

Differential Identification of *Flavobacterium* Species by Sequence Analysis of Genus-Specific Hypervariable 16S-23S rDNA Intergenic Spacer Target

¹Ahmad A. Elkamel and ^{2,3}Amr M. Mohamed

¹Fish Diseases and Management, Department of Animal Medicine,
Faculty of Veterinary Medicine, Assiut University, Egypt

²Clinical Laboratory Diagnosis, Department of Animal Medicine,
Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt

³Department of Laboratory Medicine,
Faculty of Applied Medical Sciences, Umm Al-Qura University, KSA

Abstract: The aim of the current study was to develop a molecular system for differential identification of various fish diseases caused by *Flavobacterium* species. The system uses the hypervariability of the 16S-23S rDNA Intergenic spacer region (ISR) to develop PCR-based sequence analysis assay. For this purpose, eight different 16S-23S rDNA ISR sequences of six different *Flavobacterium* species were aligned and compared to detect a hypervariable region with conserved flanking sequences as a target for amplification. The conserved flanking regions were used to design primers that target the selected hypervariable ISR sequence from all *Flavobacterium* species. American Type Collection of Cultures and other reference strains were used to assess the precision and specificity of the system. The results revealed the ability of the described molecular assay to accurately identify all the ATCC and reference *Flavobacterium* species to the strain level. In addition, two clinical isolates that were conventionally identified in the current study as *Flavobacterium psychrophilum* and *Flavobacterium columnare* were re-identified, using the molecular assay as *F. columnare* and *Flavobacterium johnsoniae*, respectively. The currently described ISR sequence analysis-based differential identification assay provides rapid and accurate identification of the different diseases causing *Flavobacterium* species and represent a useful tool for successful epidemiological studies and management of *Flavobacterium* species-caused fish diseases.

Key words: *Flavobacterium* species % 16S-23S rDNA ISR % PCR % Sequence analysis

INTRODUCTION

Yellow pigmented filamentous bacteria of the genera *Flavobacterium* is one of the most important Gram-negative bacteria groups affecting different species of fish. Fish infections caused by pathogenic *Flavobacterium* species are a major problem in the aquaculture industry worldwide, often leading to large economic losses [1]. There are currently 34 species within the genus *Flavobacterium* [2], some of which are pathogenic or regarded as opportunistic pathogens that cause diseases in plants, fish and

humans. *Flavobacterium columnare* is the causative agent of columnaris disease, with no wild or cultured freshwater fish, including ornamental fish in aquaria, are totally resistant to such disease [3]. *Flavobacterium johnsoniae* produces very similar lesions to those of *F. columnare* [4]. *Flavobacterium psychrophilum* is the etiologic agent of cold-water disease, while *Flavobacterium branchiophilum* and *Flavobacterium aquatile* causes the bacterial gill disease. Recently, there have been more emerging fish pathogens from this group [1].

Corresponding Author: Amr M. Mohamed, Clinical Laboratory Diagnosis, Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, 71515 and Department of Laboratory Medicine, Faculty of Applied Medical Sciences, Umm Al-Qura University, KSA, 7607.

Successful epidemiological studies and managing fish diseases caused by members of genus *Flavobacterium*, requires the ability to distinguish members of genus *Flavobacterium* among other yellow pigmented bacteria as well as differentiate various *Flavobacterium* species. Conventional diagnosis of *Flavobacterium* species is based on isolation of the causative agent followed by a series of biochemical tests [2, 5, 6]. Unfortunately, such approaches are time consuming and labor intensive [7]. There is no standard approach that can be used easily and economically to study these bacteria in clinical situations and this has further deterred the understanding of this complex group of organisms [3].

Recently, powerful nucleic acid-based PCR assays for rapid detection of different *Flavobacterium* species have been reported [8-11]. All developed PCR-based identification assays were, however, based on species-specific primers for the 16S rDNA sequence that may allow for detection and identification of a single *Flavobacterium* species [8, 10, 12]. Therefore, their usefulness is limited during screening and epidemiological studies.

The aim of the current study was to develop a new system for rapid and accurate identification of different pathogenic *Flavobacterium* species. The system was designed to use the hypervariability of the 16S-23S rDNA intergenic spacer region (ISR) sequence for developing PCR-sequence analysis-based molecular assay for differential identification of *Flavobacterium* species.

