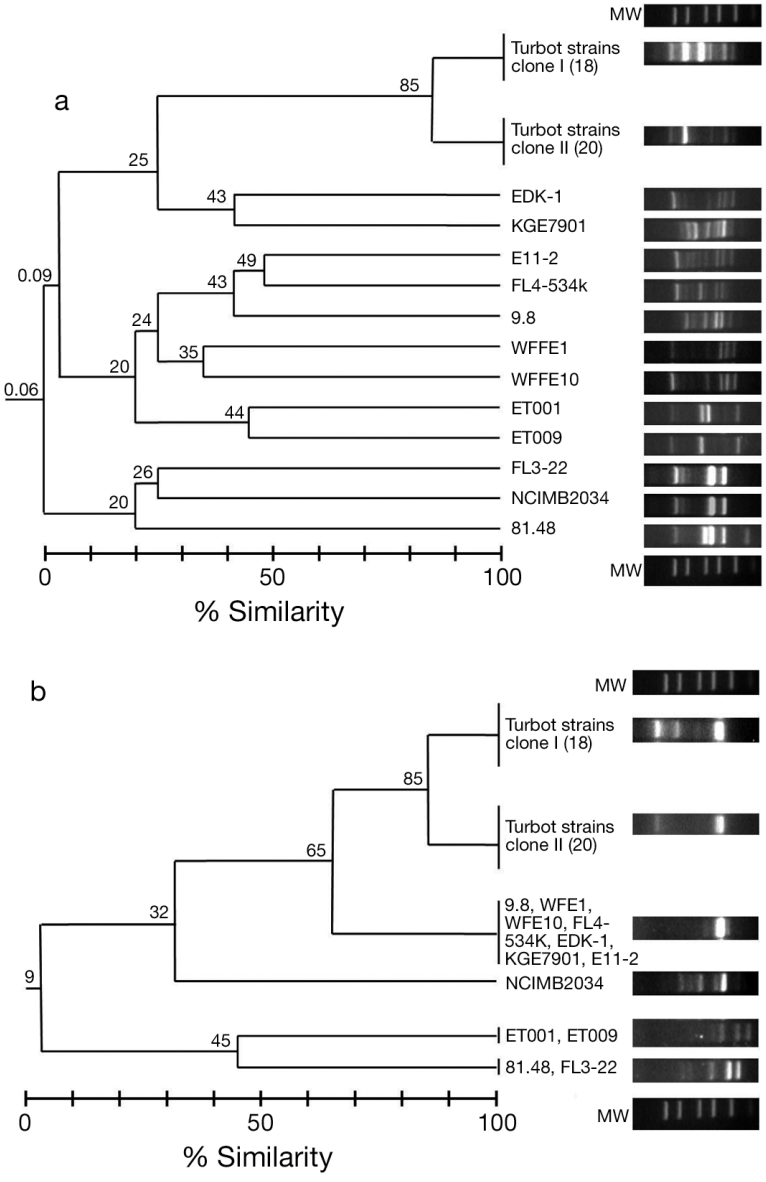

Fig. 1. *Edwardsiella tarda* strains. Randomly amplified polymorphic DNA (RAPD) profiles employing (a) primer P4 and (b) primer P5 and using Dice similarity coefficient and UPGMA analysis. Numbers in parentheses: number of strains; numbers at nodes: % similarity. Representative patterns of strains shown next to each group. Upper and lower profiles (MW): AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma)

RESULTS AND DISCUSSION

Since 2004 *Edwardsiella tarda* has become one of the most important emerging pathogens in cultured turbot in Europe, causing serious economic losses (Castro et al. 2006). Despite several studies of *E. tarda* in turbot, genetic characterization of these isolates employing molecular typing techniques has not been developed in depth. The intraspecific typing of *E. tarda* could be of great importance in the study of variants of this fish pathogen as associated with geographical area, host origin, virulence and disease outbreak.

In this study we proposed the evaluation of 4 PCR-based methods for detecting intraspecific variation within a collection of *Edwardsiella tarda* strains from turbot: RAPD, ERIC-PCR, REP-PCR and BOX-PCR, all frequently employed as molecular tools for rapid typing of microorganisms (Magariños et al. 2000, Khan et al. 2002, Romalde 2005, Rodríguez et al 2006, Mancuso et al. 2007).

