Identification and Investigation of Phenotypic and Genotypic Characteristics of Flavobacterium Psychrophilum in Fry Rainbow Trouts (Oncorhynchus mykiss) in Some Trout

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Abstract: In this study, it was aimed to identify and investigate phenotypic and genotypic characteristics of Flavobacterium psychrophilum in four rainbow trout farms in Keban district of Elazığ province. Two different primers were used to confirm 160 Flavobacterium psychrophilum with PCR after determined according to the phenotype and genotype characteristics. Subsequently, these strains were confirmed as Flavobacterium psychrophilum with DNA. Gene typing was performed with PFGE technique on strains confirmed with PCR. As a result of this, it was decided that strains obtained from four different fish farms was close relative with reference Flavobacterium psychrophilum NCIMB 19477. 

Keywords: Flavobacterium psychrophilum, Rainbow trout, Oncorhynchus mykiss, Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE)

Introduction

Bacterial fish disease in culture fishing is one of the most important factors that cause economic losses in businesses. Severity of the disease depends on the type and age of the fish. But many infections in fish can be successfully treated. However, the negativity of environmental conditions makes it difficult to protect fish from diseases and to take control measures and even makes it impossible (Austin and Austin, 1987; Buckley et al., 1998).

Psikrofiloz (Bacterial cold water disease, peduncle disease, Rainbow Trout Fry Syndrome -RTFS) is a septicaemic characterized, acute progressive, epidemic and lethal infection which is caused by F. psychrophilum and which occurs especially in hatcheries where the water temperature falls below 10°C. For this reason, it causes economical losses for enterprises. Psikrofiloz was detected for the first time in Turkey in fry rainbow trout in 1993 and it caused serious economical losses in fish production plants after it had spread in different geographical regions in the subsequent years. So far, in our country, this disease has been commonly seen in February-June and has become an important bacterial disease of fry rainbow trout (Bernardet and Kerouault, 1989; Austin, 1992; Lumsden et al., 1996; Diler et al., 2003; İspir et al., 2004; Arda et al., 2005).

F. psychrophilum resides on skin, mucosa, fins, gills, operculum, ovarian fluid and natural flora of the digestive system and causes disease as well (Diler et al., 2000; Nematollahi et al., 2003; Arda et al., 2005; Kubilay et al., 2009a; Kubilay et al., 2009b). In addition, it has been reported that the factor may infect the eggs of sick spawner fish and then the juvenile fish (Balta, 1997; Diler et al., 2003). As the environmental factors change, virulence of the factor increases too and causes infection. Because the factor infects via contact and water spreading quickly, it causes excessive deaths in rainbow trout farming enterprises and eventually significant economic losses in those enterprises (Cipriano and Holt, 2005).

Microbiological tests are often used to determine the phenotypic characteristics of Flavobacterium psychrophilum (Arda, 2000; Austin and Austin, 1987; Bernardet and Kerouault, 1989; Bernardet et al., 1996).

Polymerase Chain Reaction (PCR) is a method leading the reproduction of targeted nucleic acid as biochemically in order to determine genetic material of the required factors in a short time (Saiki et al., 1988; Mullis, 1990; Bej et al., 1991; Taylor, 1993).

Pulsed Field Gel Electrophoresis (PFGE) is used as a reference in genotypical identification because it has very high separation power and repeatable results and because it is easy to interpret. This method is based
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on the basis of cutting with restriction enzyme for the determination of genetic profile without deterioration in structural integrity of the chromosome isolated from agarose-embedded bacteria (Derbentli, 2002; Domig et al., 2003; Van Belkum et al., 2007).

There has not been an extensive research on phenotypic and genotypic characters of F. psychrophilum which is an important factor of Psikofiloz which is one of the important fish diseases in our country. In this study, it is aimed to investigate phenotypic and genotypic characteristics of this factor and to determine Flavobacterium psychrophilum bacteria in fry rainbow trout (Oncorhynchus mykiss Walbaum, 1792) in the hatcheries of some rainbow trout farming enterprises in Keinan district of Elazığ province.

**Materials and Methods**

**Bacterial isolates**

A total number of 4024 F. psychrophilum isolates were examined in this study (Table 1). The Turkey collection contained 160 isolates, isolated from diseased salmonids between December 2008 and July 2009. The type strain of F. psychrophilum (NCIMB 1947) was included in all analyses All isolates were stored at -80 °C until used.