MATERIALS AND METHODS

Fish Collection: A 100 alive African sharptooth catfish, *Clarias gariepinus* (Burchell 1822), with a wide variety of skin and gill lesions were collected from different localities of El-Ibrahemia canal, Assiut City, Egypt. Collected fish had body weight ranged from 80-120 g and lengths ranged from 20-27 cm were transported immediately to the Aquatic Animals Wet Lab., Veterinary Hospital Clinic, Faculty of Veterinary Medicine, Assiut University to be examined.

Clinical and Bacteriological Examination of fish Samples: Fish were examined for clinical signs and possible external lesions according to clinical examination procedures [13]. Opercula were removed to expose the gill tissues and samples were cultured onto cytophaga agar medium [14] and incubated at either 18° or 25°C for 48 hours. Samples from the skin mucus and fins were also cultured directly on cytophaga agar medium.

Conventional Identification of Bacterial Isolates:

Purified bacterial isolates were identified based on the colony morphology, color and cultural characteristics on cytophaga agar, microscopic examination (Gram stain and motility test), various biochemical characters that include flexirubin pigment test, oxidase, catalase, indole, vogesproskauer, methyl red, H₂S production and carbohydrate utilization (lactose, glucose, arabinose, raffinose, xylose, sucrose and maltose [15, 16].

Bacterial Strains: Four American Type Bacterial Culture Collection (ATCC) and reference strains of *Flavobacterium* species were used to evaluate the newly designed *Flavobacterium* genus-specific primers. The strains included *F. johnsoniae* (ATCC-43622) and 3 *F. columnare* (ATCC-49512, ATCC-23463 and LADL-97-376). All reference strains were obtained from the archived strain repository of the Louisiana Aquatic Diagnostic Laboratory (LADL), School of Veterinary Medicine, Louisiana state University. The ATCC instructions were carefully followed in selecting the appropriate media and temperature for growing these bacteria. The study also included three clinical isolates FAS-5, FAS-8 and FAS-9 that were conventionally identified, in the current study, as *F. psychrophilum* (FAS-5), *F. columnare* (FAS-8 and FAS-9).

Three other fish pathogens including *Aeromonas hydrophila*, *Edwardsiella ictaluri* strain LA93-146 and *Photobacterium damsela* subsp. *piscicida* strain LA91-197 were obtained from the archived strain repository of the LADL, Louisiana State University and used as control to investigate the specificity of the new primers designed.

Design of *Flavobacterium* Genus-Specific PCR

Primers: Eight 16S-23S rDNA intergenic spacer region (ISR) of six different *Flavobacterium* species were used (Table 1). The MegAlign module of the Lasergene sequence software (DNA Star, Inc., Madison, WI) was used to align the 16S rRNA gene sequences (by the CLUSTERW method [17]. Multiple alignments and comparisons were used to identify the hypervariable regions with conserved flanking regions within the eight ISR sequences used. The selected hypervariable region sequences were thoroughly reviewed to exclude any homology among the eight ISR sequences used in the current study. The conserved flanking regions were used to design both forward and reverse primers that target the selected hypervariable ISR sequence using the Primer Select module in the Lasergene sequence software (DNA Star, Inc., Madison, WI). The two designed primers were

Table 1: The *Flavobacterium* species and the accession number of the 16S-23S rDNA intergenic spacer sequences used to design the pan *Flavobacterium* genus-specific primers

Species name	Accession number (s) used
<i>F. columnare</i>	AB031216, AB031221
<i>F. johnsoniae</i>	AY75370, AY753067
<i>F. branichiophilum</i>	AY753069
<i>F. psychrophilum</i>	AY757361
<i>F. aquatile</i>	AY753066
<i>F. hydatis</i>	AY753068

aligned against The GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis tool (National Center for Biotechnology Information, Bethesda, MD) to investigate the annealing specificity of these primers only to *Flavobacteria* and that there is no cross matching any other with bacterial species.

The designed primers are FITSA-F (5'-TGA TAA TGT AGG GGT CGG CAC -3') and FITSA-R (5'-GAG AAT ATC GGA GTC GAA CC -3'). The designed primers were synthesized at the Molecular Biology Core Laboratory, Egyptian Institute for Biological Products and Vaccine Production.