Biochemical, serological and PCR analyses confirmed that the 46 isolates studied were *Edwardsiella tarda* strains belonging to the wildtype biogroup (data not shown).

Previous results employing the RAPD technique showed that in *Edwardsiella tarda* isolates from turbot, only the oligonucleotides P3, P4, P5 and P6 generated reproducible patterns with an appropriate number of amplified products suitable for accurate analysis (Castro et al. 2006). Based on these data, these 4 primers were selected to analyze the complete collection of strains.

When oligonucleotides P3 and P6 were employed, all *Edwardsiella tarda* turbot strains were placed together in the same group (Table 1). However, only primer P6, not P3, enabled separation of the turbot strains from those isolated from other fish species. Oligonucleotides P4 and P5 (Fig. 1) were the only ones that could discriminate within the turbot isolates. With these primers 2 different groups were obtained, which differed in a unique band of ~1100 bp (with primer P4) and ~1700 bp (with primer P5). These 2 RAPD types present 85% similarity (Fig. 1). These results are in agreement with those previously reported by Castro et al. (2006). As may be seen in Table 1, although the 2 RAPD groups were detected in some cases in the same rearing facility, they never coexisted in the same disease outbreak, each outbreak being

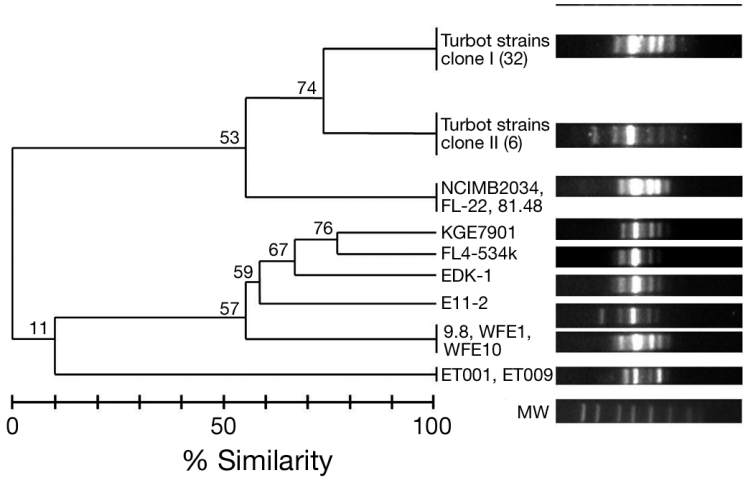

Fig. 2. *Edwardsiella tarda* strains. Repetitive extragenic palindromic-PCR (REP-PCR) profiles using Dice similarity coefficient and UPGMA analysis. Numbers in parentheses: number of strains; numbers at nodes: % similarity. Representative patterns of strains shown next to each group. Upper and lower profiles (MW): AmpliSize Molecular Ruler (50 to 2000 bp ladder, Sigma)

caused by a single group. Interestingly, after 2006, all *E. tarda* strains isolated corresponded to Group II. Additionally, in this work we report for the first time the existence of the RAPD Type II in farms located in southern Europe.

The ERIC-PCR and BOX-PCR techniques employed by Maiti et al. (2008, 2009) in comparative studies of *Edwardsiella tarda* strains were found to be discriminative and potential tools for future epizootic studies. In our study, however, these methods did not allow differentiation within *E. tarda* turbot strains, but did separate turbot isolates from those strains found in other fish hosts (Table 1).

With regard to REP-PCR, we believe this to be the first reported application of this molecular typing method to *Edwardsiella tarda* strains isolated from the environment. Our results demonstrated that this technique could discriminate within turbot strains, showing the existence of 2 different groups with a similarity of 74% between them, and which differed only in a unique band of ~1500 bp (Fig. 2). In contrast to the 2 genetic groups identified by RAPD, the 2 groups identified by REP-PCR coexisted in the same outbreak (Table 1).

Reproducibility of RAPD, ERIC-PCR, REP-PCR and BOX-PCR results was assessed by repetition in at least 3 independent assays. Excluding some variations in band intensity, no differences were observed among profiles obtained for each strain, and all band patterns proved to be highly reproducible.

