Fish Health Section

FHS NEWS – January 2022

Fish Health Section website: https://units.fisheries.org/fhs/

Fish Health Section Facebook Site: https://facebook.com/FishHealthSectionAFS

Fish Health Section Twitter feed: @AFSFishHealth

Don't forget to renew your AFS and FHS membership at https://fisheries.org/. Thank you to the members who have already renewed. Let's all try to encourage our friends to join so that 2022 can be the best year yet!

People of the Fish Health Section: Commemorating 50 years

Dear Fish Health Section members,

> Last month I asked for nominations from the membership for student members to highlight in this month's feature. I am pleased to feature one each for the east and west coast. I seek additional nominations for more students to highlight in next month's newletter.
>
> Submitted by Gary Marty, FHS President (Gary.Marty@gov.bc.ca)

Hello fish health professionals, my name is **Ben Americus**. I'm a grad student at Oregon State University in Corvallis, Oregon, working on salmon parasite research with **Stephen Atkinson** and **Jerri Bartholomew**. I also edit the Fish Health Section Website. I joined the section in 2017 to attend the Western Fish Disease Conference in Bozeman, Montana, my first science conference. A highlight of my work in fish health was collaborating with **George Schisler** of Colorado Parks and Wildlife. I got a chance to tour his lab at Parvin Lake, CO in 2018. We co-authored a paper on *Myxobolus cerebralis*, the parasite behind whirling disease; the parasite causes unusually quick mortality in juvenile Mountain Whitefish. This was a great opportunity to learn the history of whirling disease in the Western U.S., a success story for the field of fish health. In the next year, I'm looking forward to working on parasite monitoring on the Klamath River leading up to dam removal in 2023. Hopefully Klamath dam removal can be another fish health success story. After grad school, I want to work for a government agency on the West coast. Ideally this would be using 'omics to decipher fish health mysteries.

Hi everyone, my name is **Jake Veilleux**. I'm a new FHS member and 4th year veterinary student at NC State University. At my college, I've learned much about the fish world under the mentorship of

Dr. **Craig Harms** and Dr. **Gregory Lewbart**. At the recommendation of Dr. **Nora Hickey**, I joined the section this year for all the great learning and networking opportunities it provides. I have taken part in numerous fish health courses, externships, and jobs over the last several years, and hope to use those experiences to make a career in the field. Some of my favorite cases happened while externing at an aquarium in North Carolina. We were conducting annual exams on young Atlantic and yellow stingrays. After anesthetizing in MS-222, I examined and did ultrasounds on several rays, collected blood from the radial wing vessels, clipped barbs to a safe size, and read skin scrapes. Other than recommending an increase in food offered, each fish appeared to be in excellent health. Though there were no screaming health issues, routine health checks are an important part of preventative medicine. In addition to aquarium medicine, I also have a strong passion for food fish production and fisheries conservation.

Policy/Position Development Committee Update

How to request the FHS to develop a policy or position

The AFS-FHS Policy/Position Development Committee (PPDC) provides a mechanism for generating official policy/position statements by the AFS-FHS. Any member can bring a proposed issue, policy, or position statement forward to the PPDC for review. See the May 2020 FHS Newsletter for details (available on the FHS website). If you have an idea for a policy or position, don't hesitate to contact PPDC Chair Anita Kelly (amk0105@auburn.edu) or any of the Committee members listed on the FHS website.

FHS Member Contributions

New Publication: The fish pathogen *Flavobacterium columnare* represents four distinct species: *Flavobacterium columnare*, *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov. and *Flavobacterium oreochromis* sp. nov., and emended description of *Flavobacterium columnare* in Systematic and Applied Microbiology 45 (2022)

Authors: Benjamin R. LaFrentz, Stanislava Králová, Claire R. Burbick, Trevor L. Alexander, Conner W. Phillips, Matt J. Griffin, Geoffrey C. Waldbieser, Julio C. García, Fernanda de Alexandre Sebastião, Esteban Soto, Thomas P. Loch, Mark R. Liles, Kevin R. Snekvik

See attached .pdf for entire paper.