Polymerase Chain Reaction amplification of ISR target:

Total DNA of the ATCC strains, control strains, as well as clinical isolates were extracted and purified using the DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CL, USA) according to the manufacturer recommendations. PCR was used to amplify the hypervariable region of the ISR target using the currently designed pan *Flavobacterium* genus-specific primers. PCR was carried out using 5 µl template DNA (10 ng/µl) with PCR buffer mix and 0.5 µl of Phusion® DNA polymerase (New England Biolabs, Inc.) in a total reaction volume of 50 µl. PCR amplification was performed in Veriti® thermal cycler (AB, Applied Biosystems, Life Technology, USA) starting with an initial denaturation step at 95°C for 10 min., followed by 35 cycles of a denaturation at 95°C for 1 minute, an annealing at 55°C for 30 seconds and an extension step at 72°C for 45 seconds. PCR products were detected on 2% agarose gel according to the instructions of the manufacturer (UVP, Upland, CA, USA). PCR products were purified from gel for sequencing using the QIAquick® PCR purification kit (Qiagen, Valencia, CA, USA).

All previous work was done at Dr. Ronald Thune's lab, Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University. Purified PCR products were sequenced using the above forward

and reverse PCR amplification primers at the "Gene Lab", Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University.

Investigation of Primer Specificity: To confirm the specificity of the designed primers, PCR amplification of the targeted ISR sequences was performed using the genomic DNA of the ATCC and reference strains of *Flavobacterium* species as well as other bacterial fish pathogens including *A. hydrophila*, *E. ictaluri* strain LA93-146 and *P. damsela* subsp. *piscicida* strain LA91-197. The production of the expected size DNA product from given genomic DNA templates was considered a positive identity of a species of genus *Flavobacterium*.

Sequence Analysis Differential Identification of *Flavobacterium* Species: The ISR sequences of tested isolates were aligned against the *Flavobacterium* species sequences available at GenBank database (National Center for Biotechnology Information [NCBI], Washington, D.C.) using the BLAST analysis tool (<http://www.ncbi.nlm.nih.gov/blast/>).

RESULTS

Clinical Examination: The main clinical signs observed on naturally infected fish were erosions and ulceration of skin at the base of the dorsal fin and on the head and fin rot with separated fin rays, in addition to paleness of gill color and sloughing of gill filaments. Microscopic examination of wet mounts from eroded areas of infected skin or gills revealed the presence of masses of long bacterial cells.

Bacteriological Isolation and Identification: Bacteriological examination of collected samples resulted in recovery of 48 suspected *Flavobacterium* isolates from gills and skin of the 100 examined fish. Primary identification of isolates was done according to cultural and morphological characters, while conventional identification was done according to the biochemical characters. There were 36 isolates identified as *F. columnare* and 12 isolate identified as *F. psychrophilum*.

Evaluation of Designed *Flavobacterium* Genus-Specific Primers: Using the two designed *Flavobacterium* genus-specific primers, PCR amplification of the hypervariable ISR sequence target produced the expected size amplicons (210-260 base pairs, bp) from all reference

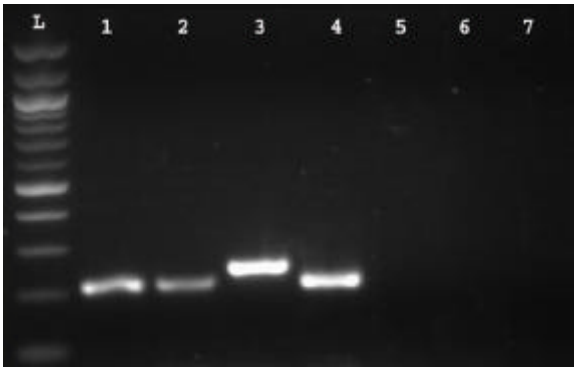


Fig. 1: PCR amplification of IGS target using the *Flavobacterium* genus-specific primers (FIGS-F/FIGS-R) from *F. columnare* strain ATCC-49512 (1), *F. columnare* strain ATCC-23463(2), *F. johnsoniae* strain ATCC-43622 (3), *F. columnare* strain LADL-97-376 (4), *Aeromonas hydrophila* (5), *Edwardsiella ictaluri* strain LA 93-146 (6) and *Photobacterium damsela* subsp. *piscicida* strain LA91-197 (7). (L) 100 pb. DNA ladder. The described primers amplified only from reference isolates of *Flavobacterium* species but not from any of the closely related other bacteria.

