The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

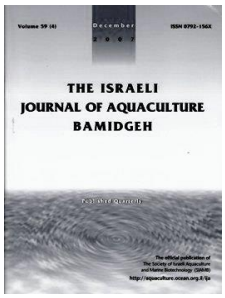
Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)
&
University of Hawai'i at Mānoa
&
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The *IJA* appears exclusively as a peer-reviewed on-line open-access journal at http://www.siamb.org.il/. To read papers free of charge, please register online at registration form.
Sale of *IJA* papers is strictly forbidden.



First Report of *Chryseobacterium* sp. from Koi (*Cyprinus carpio*) in Turkey

Behire Işıl DİDİNEN[1*] Ertan Emek ONUK[2] Türkay ÖZTÜRK[3] Seçil METİN[1] Meryem ÖZ[3] Özge ÇAYLI[1] Ayşegül KUBİLAY[1]

[1]*Egirdir Fisheries Faculty, Suleyman Demirel University, Isparta, Turkey*

[2]*Department of Aquatic Animal Diseases, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkey*

[3]*Sinop University, Faculty of Fisheries and Aquatic Sciences, 57000 Sinop, Turkey*

Keywords: *Chryseobacterium*, koi, characterization

Abstract

In this study, we isolated *Chryseobacterium* sp. from koi in Turkey. The disease outbreak occurred in fish weighing 10-300g (water temperature 9-10°C) in November 2011-February 2012. The cumulative mortality rate was approximately 55% over 4 months. Infected koi exhibited anorexia, weakness, emaciation, damage to dorsal and caudal fins, grey-white skin discoloration (1-1.5cm) in the head area, large open wounds in skin with disease progression, necropsy findings, paleness of the liver, enlarged spleen and kidney, acidic fluid in the body cavity. Samples for bacteriological examinations were collected from the kidney, liver, and spleen using sterile swabs; these samples were streaked onto Anacker ordal Agar and incubated at 18°C for 48 h. Five bacterial isolates were obtained from diseased fish. Phenotypic characteristics of the isolates were determined by conventional methods and rapid identification kits, API 20NE, and API ZYM. Comparative 16S rRNA gene sequence analysis demonstrated that isolate Sin57 belonged to the genus *Chryseobacterium*, with highest sequence similarity (98.5%) to *C. aahli* T68^T and *C. limigenitum* SUR2.

* Corresponding author. behiredidinen@hotmail.com

Introduction

The genus *Chryseobacterium* was proposed when subdividing the genus *Flavobacterium* (Vandamme et al., 1994). Members of the genus are widely distributed in various environments, including soil, plants, fresh water, marine environment, wastewater, and food sources (Pridgeon et al., 2013). The genus is pathogenic to humans and animals (Zamora et al., 2012b). Several species of the genus *Chryseobacterium* have been isolated from diseased fish. For example, *C. scophthalmum* in turbot *Scophthalmus maximus*, in Scotland (Mudarris & Austin 1989; Mudarris et al. 1994); *C. arothri* in pufferfish *Arothron hispidus,* (Campbell et al., 2008); *C. indologenes* in yellow perch *Perca flavescens*, (Pridgeon et al., 2013); *C. aahli in* lake trout *Salvelinus namaycush*, and brown trout *Salmo trutta,* in USA (Loch and Faisal, 2014); *C. piscicola* in Atlantic salmon in Chile (Ilardi and Avendaño-Herrera, 2008); *C. chaponense* in Atlantic salmon in Chile (Kämpfer et al., 2011) and in Finland (Ilardi et al., 2010); *C. oncorhynchi* (Zamora et al., 2012a); C. shigense (Zamora et al., 2012b); *C. tructae* (Zamora et al., 2012c); and *C. viscerum* (Zamora et al., 2012d) in rainbow trout in Spain have been reported. In fish, species of the genus *Chryseobacterium* are associated with skin and muscle ulcerations (Bernardet et al., 2005; Ilardi et al., 2010; Kämpfer et al., 2011), gross yellowish skin lesions below the dorsal fin as well as the caudal peduncle (Pridgeon et al., 2013), hemorrhage of the eyes, skin, gill sand jaw, necrosis, and hemorrhage of the brain, stomach, intestine, liver, kidney, and ascites within the peritoneum (Mudarris & Austin, 1989).