a b s t r a c t

Flavobacterium columnare is the causative agent of columnaris disease in freshwater fish and four discrete genetic groups exist within the species, suggesting that the species designation requires revision. The present study determined the taxonomic status of the four genetic groups of F. columnare using polyphasic and phylogenomic approaches and included five representative isolates from each genetic group (including type strain ATCC 23463T; genetic group 1). 16S rRNA gene sequence analysis revealed genetic group 2 isolate AL-02-36T, genetic group 3 isolate 90-106T, and genetic group 4 isolate Costa Rica 04-02-TNT shared less than <98.8 % sequence identity to F. columnare ATCC 23463T. Phylogenetic analyses of 16S rRNA and gyrB genes using different methodologies demonstrated the four genetic groups formed wellsupported and distinct clades within the genus Flavobacterium. The average nucleotide identity (ANI) and digital DNA-DNA hybridization (GGDC) values between F. columnare ATCC 23463T, genetic group 2 isolate AL-02-36T, genetic group 3 isolate 90-106T, and genetic group 4 isolate Costa Rica 04-02-TNT were less than 90.84% and 42.7%, respectively. Biochemical and physiological characteristics were similar among

the four genetic groups; however, quantitative differences in fatty acid profiles were detected and MALDI-TOF analyses demonstrated numerous distinguishing peaks unique to each genetic group. Chemotaxonomic, MALDI-TOF characterization and ANI/GGDC calculations afforded differentiation between the genetic groups, indicating each group is a discrete species. Herein, the names F. covae sp. nov. (AL-02-36T), F. davisii sp. nov. (90-106T), and F. oreochromis sp. nov. (Costa Rica 04-02-TNT) are proposed to represent genetic groups 2, 3, and 4, respectively.

Call for New Content

Do you have a puzzling diagnostic case? Or do you have a new diagnostic finding that might interest others? How about newly published research? I am starting a new feature to highlight Fish Health Section Members' current conundrums and accomplishments. Think about what you could submit and contact me at stacy.a.strickland@odfw.oregon.gov with your ideas. Limit to be around 1 page including graphs/pictures. Especially during these virtual meeting and conference times, let's share our work and keep our peers up-to-date on what's happening in the world of fish health!

Thanks in advance for your submissions!

Stacy Strickland, AFS-FHS Newsletter Editor

MEETINGS, WORKSHOPS AND COURSES

61st AFS FHS Western Fish Disease Workshop
May 16-18, 2022, with a day of CE on May 19
Hood River, Oregon
Best Western Hood River Inn and Conference Center

Save the date! Come to Oregon!

Registration and call for abstracts will come late winter. Keep an eye out on the website for more information. https://units.fisheries.org/fhs/wfdw/

9th International Symposium on Aquatic Animal Health (ISAAH)
Santiago, Chile
September 4-8, 2022

RE: Call for Special Sessions

The 9th International Symposium on Aquatic Animal Health (9th ISAAH) will be held September 4 – 8, 2022, in Santiago, Chile. The ISAAH is a truly unique event, held every four-years, and hosted in conjunction with the Fish Health Section of the American Fisheries Society. The ISAAH typically attracts 300–400 aquatic animal health professionals from around the world covering all manners of discipline and scientific inquiry. This is an exciting event you won't want to miss!

As a global forum for interdisciplinary collaboration and communication, ISAAH aspires to create an environment of fellowship, to learn and share the latest groundbreaking research, with a vision of building a better future for aquatic animal health professionals. The 9th ISAAH will bring together scientists and aquatic animal health specialists from across the globe, to open new avenues of research and help foster international collaborations. This will also be the first time ISAAH is held outside of North America!

The organizing committee is still taking suggestions for special sessions. If you have a topic for consideration or would like to organize a session of particular interest, please contact Matt Griffin (matt.griffin@msstate.edu) with the subject heading "ISAAH 2022 Special Sessions".

The following topics have already been submitted for consideration:

WAVMA/AAFV (Continuing Education Opportunity)
Zebrafish/Lab Animal Models
Genomic Applications in Fish Health
Immune modulation in Fish Health Management
Microbiomes: Applications in Fish Health
Polymicrobial Infections
Parasite Life Cycles
Applications of Modeling in Aquatic Animal Health
Emerging Diseases
Myxozoa
Climate Change: Impacts on Fish Health

We look forward to hearing from you and hope to see you in Santiago!

The Organizing Committee
Fernando Mardones, School of Veterinary Medicine, Pontifical Catholic University, Chile
Marilia Salgado Caxito, School of Veterinary Medicine, São Paulo State University, Brazil
Natalia Zimin-Veselkoff, School of Veterinary Medicine, Pontifical Catholic University, Chile
Matt Griffin, College of Veterinary Medicine, Mississippi State University, USA.
Esteban Soto, School of Veterinary Medicine, UC Davis, USA

Interdisciplinary PhD Programme in Veterinary Medicine

https://www.cityu.edu.hk/jcc/education/postgraduate-programmes/interdisciplinary-phd-programme-veterinary-medicine

City University of Hong Kong (CityU) is a dynamic, fast-growing university that is pursuing excellence in research and professional education. As a publicly-funded institution, the University is committed to nurturing and developing students' talents and creating applicable knowledge to support social and economic advancement. Currently, the University offers postgraduate research programmes in various disciplines, including business, creative media, energy, engineering, environment, humanities, law, science, social sciences, and other strategic growth areas, including veterinary medicine.