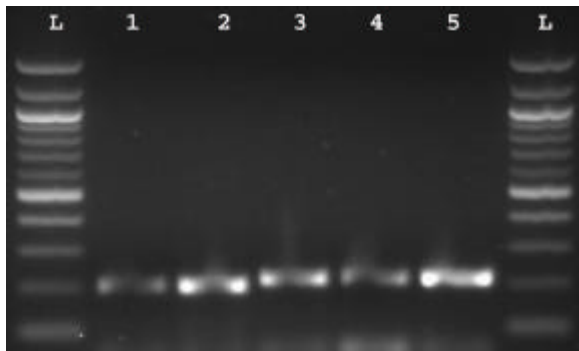


Fig. 2: PCR amplification of IGS target from 3 *F. columnare* reference isolates: ATCC-49512 (1), ATCC-23463(2) and LADL-97-376 (3), and from the 2 clinical isolates FAS -8 (4) and FAS-5(5). (L) 100 pb. DNA ladder.

strains of *Flavobacterium* species but did not yield any amplified product from the genomic DNA of other fish bacterial pathogens (Fig.1). Clinical isolates previously isolated and conventionally identified as *Flavobacterium*, showed amplicons of the expected size for members of genus *Flavobacterium*. Isolates FAS-5 and FAS-8 produced amplicons comparable to that generated from *F. columnare* ATCC-49512, ATCC-23463 and LADL-97-

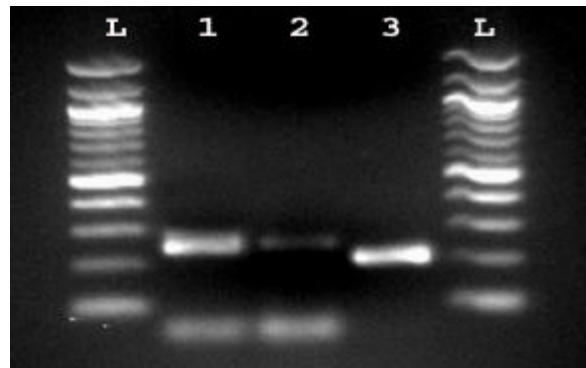


Fig. 3: PCR amplification of IGS target from 2 reference isolates; *F. johnsoniae* strain ATCC-43622 (1) and *F. columnare* strain ATCC-49512 (3), and from the clinical isolate FAS -9 (2). (L) 100 pb. DNA ladder.

376 reference strains (Fig. 2), while amplicons from isolate FAS-9 was comparable to that generated from *F. johnsoniae* ATCC-43622 reference strain (Fig. 3).

Sequence Analysis-Based Differential Identification of *Flavobacterium* Species: BLAST sequence analysis of generated ISR sequence from tested strains provided the accurate identification of all reference strains. The BLAST sequence analysis for the previously identified clinical isolates was, however, able to confirm the identity of only one isolate FAS-8, as *F. columnare*. On the other hand, the other two clinical isolates, FAS-5 and FAS-9 that were originally identified based on conventional biochemical characters as *F. psychrophilum* and *F. columnare*, respectively, were re-identified as *F. columnare* and *F. johnsoniae*, respectively based on the ISR sequence analysis-based differential identification.

DISCUSSION

Fish infections caused by pathogenic *Flavobacterium* species affects countless varieties of fish worldwide and considered one of the most economically important fish diseases that affect aquaculture industry [1]. They have been found in connection with external symptoms on fish, like gill or jaw erosion, fin or tail rot, or saddle like skin lesions [3]. All fish worldwide are susceptible to some form of disease caused by *Flavobacterium* spp. The current study describes a new sequence analysis-based assay for differential identification of various species and strains of genus *Flavobacterium*. The assay includes PCR amplification of the hypervariable 16S-23S rDNA intergenic spacer region

(ISR) sequence using newly designed *Flavobacterium* genus-specific primers. This would represent a useful tool for successful epidemiological studies and managing fish diseases caused by members of genus *Flavobacterium*. This two-step assay is designed to use *Flavobacterium* genus-specific primers to amplify a hypervariable target within the ISR region. Having a PCR product indicates a *Flavobacteria* species is being investigated and sequence analysis of such product provides the accurate identification of the species even to the strain level.