Many chryseobacteria isolated from diseased fish are usually identified only at the genus level due to difficulty of correct identification by phenotypically based laboratory methods alone; this limits knowledge of the diversity of species associated with fish disease (Zamora et al., 2012b). Presumptive identification of a *Chryseobacterium* sp. is often based upon phenotypic characters such as Gram negative, non-motile rods that produce bright yellow colonies due to the presence of flexirubin-type pigments, possess oxidase and catalase activities, and produce a *Chryseobacterium* spp. profile. Definitive identification is based upon polyphasic characterization, fatty acid profiling, and sequence/phylogenetic analyses. Additional techniques include matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and PCR amplification of the 16S and internal transcribed spacer (ITS) rDNA and subsequent sequence analysis (Loch and Faisal, 2015).

The aim of this study was the characterization of *Chryseobacterium* sp. isolated from diseased koi (*Cyprinus carpio*) for the first time in Turkey.

Material and Methods

Sampling. The disease was observed in koi (*Cyprinus carpio*) from November 2011-February 2012 at 9-10^{o}C. Five moribund diseased fish ranging from 10-300 g were collected from an ornamental pond for bacterial isolation and then all remaining fish were treated with enrofloxacin bath (2mg/L) for 7 days.

Isolation and Phenotypic Characterization. Bacterial isolations were performed from the kidney, liver, and spleen. All samples were streaked on Anacker Ordal agar (AOA). Plates were incubated at 18°C for 48 h. Based on morphology, only one type of colony growth in internal organs could be determined after 48 hours. A presumptive identification of the isolates (n=5) was performed using the following tests: Gram reaction, oxidase activity, and catalase production, oxidative and fermentative degradation of glucose with O/F basal medium supplemented with 1% glucose, presence of flexirubin-type pigments, brown pigment production on tyrosine agar, congo red absorbsion, hydrolysis of lecithin, starch, gelatin and casein, growth at different temperatures (5^{o}C, 20^{o}C, 30^{o}C, 37^{o}C, 42^{o}C), growth on 3%, 4.5%, 6.5% NaCl, growth on Triptic Soy Agar and MacConkey Agar. Additional tests were performed using API 20 NE and API ZYM.

Sensitivity to antibiotics. Antibiotic susceptibilities of *Chryseobacterium* sp. strains were performed by the disc diffusion method in Anacker Ordal agar (AOA). The antibiotic sensitivity discs included amoxicillin (25 µg), ampicillin (10 µg), ciprofloxacin (5 µg), clindamycin (2 µg), enrofloxacin (5 µg), erythromycin (15 µg), florfenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), neomycin(10 µg), lincomycin(2 µg), penicillin G (10 µg), rifampicin(5 µg), streptomycin (10 µg), sulphadiazine(300 µg), tobramycin (10 µg) and trimethoprim (5 µg); Agar plates were incubated at 18^{o}C for 48 h. The diameters

of the growth inhibition zones were measured and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations.

16S rRNA gene sequencing and data analysis. To determine the phylogenetic position of strain Sin57 isolated from kidney, 16S rRNA gene sequencing was performed by the Macrogen DNA sequencing service (Seoul, Korea). PCR amplification of the 16S rRNA gene of the isolate was conducted using universal primers 27F and1492R, and PCR products were purified. The 16S rRNA gene sequence was carried out directly on the purified fragments with an ABI PRISM 3100 sequencer (Applied Biosystems) per manufacturer's recommendations using universal primers 518F and 800R. The resulting sequence was verified by forward and reverse comparisons, assembled and edited with Contig Express in Vector NTI Advance 11.5 (Invitrogen). The obtained consensus sequence (1448 bp) was compared with previously published data in GenBank (Altschul et al., 1990) (http://www.ncbi.nlm.nih.gov/genbank) and EzTaxon-e database (Kim et al., 2012) and aligned with previously characterized sequences of closely related members of the genus *Chryseobacterium* and a range of *Chryseobacterium* species derived from fish and water, using ClustalW in Mega 5.0 multiple sequence alignments (Thompson et al. 1994).