Data obtained in all molecular typing methods were employed to develop a single similarity matrix that was

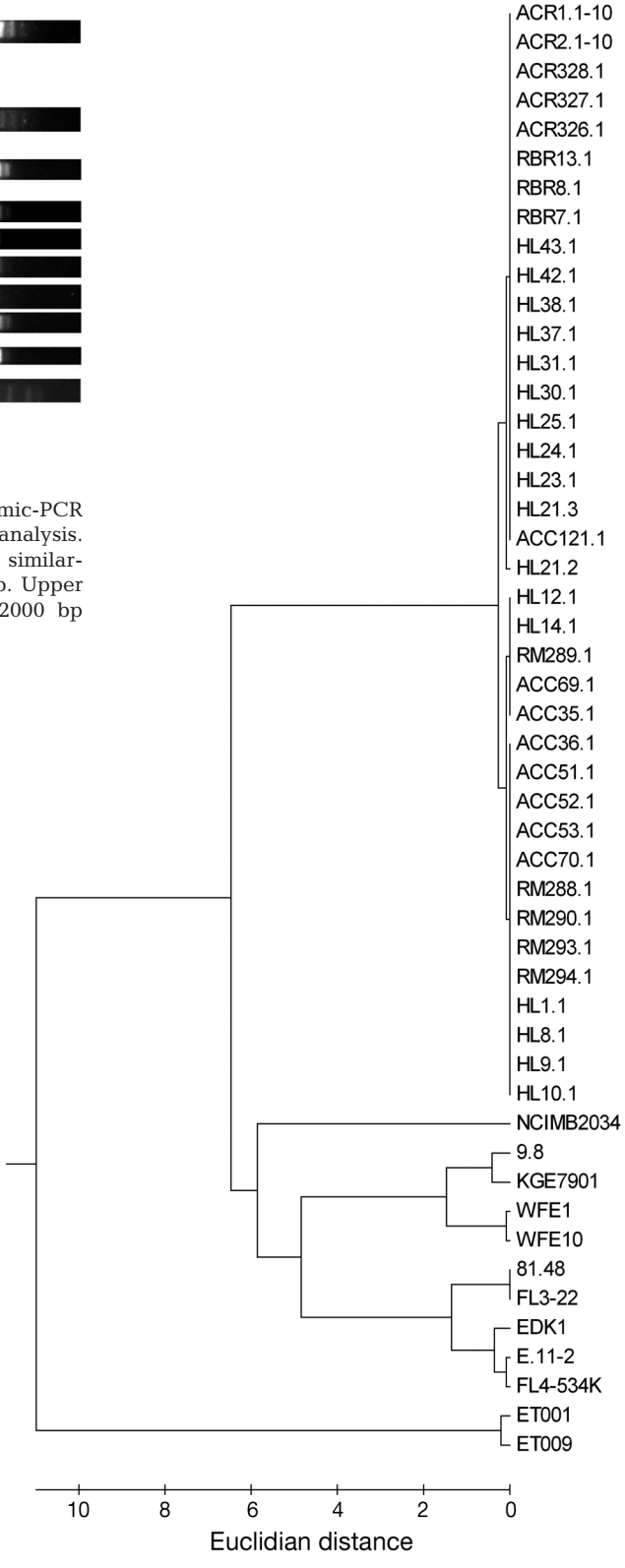

Fig. 3. *Edwardsiella tarda* strains. Composite tree generated using combined data for *E. tarda* isolates from turbot from all molecular typing methods tested, and using BioNumerics

used to generate a composite dendrogram (Fig. 3). The results confirmed that turbot *Edwardsiella tarda* isolates are a highly homogeneous group, regardless of their origin. Interestingly, this homogeneity strongly contrasts with the heterogeneity observed among strains from other sources.

In sum, the evaluation of 4 PCR-based techniques for the study of the intraspecific genetic diversity of *Edwardsiella tarda* from turbot demonstrated that, although a low genetic variability was found within these isolates, the RAPD assays employing primers P4 and P5 and REP-PCR method are the most appropriate as typing methods in *E. tarda* from this marine fish.

Acknowledgements. This work was supported in part by grant no. 08MMA018E from the Xunta de Galicia and grant no. AGL2009-08859 from the Ministerio de Ciencia e Innovación (Spain).