**Table 1.** I, II, III, IV of farm isolated samples taken from the number and percentage of Flavobacterium psychrophilum.

<table>
<thead>
<tr>
<th>SAMPLE TAKEN PLACE</th>
<th>EXAMPLE FROM FARMS</th>
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<tr>
<td></td>
<td>I. farm</td>
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<tr>
<td>Eggs</td>
<td>40</td>
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<tr>
<td>Ovarian Liquid (2 ml)</td>
<td>40</td>
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<tr>
<td>Sperm (2 ml)</td>
<td>50</td>
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<tr>
<td>Eggs Washed With Povidin/Iodin Solution</td>
<td>40</td>
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<tr>
<td>Fertilized Eggs</td>
<td>50</td>
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<tr>
<td>Eyed Eggs</td>
<td>60</td>
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<tr>
<td>Larvae with Egg Sac</td>
<td>100</td>
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<td>Fry Under 5 g</td>
<td>100</td>
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<td>Healthy Fry over 5 g</td>
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<td>Healthy of newly dead and Suspicious from disease fry over 5 g</td>
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<td>Intestine</td>
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<tr>
<td>Food</td>
<td>30</td>
</tr>
<tr>
<td>Water</td>
<td>64</td>
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Culture media

For grown of *Flavobacterium psychrophilum*; Tryptone Yeast Extract Salt Agar (TYES-A), (Holt, 1987; Michel et al., 1999) (0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, 1.1% agar pH 7.2) and Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar (EAO+FBS+A) (0.5% tryptone, 0.05% yeast extract, 0.05% beef extract, 0.02% sodium acetate, 0.02% calcium chloride, 0.05% magnesium sulfate, and 5% fetal bovine serum, 1% agar) (Starliiper vd., 2007), were used.

Biochemical and physiological characterization

*Flavobacterium psychrophilum* isolates were identified and characterized using the following tests, as described by Bernardet (1989): catalase, cytochrome oxidase, absorption of Congo red, presence or absence of fexirubin-type pigment, production of acid from glucose, fructose, galactose, mannose and glycerol, production of amylase and caseinase, tyrosine hydrolysis, reduction of nitrate and production of hydrogen sulphide using commercial lead acetate paper strips.

Gram staining was carried out according to Collins & Lyne (1976). Gliding motility was tested by direct microscopy of agar cultures (24±48 h). A covesipl was placed on the agar at the edge of the bacterial growth. Gliding motility was assessed by phase-contrast microscopic examination of a fresh TYES broth culture and by the hanging drop technique (Bernardet et al. 2002). Anaerobic growth of the isolates was tested on agar plates incubated in an anaerobic jar for 7 days. Growth of the isolates was tested on Tryptone Yeast Extract Salt Agar (TYES-A), (Holt, 1987; Michel et al., 1999) and Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar (EAO+FBS+A) (Starliiper vd., 2007) for 7 days. Growth at different temperatures was determined by inoculating bacterial cells in early log-phase into TYES and incubating the cultures at 5, 20 and 25 °C for 7 days. The growth was evaluated by eye. Production of gelatinase and elastinase was tested using AO agar supplemented with 1% gelatine and 0.05% elastine, respectively. The plates were streak inoculated and observed for subsequent clearance around the colonies during 14 days. Esculin hydrolysis was tested in TYES containing 0.1% esculin and 0.05% ferric citrate. The tubes were incubated for 7 days and a dark brown or black colour was considered as a positive result. Lysis of cells was tested on TYES with added washed and autoclaved yeast cells (bakers yeast). Clearing around colonies after 14 days of incubation indicated lysis of the yeast cells. All tests were carried out at 15 °C unless otherwise indicated.

DNA Extraction

For the DNA extraction from cultures; a few represen-tative colonies from pure cultures were transferred into an Eppendorf tube containing 300 μl distilled water. The bacterial suspension was treated with 300 μl TNES buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.2% SDS) and 5 μl proteinase K (200 μg ml⁻¹), and was kept at 37 °C for 2 h. Following 10 min of boiling, an equal amount of phenol: chloroform:isoamyl alcohol (25:24:1) was added to the suspen-sion. The suspension was shaken vigorously by hand for 5 min and then centrifuged at 13.000 g for 10 min. The upperphase was carefully transferred into another Eppen-dorf tube and 3 M sodium acetate (0.1 volumes) and 95% ethanol (2.5volumes) were added to the suspension, which was leftat –20 °C overnight to precipitate the DNA. The pellet, ob-tained following the centrifugation at high speed for 10min was washed twice with 90 and 70% ethanol respectively, each step was followed by 5 min centrifugation. Finally, the pellet was dried and resuspended in 50 μl distilled water.