Elizabethkingia miricola GTC 862^T, *Elizabethkingia meningoseptica* ATCC 13253^T and *Empedobacter brevis* LMG 4011^T were used as an outgroup. Phylogenetic relationships of the strains were estimated using the neighbor joining (NJ) method in Mega 5.0 (Tamura et al. 2011). NJ analysis was performed using a Kimura two-parameter correction model (Saitou and Nei, 1987) and pairwise deletion option for gaps. Confidence in the NJ trees was determined by analyzing 1,000 bootstrap replicates (Felsenstein, 1985) using the Mega program. The sequence of strain Sin57 has been deposited in GenBank databases under accession numbers KX352242.

Results

This is the first report of *Chryseobacterium* sp. from koi (*Cyprinus carpio*) in Turkey. The outbreak was observed in koi in November 2011-February 2012 at 9-10^{o}C. Cumulative mortality attributed to this pathogen was 50% within 4 months. Clinical signs in sick fish included anorexia, weakness, emaciation, damage in dorsal and caudal fins, grey-white skin discoloration(1-1.5cm) in the head area, gross skin lesions in the head, dorsal (Fig.1) and peduncle areas with disease progression and yellowish lesions on the surface (Fig.2). Internal symptoms included paleness of the liver, enlarged spleen and kidney, acidic fluid in the body cavity.

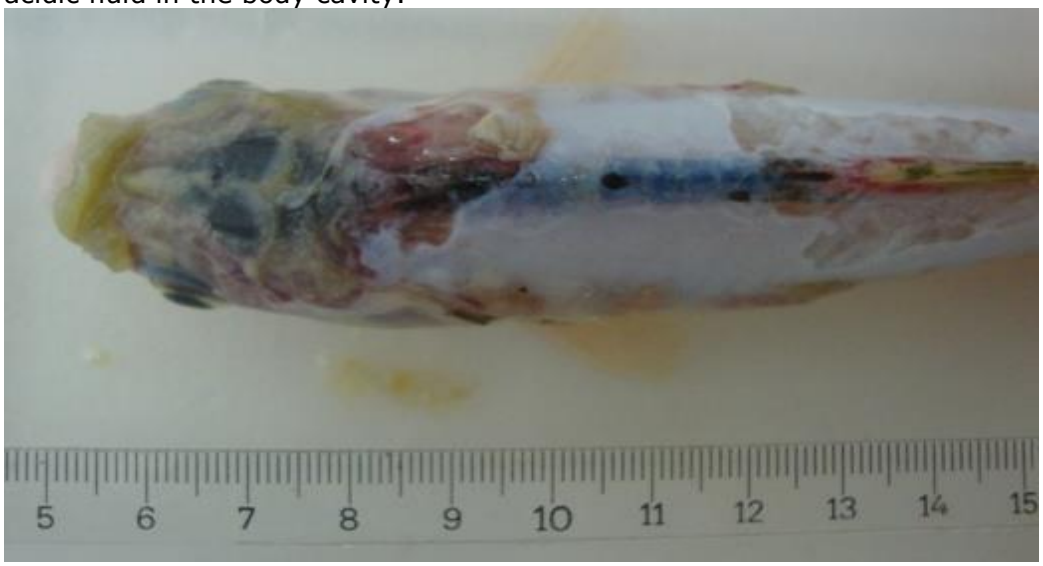


Fig.1 Gross skin lesions in head and dorsal area skin in koi infected with *Chryseobacterium* sp.