LITERATURE CITED

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

Austin B, Austin DA (1999) Characteristics of the diseases. In: Austin B, Austin DA (eds) Bacterial fish pathogens: diseases of farmed and wild fish. Springer-Verlag, Heidelberg, p 15–46

Bennasar A, Luna G, Cabrer B, Lalucat J (2002) Rapid identification of *Salmonella typhimurium, S. enteritidis* and *S. virchow* isolates by polymerase chain reaction based fingerprinting methods. Int Microbiol 3:31–38

Bruant G, Watt S, Quentin R, Rosenau A (2003) Typing of nonencapsulated *Haemophilus* strains by repetitive-element sequence-based PCR using intergenic dyad sequences. J Clin Microbiol 41:3473–3480

Castro N, Toranzo AE, Barja JL, 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 AE, Núñez S, Osorio CR, Magariños B (2010) Evaluation of four polymerase chain reaction primer pairs for the detection of *Edwardsiella tarda* in turbot. Dis Aquat Org 90:55–61

Chowdhury SP, Khanna S, Verma SC, Tripathi AK (2004) Molecular diversity of tannic acid degrading bacteria isolated from tannery soil. J Appl Microbiol 97:1210–1219

Dice LR (1945) Measures of the amount of ecological association between species. Ecology 26:297–302

Hahm BK, Maldonado Y, Schreiber E, Bhunia AK, Nakatsu CH (2003) Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR, rep-PCR, PFGE, ribotyping and AFLP. J Microbiol 53:387–399

Khan AA, McCarthy S, Wang RF, Cerniglia CE (2002) Characterization of United States outbreak isolates of *Vibrio parahaemolyticus* using enterobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3:K6 isolates. FEMS Microbiol Lett 206:209–214

Magariños B, Toranzo AE, Barja JL, Romalde JL (2000) Existence of two geographically-linked clonal lineages in the bacterial fish pathogen *Photobacterium damselae* subsp. *piscicida* evidenced by random amplified polymorphic DNA analysis. Epidemiol Infect 125:213–219

Maiti NK, 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 NK, Mandal A, Mohanty S, Mandal RN (2009) Phenotypic and genetic characterization of *Edwardsiella tarda* isolated from pond sediments. Comp Immunol Microbiol Infect Dis 32:1–8

Mancuso M, Avendaño-Herrera R, Zaccone R, Toranzo AE, Magariños B (2007) Evaluation of different DNA-based fingerprinting methods for typing *Photobacterium damselae* ssp. *piscicida*. Biol Res 40:85–92

Marques ASA, Marchaison A, Gardan L, Samson R (2008) BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae–P. viridiflava* group. Genet Mol Biol 31:106–115

Nucci C, da Silveira WD, da Silva Correa S, Nakazato G, Bando SY, Ribeiro MA, Pestana de Castro AF (2002) Microbiological comparative study of isolates of *Edwardsiella tarda* isolated in different countries from fish and humans. Vet Microbiol 89:29–39

Rodríguez JM, López-Romalde S, Beaz R, Alonso MC, Castro D, Romalde JL (2006) Molecular fingerprinting of *Vibrio tapetis* strains using three PCR-based methods: ERIC-PCR, REP-PCR and RAPD. Dis Aquat Org 69:175–183

Romalde JL (2005) Application of DNA fingerprinting techniques to the study of fish and shellfish pathogens. In: Read MM (ed) Trends in DNA fingerprinting research. Nova Science Publishers, New York, NY, p 163–191

Romalde JL, Magariños B, Villar C, Barja JL, Toranzo AE (1999) Genetic analysis of turbot pathogenic *Streptococcus parauberis* strains by ribotyping and random amplified polymorphic DNA. FEMS Microbiol Lett 179:297–304

Tacão M, Alves A, Saavedra MJ, Correia A (2005) BOX-PCR is an adequate tool for typing *Aeromonas* spp. Antonie van Leeuwenhoek 88:173–179

Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). Meth Cell Mol Biol 5: 25–40

Yamada Y, Wakabayashi H (1998) Enzyme electrophoresis, catalase test and PCR-RFLP analysis for the typing of *Edwardsiella tarda*. Fish Pathol 33:1–5

Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: August 31, 2010; Accepted: March 29, 2011
Proofs received from author(s): June 20, 2011