Polymerase Chain Reaction (PCR)

For the PCR analysis of *F. psychrophilum* suspected isolates, the reaction mixture was prepared in a total volume of 50 μl, consisting of 5 μl DNA, 10x PCR buffer (750 mM Tris/HC1, 200mM (NH4)2SO4, 0.1% Tween 20), 5 μl 25 mM MgCl2, 250 μMeach of deoxynucleoside triphosphates. 1.25 U *Ta*q DNAPolymerase (MBI Fermentas) and 20 pmol each of primerrerived from 16S rDNA gene of F. *psychrophilum* PSY1 190–206 (5’-GGT GGC ATC AAC ACA CT-3’) ve PSY2 1278–1262 (5’- CGATCC TAC TTG CGT AG-3’) 50 pmol and 5μl target DNA (Suzuki vd., 2008). The PCR analysiswas performed in a thermal cycler with an initial denatura-tion step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 second, 51°C for 90 second and 72°C for 2 min, then, a last step of extension at 72°C for 5 min. PCR products were separated by electrophoresis in 2% (w/v) agarose gels and visualized by ethidium bromide staining. A 100 bp DNA ladder (MBI Fermentas) molecular mass marker was used to evaluate the size of bands.

A reference strain of *Flavobacterium psychrophilum* NCIMB 1947 which was obtained from Centro de Biotecnologia Animal Laboratory in Spain was used in order to determine the most suitable DNA extraction procedure and to optimize PCR.

Pulsed-field gel electrophoresis (PFGE)

The 160 isolates of *F. psychrophilum* were analysed by PFGE, which was performed as described previously (Barrett et al. 1994; Izumiya et al. 1997) with minor modifications. Single colonies of *F. Psychrophilum* were inoculated onto Enriched Anacker and Ordal + Fetal Bovine Serum+ Agar
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(EAO+FBS+A ) (Starliper vd., 2007) and incubated at 15 °C for 72 h. Flavobacterium psychrophilum grown on EAO+FBS+A was suspended in 3 ml of sterilized distilled water (Arai, et al., 2007), Bacterial cells from 1ml aliquots were harvested, washed twice with cell suspension buffer (CSB: 100 mM Tris,100 mM EDTA, pH 8.0) and resuspended in the same buffer until an optical density (OD) of 1.2 was obtained. The OD was measured at 610 nm. Bacterial suspensions were incubated with 20 μl Proteinase K (20 mg/ml) 37 °C for 15 min, mixed with an equal volume of 1% chromosomal grade agarose (Seakem GTG agarose, Cambrex Corporation) and immediately loaded into a disposable plug mould (Bio-Rad Laboratories) and allowed to solidify at room temperature. Bacterial cells in each plug were then lysed in lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) and 25 μl proteinase K (20 mg/ml) for 2–3 h at 56 °C with shaking. Prior to digestion with restriction enzyme, the plugs were washed twice for 30 min with distilled water at 50 °C and four times for 15 min with Tris–EDTA buffer (TE: 10 mm Tris–HCl [pH 8], 0.1 mm EDTA). The plugs were stored in TE at 4 °C until use. XhoI and BlnI of restriction endonucleases were used in the study. The DNA in the plugs were cut in half, the individual pieces were incubated in 200 IL of buffer for each enzyme at 4 °C for 30 min. The DNAs in each plug were digested with 50 units of each restriction endonuclease at 37 °C for 4 h. PFGE was performed using a CHEF DR II apparatus (Bio-Rad Laboratories), with 1% agarose gel in 0.5x TBE (Tris-borate-EDTA) buffer at 6 V cm⁻¹ at 12 °C. For separation of the whole genome, a linearly ramped switching time from 1 to 12 s was applied for 18.5 h. After PFGE, the gels were stained with ethidium bromide (10mg/ml) and viewed and photographed under UV light. Digital images were stored electronically as TIFF files. After visual inspection of the banding patterns obtained by PFGE, computer analyses were carried out using BioD 1++ (infinity capt software, Vilber Lourmat, Fransa). A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA), with the Dice coefficient and a tolerance of 1%.