Conventional identification of species of *Flavobacteria* requires detailed biochemical testing including optimum growth temperature, morphology of the colonies, sugar utilization, gelatin degradation, production of chondroitinase, catalase and hydrogen sulfide, salinity tolerance and chromoshift from yellow to red in the presence of 3% sodium hydroxide [1]. The above procedures, however, take at least 7–10 days and are not practical in the field [7]. Besides, these methods are burdensome and often inconclusive. The newly designed assay is not only a time saving assay when compared to conventional biochemical analysis, but it also and importantly, more precise and accurate. This is supported by the accurate identification of all ATCC and reference strains to the strain level based on the *Flavobacterium* genus-specific ISR BLAST sequence analysis as described. It was also supported by re-identification of the previously misidentified clinical isolates (FAS-5, FAS-8 and FAS-9). The current assay confirmed the identity of only one isolate (FAS-8) as *F. columnare*, while the other two isolates (FAS-5 and FAS-9) were misidentified as *F. psychrophilum* and *F. columnare*, respectively. These two clinical isolates were re-identified as *F. columnare* and *F. johnsoniae*, respectively.

The described assay successfully amplified the hypervariable ISR sequence from all tested ATCC and reference strains and clinical isolates of various *Flavobacterium* species but not from any of the tested other fish pathogenic bacteria. These findings, in addition to the absence of nucleotide sequence matching between the two primers designed for *Flavobacterium* and any other bacterial species in the Gene Bank database, confirm the specificity of the described primers to genus *Flavobacterium*.

All the DNA-based diagnostic approaches previously developed are species specific that can identify some members of genus *Flavobacterium* on individual basis [8, 10, 12]. This limits the use of such

approaches in both diagnostic and epidemiological studies where no specific *Flavobacterium* species is being expected. Sequence analysis of specific genetic elements could be useful for the differential identification of suspected bacterial species.

The 16S rDNA gene is highly conserved and contains both genus and species-specific sequences that could be used for the differential identification of bacterial species [18-21]. The product of 16S rDNA gene, however, serves a vital function for bacteria; therefore the species variability in the sequence of this gene is inherently limited. This resulted in identical or highly homogenous 16S rDNA sequences among some of the *Flavobacterium* species making the species differentiation difficult [20, 22]. The ISR sequence, between the 16S rDNA and the 23S rDNA, has been reported as a hypervariable target that has larger differentiating ability at both the genus and species levels than the 16S rDNA target [23] and the new assay developed in the current study uses this hypervariability power. First, the ISR sequence is genus-specific and highly conserved for each genus [23] and thus was used to identify *Flavobacteria* among other genera using the genus-specific *Flavobacterium* primers. This was indicated by the finding that only members of genus *Flavobacterium*, but not other genera were amplified by the new specific primers. Second, the hypervariability of the ISR sequence among various species within the same genus made it possible to differentiate species of *Flavobacteria* as was shown by sequencing of the PCR product of the various species of *Flavobacterium* studied.

Restriction fragment length polymorphism (RFLP) and sequence analysis of the ISR were useful in identifying only *F. columnare* strains among other *Flavobacterium* spp. This differentiation ability was found useful in epidemiological studies and disease management in only *F. columnare* infections [20]. In contrast, results of the current study clearly show that the newly developed assay enables the identification of more than one species of *Flavobacterium* using the same primers.

In conclusion, the currently described ISR sequence analysis-based differential identification assay comes handy when there is flavobacterial infection with inconclusive conventional identification or more than one species are expected in comprehensive or epidemiological surveys. The described assay provides for rapid and accurate identification of *Flavobacterium* species and it has an advantage over previously described DNA-based assays in being more conclusive in targeting different pathogenic members of genus *Flavobacterium*.

Therefore, it represents a useful tool for successful epidemiological studies and management of *Flavobacterium* species-caused fish diseases that would provide crucial knowledge for effective control approaches of such diseases.

ACKNOWLEDGMENT

The authors would like to indeed thank Dr. Ronald Thune and Dr. John Hawke, Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, for their help and support.