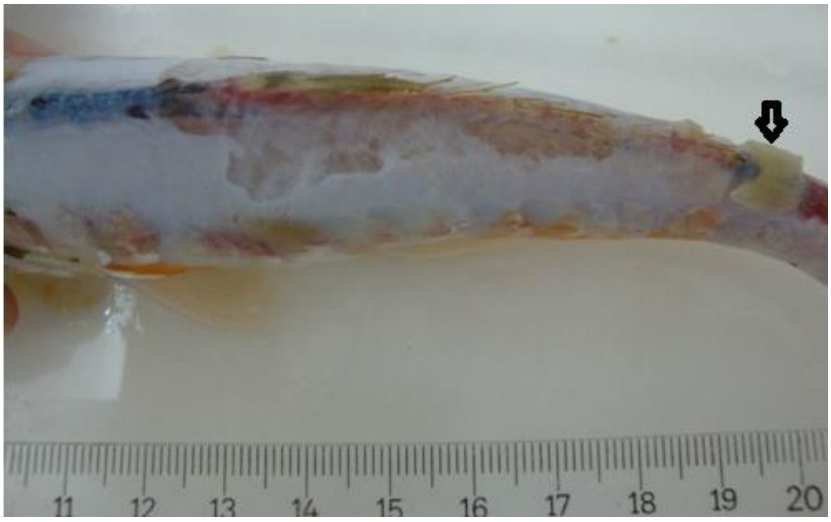


Fig.2 Yellowish lesion in pedincule area in koi infected with *Chryseobacterium* sp.

Gram-negative, rod-shaped bacteria were recovered from the liver, kidney, and spleen of fish. All isolates produced shiny, round, yellow-pigmented colonies on Anacker Ordal Agar after incubation at 18^0C for 48 h, under aerobic conditions. Phenotypically, all isolates were catalase and oxidase positive, non-motile, neither oxidative nor fermentative, congo red absorption negative, produce of flexirubin-type pigment and produce of brown pigment on tyrosine agar, growth at 5-30°C on Triptic Soy Agar(TSA) positive, growth at 37°C, 42°C on 3%, 4.5%, 6.5% and MacConkey Agar negative, hydrolysis of casein and lecithin positive, hydrolysis of gelatin and starch negative. Phenotypic characteristics of the isolates performed by API 20 E and API ZYM are represented in Tables 1 & 2.

Table 1. Phenotypic characteristics of *Chryseobacterium* sp. isolates by conventional methods and API 20 NE

Gr staining	-	Congo red absorption	-
Motility	non-motile	Production of flexirubin pigment	+
Oxidase	+	Nitrate reduction*	+
Catalase	+	Indole production*	-
O/F Test	-/-	Glucose acidification*	-
Hydrolysis of :		Arginine dihydrolase*	-
Lecithin	+	Urea hydrolysis*	-
Starch	-	Esculin hydrolysis*	+
Gelatin	-	Gelatin hydrolysis*	-
Casein	+	p-Nitropyhenyl-ßD Galactopyranoside*	-
Growth at:		Glucose assimilation*	-
5°C	-	Arabinose assimilation*	-
20 °C	+	Mannose assimilation*	-
30 °C	+	Mannitol assimilation*	-
37 °C	-	N-Acetyl Glusomine*	-
42 °C	-	Maltose Glusomine*	-
3 % NaCl	-	Gluconate Glusomine*	-
4.5 % NaCl	-	Caprate Glusomine*	-
6.5 % NaCl	-	Adipate Glusomine*	-
Growth on TSA		Malate Glusomine*	-
Growth on MacConkey Agar	-	Citrate Glusomine*	-
Brown pigment on tyrosine agar	+	Phenyl acetate Glusomine*	-

*Performed by API 20 NE

Table 2. Phenotypic characteristics of *Chryseobacterium* sp. isolates by API ZYM

Alkaline phosphatase	+
Esterase	-
Esterase lipase	-
Lipase	-
Leucine arylamidase	+
Valine arylamidase	+
Cystine arylamidase	-
Trypsin	+
α-Chymotrypsin	-
Acid phosphatase	+
Naphthol-AS-BI-phosphohdrolase	+
α -Galactosidase	-
β-Galactosidase	-
β-Glucuronidase	-
α -Glucosidase	-
β- Glucosidase	+
N-acetyl-β-glucosaminidase	+
α -Mannosidase	-
α -Fucosidase	-

Antibiotic sensitivity of *Chryseobacterium* sp. isolates are given in Table 3. All isolates were sensitive to enrofloxacin, ciprofloxacin, erythromycin, gentamicin, kanamycin, lincomycin, rifampicin, streptomycin and trimethoprim; they were resistant to ampicillin, amoxicillin, florfenicol, neomycin, tobramycin, clindamycin, penicillin G, sulphadiazine.