Results

Physiological and biochemical reactions

The F. psychrophilum isolates were rather homogeneous in their physiological and biochemical reactions and were identical to those of the type strain NCIMB 1947T. Positive reactions were obtained for catalase (Weak +), cytochrome oxidase and presence of fexuirin-type pigment, Oxidatio, Hemolysis, gelatine, tyrosine, Tween 20, Tween 80 and casein hydrolysis, growth at 5 and 20 °C and growth on Plate Count Agar and Mueller-Hinton Agar. Negative reactions occurred for Gram stain, Motion, Congo red adsorption, Indole, Metil Red, Simmons sitrate, Üreaz, H²S, ONPG, OD, Voges Proskauer, Oxidase Nitrate, starch Hydrolysis esculin hydrolysis, acid production from carbohydrates, 0°C growth, 30°C growth, 37°C growth and anaerobic growth. Variable reactions were seen for growth at 25 °C (16 of 160), growth in TSB (32 of 160), growth in TSA(32 of 160) and Fermentation (48 of 160).

Specificity of the primers

The primers PSY1190–206 and PSY2 1278–1262 generated a fragment of identical size (1089 bp) from all tested strains of F. psychrophilum including the type strain NCIMB 1947T (Fig. 1).

![Figure 2. The agarose gel that shows 1089 bp length bands that belongs to F. psychrophilum type formed as a result of PCR analysis using PSY1 190–206, PSY2 1278–1262 primer pairs of DNA samples; 1-12: Samples of F.](image-url)
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*psychrophilum* isolated from enterprises; M: 100 bp molecular marker; N: negative control; P: Positive control reference strain.

**PFGE analysis**
Isolates that were identified as *F. psychrophilum* from egg for Molecular sub-Identification of *Flavobacterium psychrophilum* with Pulsed Gel Electrophoresis, ovarium fluid, egg disinfectated with providine/iodine, fertilized eggs, observed eggs, marsupial fry, fry lighter than 5 grams, spleen of fry heavier than 5 grams, kidney of fry heavier than 5 grams, liver of fry heavier than 5 grams, intestine of fry heavier than 5 grams were subjected to PFGE analysis with Xhol enzyme. As a result of PFGE analysis, the same band profile was detected in all the isolates obtained from four different enterprises (Figure 3).

![Figure 3](image-url)

**Figure 3.** The bands on agarose gel as a results of cutting Choromosal DNA of *F. psychrophilum* with Xhol enzyme (A); P: *F. psychrophilum* NCIMB 1947 reference strain (B); 1: egg; 2: Ovarian fluid; 3: Egg disinfected with Povidine/iodine; 4: fertilized egg; 5: observed egg; 6: Marsupial fry; 7: fry rainbow trout lighter than 5 grams; 8: spleen of fry rainbow trout heavier than 5 grams; 9: Spleen of fry rainbow trout heavier than 5 grams; 10: liver of fry rainbow trout heavier than 5 grams; 11: kidney of fry rainbow trout heavier than 5 grams; 12: intestine of fry rainbow trout heavier than 5 grams.

DNA isolation from agarose molds containing 160 pieces of *F. psychrophilum* confirmed by PCR and 3 pieces of *F. psychrophilum* NCIMB 1947 reference strain was performed. 12 samples were chosen among these agarose mold DNA. Along with these samples, agarose molds containing 3 reference strain (P) were subjected to PFGE analysis with BlnI enzyme (Figure 4). At the end of PFGE analysis, the same band profile was detected in all of the isolate obtained from four different enterprises.
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Figure 4. The bands on agarose gel as a result of cutting chromosomal DNA of *F. psychrophilum* with BlnI enzyme (A); P: psychrophilum NCIMB 1947 reference strain (B); 1: egg; 2: Ovarian fluid; 3: Egg disinfected with Povidone iodine; 4: fertilized egg; 5: observed egg; 6: Marsupial fry; 7: fry rainbow trout lighter than 5 grams; 8: spleen of fry rainbow trout heavier than 5 grams; 9: Spleen of fry rainbow trout heavier than 5 grams; 10: liver of fry rainbow trout heavier than 5 grams; 11: Kidney of fry rainbow trout heavier than 5 grams; 12: intestine of fry rainbow trout heavier than 5 grams.