REFERENCES

1. Plumb, J.A. and L.A. Hanson, 2011. Health maintenance and principal: microbial diseases of cultured fishes. 3rd ed., Ames, Iowa: Wiley-Blackwell.
2. Bernardet, J.F., P. Segers, M. Vancanneyt, F. Berthe, K. Kersters and P. Vandamme, 1996. Cutting a Gordian Knot: Emended Classification and Description of the Genus *Flavobacterium*, Emended Description of the Family *Flavobacteriaceae* and Proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). International Journal of Systematic Bacteriology, 46(1): 128-148.
3. Shotts, E.B. and C.B. Starliper, 1999. Flavobacterial Diseases: Columnaris Disease, Coldwater Disease and Bacterial Gill Disease, in Fish diseases and disorders. Volume 3, Viral, bacterial and fungal infections, P.T.K. Woo and D.W. Bruno, Editors. CABI publ.: Oxon, pp: 559-576.
4. Carson, J., L.M. Schmidtke and B.L. Munday, 1993. *Cytophaga johnsonae*: a putative skin pathogen of juvenile farmed barramundi, *Lates calcarifer* Bloch. Journal of Fish Diseases, 16(3): 209-218.
5. Bernardet, J.F., Y. Nakagawa and B. Holmes, 2002. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. International Journal of Systematic and Evolutionary Microbiology, 52(3): 1049-1070.
6. Shoemaker, C.A., C.R. Arias, P.H. Klesius and T.L. Welker, 2005. Technique for Identifying *Flavobacterium columnare* Using Whole-Cell Fatty Acid Profiles. Journal of Aquatic Animal Health, 17(3): 267-274.
7. Yeh, H.Y., C.A. Shoemaker and P.H. Klesius, 2006. Sensitive and rapid detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus* by a loop-mediated isothermal amplification method. Journal of Applied Microbiology, 100(5): 919-925.
8. Darwish, A.M., A.A. Ismaiel, J.C. Newton and J. Tang, 2004. Identification of *Flavobacterium columnare* by a species-specific polymerase chain reaction and renaming of ATCC43622 strain to *Flavobacterium johnsoniae*. Molecular and Cellular Probes, 18(6): 421-427.
9. Dobson, S.J., R.R. Colwell, T.A. McMeekin and P.D. Franzmann, 1993. Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of *Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antarctic lake. International Journal of Systematic Bacteriology, 43(1): 77-83.
10. Flemming, L., D. Rawlings and H. Chenia, 2007. Phenotypic and molecular characterisation of fish-borne *Flavobacterium johnsoniae*-like isolates from aquaculture systems in South Africa. Research in Microbiology, 158(1): 18-30.
11. Welker, T.L., C.A. Shoemaker, C.R. Arias and P.H. Klesius, 2005. Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*. Diseases of Aquatic Organisms, 63(2-3): 129-138.
12. Bader, J.A., C.A. Shoemaker and P.H. Klesius, 2003. Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. Journal of Microbiological Methods, 52(2): 209-220.
13. Stoskopf, M.K., 1993. Fish medicine. Philadelphia: W.B. Saunders Co.
14. Anacker, R.L. and E.J. Ordal, 1959. Studies on the myxobacterium *Chondrococcus columnaris*. I. Serological typing. Journal of Bacteriology, 78(1): 25-32.
15. Austin, B. and D.A. Austin, 2007. Bacterial fish pathogens disease of farmed and wild fish. Dordrecht; Chichester: Springer; Published in association with Praxis Pub.
16. McDaniel, D., 1979. Procedures for the detection and identification of certain fish pathogens. [Bethesda, Md.]: American Fisheries Society, Fish Health Section.

17. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22): 4673-4680.
18. Bader, J.A. and E.B. Shotts, 1998. Determination of phylogenetic relationships of *Flavobacterium psychrophilum* (*Flexibacter psychrophilus*), *Flavobacterium columnare* (*Flexibacter columnaris*) and *Flexibacter maritimus* by sequence analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction. *Journal of Aquatic Animal Health*, 10(4): 320-327.
19. Bosshard, P.P., R. Zbinden, S. Abels, B. Boddingtonhaus, M. Altwegg and E.C. Bottger, 2006. 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting Gram-negative bacteria in the clinical laboratory. *Journal of Clinical Microbiology*, 44(4): 1359-1366.
20. Darwish, A.M. and A.A. Ismaiel, 2005. Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S-23S rDNA spacer. *Molecular and Cellular Probes*, 19(4): 267-274.
21. Gherna, R. and C.R. Woese, 1992. A partial phylogenetic analysis of the "flavobacter-bacteroides" phylum: basis for taxonomic restructuring. *Systematic and Applied Microbiology*, 15(4): 513-521.
22. Triyanto, A. and H. Wakabayashi, 1999. Genotypic Diversity of Strains of *Flavobacterium columnare* from diseased Fishes. *Fish Pathology*, 34(2): 65-71.
23. Aakra, A., J.B. Utaker and I.F. Nes, 1999. RFLP of rRNA genes and sequencing of the 16S-23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. *International Journal of Systematic Bacteriology*, 49(1): 123-130.