Table 3. Antibiotic susceptibilities of *Chryseobacterium* sp. isolates

Ampicillin (AMP 10)	R
Amoxycillin (AML 25)	R
Clindamycin (DA 2)	R
Ciprofloxacin (CIP 5)	S
Enrofloxacin (ENR 5)	S
Erythromycin (E 15)	S
Florfenicol (FFC 30)	R
Gentamicin (CN 10)	S
Kanamycin (K 30)	S
Lincomycin (MY 2)	S
Neomycin (N 10)	R
Penicilin G (P 10)	R
Rifampicin (RD 5)	S
Streptomycin (S 10)	S
Sulfadiazine(SD 300)	R
Tobramycin (TOB 10)	R
Trimethoprim (W 5)	S

R: resistant, S: susceptible

In phylogenetic analyses based on 16S rRNA gene sequences, the novel strain appeared to belong to the genus *Chryseobacterium* (Fig.3). Strain Sin57 was found to be most closely related to recently described species *C. aahli* T68^T and *C. limigenitum* SUR2 (98.5% 16S sequence similarity), *C. yeoncheonense* DCY67 (98.1%), *C. gambrini* 5-1St1a^T (97.00%) and *C. aquaticum* 10-46^T (96.77%). Sequence similarities between the novel strain and other members of the genus *Chryseobacterium* were found to be 96.66% and below.