When the profile analysis of the bands was performed using gel comparison software system, it was revealed that there was an 80% relationship between *F. psychrophilum* strain and reference strains (Figure 5). Accordingly, it was observed that there was a close relation between *F. psychrophilum* reference strain and isolates obtained from the enterprises. The reference strain profile of *F. psychrophilum* was identified as Type A and the isolates taken from the enterprises were identified as Type A1 as they had the same profile.

Figure 5. PFGE profile of *F. psychrophilum* reference strain and *F. psychrophilum* obtained from the enterprises. 1,8,15: *F. psychrophilum* NCIMB 1947 reference strain; 2,3,4,5,6,7,9,10,11,12,13,14: *F. psychrophilum*
Discussion and Conclusions
In this research, primarily the isolation and identification of *F. psychrophilum*, one of the fry trout that is healthy or suspicious of disease found in four different rainbow trout plants located in Keban District of Edirne Province, was practiced through the common culture method. The typology of *F. psychrophilum*, identified by the Polymerase Chain Reaction technique was carried out by the Pulsed Field Gel Electrophoresis technique regarded as the golden standard of molecular typology methods and the affinity between the isolates and *F. psychrophilum* brought from abroad, was determined by the reference strain. In the end, phenotypic and genotypic features of *F. psychrophilum* were found out.

In their studies, Schmidt *et al.* (2000) homogenizing the fish samples in the stomacher, stated that they had prepared those samples for bacteriological culture. In another research (Yıldırım, 2007), the fish material was pestled in porcelain mortar and used for bacterial culture. Because it was more sterile and gave result in a short time, the stomacher homogenizer was also preferred in this research. Accordingly, 1835 eggs in total, eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g and fish food samples taken from four plants where the research was carried out, were homogenized successfully in the stomacher and used for bacteriological culture.

In their studies, Sander and Fryer (1988), Bernardet and Kerouault (1989), Crump *et al.*, (2001) Wiklund *et al.*, (1994), Lorenzen *et al.*, (1997), Bowser (1999), Dalsgaard and Madsen (2000), Korun and Timur (2001), Nemattollahi *et al.*, (2003), Diler *et al.*, (2003), Ispir *et al.*, (2004) and Cipriano and Holt (2005); incubated CA, MAOA and TYES-A mediums at 15-20°C for seven days, that they cultured in order to isolate *F. psychrophilum*. In these mediums, mid-fluffy, convex, circular and yellow colonies with thin spreading edges were produced and inside the painted preparates made by these colonies they specified the presence of Gram negative bacilli-shaped, gliding bacteria with 1,20-1,30 μm length. Findings of the researchers relevant to the colony structure and active features of 160 *F. psychrophilum*, isolated from trout plants in Keban district.

It was stated that, the colonies formed by the isolated *F. psychrophilum* in the researches carried out, appeared yellow, the oxidase test was negative, flexirubin-type pigment production test was orange in colour, gelatin and casein hydrolisation test was positive, congo red test and the acid production from H₂S and carbohydrates was negative (Wiklund *et al.*, 1994; Lorenzen *et al.*, 1997; Madetoja *et al.*, 2001; Cipriano and Holt 2005; Ekman, 2003). In this study, in the identification of *F. psychrophilum*, isolated from the samples of eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g, and from spleen, liver, kidney, guts and water samples of fry trout bigger than 5 g, healthy, suspicious of disease or just died, these distinctive biochemical tests were successfully carried out and similar results were acquired.

In similar studies (Diler *et al.*, 2003; Korun and Timur 2001; Diler *et al.*, 2005) reported that the oxidase test was negative, and in some of them (Bernardet and Kerouault 1989; Ostland *et al.*, 1997; Elsayed *et al.*, 2006), it gave positive results. It was determined that all the strains isolated in this study was oxidase negative. This supports the idea of phenotypic differences between *F. psychrophilum* strains.

In the identification of *F. psychrophilum*, Cipriano and Holt (2005) and Diler *et al.*, (2005) stated that O/F test was negative, Ispir *et al.*, (2004) stated that the oxidase test was negative and the fermentation test was positive. In this study, it was found out that the fermentation test was both positive and negative for some strains. It was determined that O/F test of *F. psychrophilum* reference bacteria strain was negative.