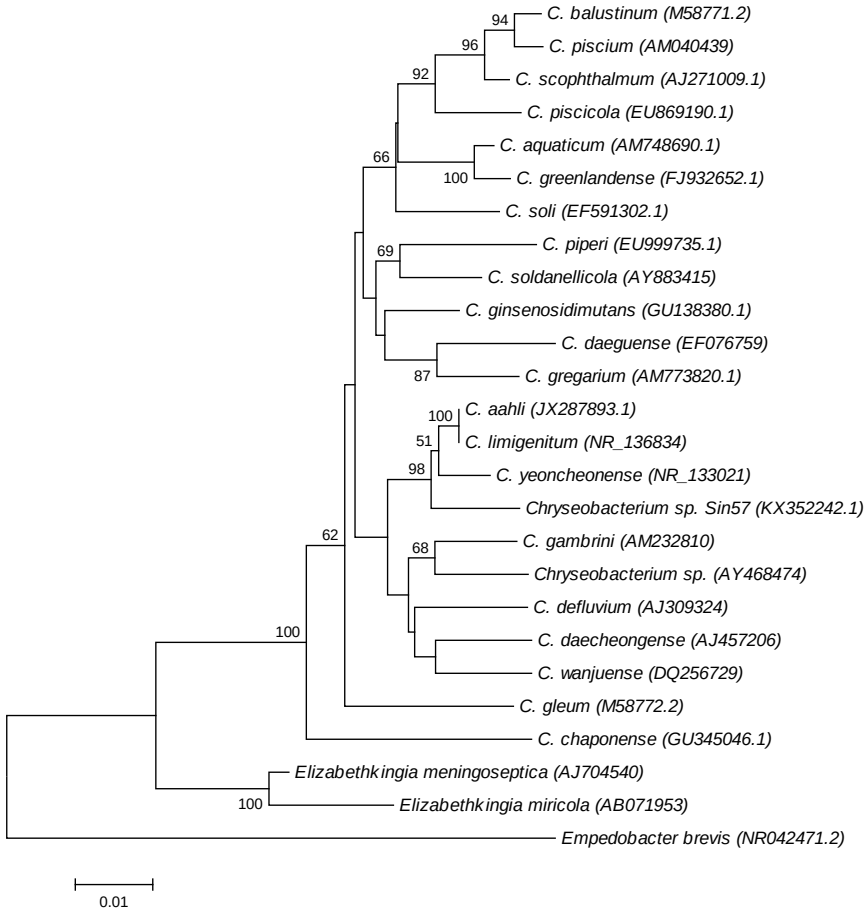


Fig.3 Phylogenetic tree based on 16S rRNA gene sequence comparison, obtained with the neighbor-joining algorithm, showing the *Chryseobacterium* sp. Sin57 with related taxa. Bootstrap values (expressed as a percentage of 1000 replications) >50% are given at the branching points. Members of the genera *Elizabethkingia* and *Empedobacter* used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Discussion

Species of the genus *Chryseobacterium* caused lethargy, gross external lesions (ulcers, skin and muscle lesions on the flank or in the peduncle area), surface hemorrhaging, distended abdomen, and general signs of septicemia in freshwater and marine fish (Mudarris & Austin, 1989; Bernardet et al., 2005; Ilardi & Avendaño-Herrera, 2008; Kämpfer et al., 2011; Pridgeon et al., 2013). In the present study, similar clinical signs were observed in koi.

In the present study, *Chryseobacterium sp.* Sin57 exhibited the high 16 S rRNA sequence similarity to *C. aahli* T68^T isolated from the kidney of lake trout *Salvelinus namaycush*, (Loch and Faisal, 2014). In addition, most of the phenotypic characteristics of *Chryseobacterium sp.* isolates from koi were consistent with *C. aahli* T68^T. However, some phenotypic characteristics of *Chryseobacterium* sp. isolates (nitrate reduction, utilize of p-Nitropyhenyl-ßD galactopyranoside, hydrolysis of aesculin, D-glucose, L-arabinose, and D-mannose; activities of urease, arginine dihydrolase, esterase, esterase lipase, cystine arylamidase, α-chymotrypsin and α-glucosidase) were different to *C. aahli* T68^T.

Chryseobacterium spp. are naturally resistant to a wide spectrum of antibiotics, including tetracyclines, erythromycin, linezolid, polymyxins, aminoglycosides, chloramphenicol, and many β-lactams, while also being intermediately sensitive to vancomycin and clindamycin and varying in their sensitivity to trimethoprim-sulfamethoxazole (Loch and Faisal, 2015). Among the 65 isolates obtained from aquatic habitats, 89% were resistant to polymyxin-B, 97% were resistant to ampicillin, 62% were resistant to erythromycin, and 54% were resistant to oxytetracycline, while 21.5% and 41.5% were resistant and moderately resistant to florfenicol (Michel et al. 2005).

Additionally, 69% of the isolates were sensitive to trimethoprim-sulfamethoxazole. In the present study, we also found that *Chryseobacterium sp.* isolates were resistant to β-lactams (amoxicillin, ampicillin, penicillin G), aminoglycosides (neomycin, tobramycin), florfenicol and erythromycin.

In this study, *Chryseobacterium* sp. was isolated from koi *Cyprinus carpio*, for the first time in Turkey. Comparative 16S rRNA gene sequence analysis demonstrated that *Chryseobacterium* sp. Sin57 had the highest sequence similarity (98.5%) to *C. aahli* T68^T and *C. limigenitum* SUR2.

References

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J., 1990. Basic local alignment search tool. *J Mol Biol.*, 215:403-410.

Bernardet J.F., Vancanneyt M., Matte-Tailliez O., Grisezc, L., Tailliez P., Bizet C., Nowakowski M., Kerouault B. and J. Swings, 2005. Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. *Syst Appl Microbiol.*, 28:640–660.

Campbell S., Harada R.M. and Q.X. Li, 2008. *Chryseobacterium arothri* sp. nov., isolated from the kidneys of a pufferfish. *Int. J Syst Evol Microbiol.*, *58*(1): 290-293.

Felsenstein J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

Ilardi P. and R. Avendaño-Herrera, 2008. Isolation of Flavobacterium- like bacteria from diseased salmonids cultured in Chile. *Bull Eur Assoc Fish Pathol.*, 28: 175–184.

Ilardi P., Abad J., Rintamäki P., Bernardet J.F. and R. Avendaño-Herrera, 2010. Phenotypic, serological and molecular evidence of *Chryseobacterium piscicola* in farmed Atlantic salmon, *Salmo salar* L., in Finland. *J Fish Dis.*, *33*(2):179.

Kämpfer P., Fallschissel K. And & R. Avendaño-Herrera, 2011. *Chryseobacterium chaponense* sp. nov., isolated from farmed Atlantic salmon (*Salmo salar*). *Int J Syst Evol Microbiol.*, 61:497–501.

Kim O.S., Cho Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H., Yi H., Won S. and J. Chun, 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol*, 62, 716-721.

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.

Loch T.P. and M. Faisal, 2014. *Chryseobacterium aahli* sp. nov., isolated from lake trout (*Salvelinus namaycush*) and brown trout (*Salmo trutta*), and emended descriptions of *Chryseobacterium ginsenosidimutans* and *Chryseobacterium gregarium*. *Int J Syst Evol Microbiol.*, *64*(5), 1573-1579.

Loch T.P. and M. Faisal, 2015. Emerging flavobacterial infections in fish: A review. *J Adv Res.*, *6*(3), 283-300.

Michel C., Matte-Tailliez O., Kerouault B. and J.F. Bernardet, 2005. Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant *Chryseobacterium* isolates from fish and aquatic habitats. *J Appl Microbiol.*, *99*(2): 323-332.

Mudarris M. and B. Austin, 1989. Systemic disease in turbot *Scophthalmus maximus* caused by a previously unrecognized Cytophaga-like bacterium. *Dis Aquat Organ.*, 6:161–166.

Mudarris M., Austin B., Segers P., Vancanneyt M., Hoste B. and J.F. Bernardet, 1994. *Flavobacterium scophthalmum* sp. nov., a pathogen of turbot (*Scophthalmus maximus* L.). *Int J* Syst *Bacteriol.*, 44: 447–453.

Pridgeon J.W., Klesius P.H. and J.C. Garcia, 2013. Identification and virulence of *Chryseobacterium indologenes* isolated from diseased yellow perch (*Perca flavescens*). *J Appl Microbiol.*, *114*(3), 636-643.

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

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and S. Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol.*, 28:2731-2739.

Zamora L., Fernández-Garayzábal J.F., Palacios M.A., Sánchez-Porro C., Svensson-Stadler L.A., Domínguez L., Moore E.R.B., Ventosa A. and A.I. Vela, 2012a. *Chryseobacterium oncorhynchi* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Syst Appl Microbiol.*, *35*(1):24-29.

Zamora L., Vela A.I., Palacios M.A., Domínguez L. and J.F. Fernández-Garayzábal, 2012b. First isolation and characterization of *Chryseobacterium shigense* from rainbow trout. *BMC Vet Res.*, *8*:77.

Zamora L., Vela A.I., Palacios M.A., Sánchez-Porro C., Moore E. R., Domínguez L., Ventosa A. and J.F. Fernández-Garayzábal, 2012c. *Chryseobacterium tructae* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Syst Appl Microbiol.*, 35: 315-319.

Zamora L., Vela A. I., Palacios M. A., Sánchez-Porro C., Svensson-Stadler L. A., Domínguez L., Moore E. R., Ventosa A. and J.F. Fernández-Garayzábal, 2012d. *Chryseobacterium viscerum* sp. nov., isolated from diseased fish. *Int J Syst Evol Microbiol.,* 62:2934–2940.

Vandamme P., Bernardet J.F., Segers P., Kersters K. and B. Holmes, 1994. New perspectives in the classification of flavobacteria: description of Chryseobacterium gen. nov., Bergeyella gen. nov., and Empedobacter nom. rev. *Int J Syst Evol Microbiol.*, 44:827–831.