In a study (Lorenzen *et al.*, 1997), *F. psychrophilum* did not reproduce at 25 °C, and in another study (Bernardet and Kerouault 1989), again this determinant rarely reproduced at 25 °C and did not reproduce over 25 °C. Lorenzen *et al.*, (1997) stated that 18 *F. psychrophilum* out of 25, rarely reproduced at 25 °C, however did not reproduce at 30 °C, Schmidtke and Carson (1995) stated that 18 isolates out of 20, rarely reproduced at 25 °C and did not reproduce at 30 °C. Finally, Dalsgaard and Madsen (2000) determined that this determinant reproduced at 15, 20 and 25 °C. It was observed that, the research subject, 160 strains isolated from the trout plants in Keban, reproduced at 15, 20 and 25 °C, thus it partially showed similarity with the findings of Dalsgaard and Madsen (2000). And it also showed parallelism with the results of Lorenzen *et al.*, (1997) when it did not reproduce at 25 °C.

Lorenzen *et al.*, (1997), reported that *F. psychrophilum* did not reproduce in 2% NaCl, although it reproduced in 1% NaCl. Diler *et al.*, (2003) reported that this determinant reproduced in 0.5% NaCl, and did not reproduce in 1% and 2% NaCl, and Nemattollahi *et al.*, (2003) reported that *F. psychrophilum* reproduced in 0.8-1% and 2% NaCl. Accordingly, it was determined that this bacteria did not reproduce in 2% salinity or over.
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975 samples from plant no I; 1040 from plant no II; 1000 from plant no III; and 1040 from plant no IV were analyzed. Total 4024 samples taken from these four plants are thought to be sufficient in terms of analyzing the phenotypic and genotypic features of F. psychrophilum.

The presence rates of F. psychrophilum in trout plants were different. 45 (4.62 %) F. psychrophilum were isolated from plant no I; 30 (2.88%) from plant no II; 43 (4.3%) from plant no III; and 42 (4.15%) F. psychrophilum were isolated from plant no IV. However, differences in these rates were not regarded as significant statistically (P>0.05, F3,68=1.05).

This determinant, causing fishes to get sick through the adverse changes in the environmental conditions, situated in the ovarian fluid of fishes and in the natural flora of the nervous system, (Sarreyüpoğlu, 1984; Austin and Austin, 1987; Holt et al., 1993; Gonzalez et al., 1999; Cengizler, 2000; Gonzalez et al., 2001; Nematollahi et al., 2003; Hatha et al., 2005; Arda et al., 2005; Skrodenyte-Arbaciauskiene et al., 2006; Kubilay et al., 2009a; Kubilay et al., 2009b). Determinants, being isolated in both water and fish samples, show parallelism with the results of researchers studying on this subject. F. psychrophilum was not isolated from the fish food taken from all four plants. And this claims that F. psychrophilum did not pass on fishes through feed.

The isolation of F. psychrophilum from the ovarian fluid of rainbow trout spawners on the plants in Keban, showed parallelism with the previous studies (Holt, 1987; Rangdale et al., 1996; Madsen and Dalsgaard, 2008; Kubilay et al., 2009a). Holt (1987), Ekman et al., (1993) and Madetoja et al., (2002) noted that spawners were the carriers of F. psychrophilum pathogeny.

Rangdale et al., (1996) could not isolate this bacteria from the sperm. As a result of this study, the isolation of F. psychrophilum from the sperms of male spawners could not be carried out as the researcher noted. Isolation of F. psychrophilum without any signs of disease on the spawners examined, confirmed the possibility that male spawners on the plants in Keban could be the carrier of this determinant.

The disease that F. psychrophilum caused, progressed in different mortality rates when compared to fish species. 50% mortality was observed for Coho Salmon fishes because of this determinant (Holt, 1987). It was reported that the disease occurred on rainbow trout fry after two months they began to feed and the mortality rate was 75% (Lorenzen et al., 1991; Bruno, 1992). In this research, in four plants, the death of fry after 20-50 days they began to feed, confirmed that the disease could occur within the first months of the fry. However, it was observed that the mortality rates in the plants were 20% less in plant no.I; 30% in plant no.II; 35% in plant no.III; and 40% less in plant no.IV than the rates that the researchers noted (Holt, 1987; Lorenzen et al., 1991; Bruno, 1992). It was understood as, there was a relation between the differences in the water quality of plants and the mortality of disease, and the mortality was low in the plants where the water quality was high.

In some studies (Austin, 1992; Bernardet and Kerouault 1989; Bruno, 1992; Bustos et al., 1995; Lorenzen et al., 1991; Toranzo and Barja, 1993; Wiklund et al., 1994; Didinen et al., 2005), they observed that in sick and dead fishes, on ventral cavity, depending on the fluid, bloating, exophthalmos, condensation on the skin, white lesions were shaped on the dorsal and kaudal fin of several fishes and in the further phases of the disease, the kaudal fin was completely ruined, radiuses appeared, the liver and kidney faded and the spleen was grew. Similar results were acquired in the trout plant where the research was carried out.

In previous years, in a study carried out in the plants located in Keban district, on fry rainbow trout F. psychrophilum was isolated (İspir et al., 2004). In these studies carried out in different time limes, the isolation of F. psychrophilum from the samples of eggs disinfected with povidone/iodine solution, fertilized egg, controlled egg, marsupial fry, fry smaller than 5 g, and from spleen, liver, kidney, guts and water samples of fry trout bigger than 5g, healthy, suspicious of disease or just died, proved that the contamination carried on through this determinant on the plants in this region.

It was stated that the limited number of microorganisms of PCR technique found in pure and mixed cultures, could be identified within a day, thus it was more advantageous than the culture and serologic tests (Lin and Tsen, 1996). The use of specific primer for PCR amplification made identification easier. In this research, the wrong positive reaction of the bacteria was prevented using two different primer pairs; Flavobacterium type specific GYRA-FP1F and GYRA-FP1R; F. psychrophilum type specific PSY1 190–206 and PSY2 1278–1262. Conducting this technique along with negative (E.coli) and positive controls (F. psychrophilum NCIMB 1947) referans in each step, proved that PCR technique was successfully carried out.

In their study, Chen et al., (2008), reported the isolation and identification of F. psychrophilum from 139 samples taken from two fish species in two different regions, on fifteen different plants. Then, they exposed these isolates to PFGE analysis with SaceI enzyme, and in reference to the tape profile formed, they found out a 88% affinity among rainbow trout isolates and 70% among Coho salmon isolates. In his study, Del Corro., (2010); isolated 25
F. psychrophilum from the kidney and spleen of the trout in five different regions on twelve different plants. These isolates were exposed to PFGE analysis with seventeen enzymes (Apal, BamHI, BglII, HindIII, KpnI, MluI, NcoI, NdeI, NotI, PvuI, SalI, SmaI, Spel, Stul, XbaI and XhoI) and amongst them, Stul enzyme, having the best tape profile, was preferred. With regard to the tape profiles formed as a result of PFGE analysis, 80% affinity was found out among the isolates. Arai et al., (2007) performed the isolation and identification of 81 F. psychrophilum for three fish species in different regions. These isolates were exposed to PFGE analysis with eight enzymes (BlnI, XhoI HindIII, SmaI, SacII, KpnI ve HindIII) and amongst them, BlnI and Stul enzymes, having the best tape profile, were preferred. With regard to the tape profiles formed as a result of PFGE analysis, 80% affinity was found out among the isolates. In this study, it was found out that there was 80% affinity between F. psychrophilum strains and reference strains isolated in different plants. The plants situated in the same region and water resources having the same origin, could explain the similar profiles formed by F. psychrophilum strains isolated in the plants. In addition, enzyme preferences might also have a role acquiring these results.

In conclusion, in this study, the isolation and identification of F. psychrophilum, causing disease on rainbow trout in our country and many countries in the world, especially causing enormous losses of fry trout and the determinant of Psychrophylus, was practiced through the common culture method. However, the isolation of this determinant is difficult and it takes a long time. Moreover, the isolated F. psychrophilum can be kept in a refrigerator or deep freezer for a short while. Therefore, using PCR, a molecular technique delivering quick results on the diagnosis of bacterial diseases, the identification of F. psychrophilum as molecular was performed for the first time in our country. Thus, the diagnosis of F. psychrophilum produced by the culture, through PCR technique in a short time, and the timely treatment, huge financial losses would be prevented in fishing plants.

In this study, the sub-typology of F. psychrophilum through Pulsed Field Gel Electrophoresis technique was successfully performed. The success of this study will cast light upon more original and comprehensive future studies in aquaculture sector towards the epidemiology of fish disease determinants observed in all our regions.

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