The Jockey Club College of Veterinary Medicine and Life Sciences (JCC) was launched in spring 2014 in collaboration with Cornell University's College of Veterinary Medicine. The JCC is the first of its kind in Hong Kong and is envisioned as a centre of excellence in animal health education, research and discovery, and clinical care in China and the Asia-Pacific region. A key part of CityU's Life Sciences Initiative, the college offers a postgraduate research programme leading to interdisciplinary PhD degrees in veterinary medicine.

This is an interdisciplinary programme open to outstanding graduate students who wish to conduct state-of-the-art basic, clinical and translational life sciences research alongside research professionals. The programme is student-centred and led by faculty who are accessible, engaged and committed to ensuring that our postgraduate students reach their full potential in research, teaching and professional development. The students will participate in research programmes leading to PhD degrees in one of the following areas:

- Comparative Biomedical Sciences
- Immunology and Infectious Disease
- Molecular and Systemic Neuroscience
- Public Health and Epidemiology

JOBS/GRADUATE ASSISTANTSHIPS

Professional Services Veterinarian, Aquaculture (Remote)

Merck Animal Health
Boise, ID
Link: https://jobs.merck.com/us/en/job/R159465/Professional-Services-Veterinarian-Aquaculture-Remote

The Professional Services Veterinarian role is field based. The position is designed to support the technical needs of the sales regions and the customers within those regions. The individual will interact with our customer base as well as university, state, and federal aquaculturists, fish health professionals, veterinarians, & researchers in both Canada and the U.S. on a technical level. The successful candidate will analyze data, give presentations at various local and national venues, and train sales representatives on product use and the use of technical data during sales calls. The Professional Services Veterinarian will also coach Sales Representatives on technical selling, partnering with them to promote and sell Biomark, AGO, and Vaki brands, products and services. This role will also assist in the preparation of presentations and serve in a technical capacity on marketing brand teams.

Paid Internship with US Fish and Wildlife Service

The US Fish and Wildlife Service is partnering with MANRRS to provide students at (1) Oregon State University, (2) University of Idaho, and (3) Washington State University with an opportunity to step into a career with our agency!

The MANRRS internship program is a **diversity-seeking, program** for interested students **from diverse backgrounds, including women and historically under-represented populations such as Black, Hispanic/Latino, Asian and Pacific Islander, and Native American.**

This program is a pipeline into the U.S. Fish and Wildlife Service. Students joining this internship program may have the ability to be directly placed into a permanent job with the agency following their graduation. All majors are encouraged to apply!

About the summer internship

-Weekly stipend of $540. Housing is provided, and rent is reimbursed up to $1500/month at sites where no housing is available.

-Projects are available in the Pacific Northwest and Pacific Islands (Hawaii).

-Conservation projects range from biological field work to environmental policy, and community outreach.

See the list of available projects here:

https://drive.google.com/file/d/1eNoXJ6QJtlhMwhkaUAk10X-NY6z6W7uK/view?usp=sharing

To be eligible, students must:

(1) Be an undergraduate -**AND-** not graduate before December 2023.

(2) Be available for 12 weeks in Summer 2022

(3) Join your campus MANRRS chapter. Learn more about MANRRS here --> MANRRS.org

(4) Be a US Citizen, 18 years or older

To apply:

Eligible students should email their campus MANRRS advisors (contact info below) to be referred to the program hiring managers. Students will be evaluated for positions on a rolling basis, so you are encouraged to act on this opportunity ASAP.

In your email, please indicate (1) your major, (2) expected graduation date, and (3) your first, second and third project choice

Oregon State University students should contact Wanda Crannell at Wanda.Crannell@oregonstate.edu

University of Idaho students should contact Chloe Wardropper at cwardropper@uidaho.edu

Washington State University students should contact Colette Casavant at colette.casavant@wsu.edu

For more information:

If you have questions regarding the MANRRS internship, U.S. Fish and Wildlife Service or how this internships may lead to a permanent position, please contact Nicole Hams (nicole_hams@fws.gov) or Chelsea McKinney (chelsea_mckinney@fws.gov).

PhD Programs in Aquatic Animal Health
City University
Hong Kong

City-University in Hong Kong want to fill Two PhD openings for the 2022 Fall semester. One of these positions is an interdisciplinary PhD program with collaboration in Cornell Veterinary College, USA, where the candidate will spend three years in CityU in Hong Kong and one year in Cornell. https://www.cityu.edu.hk/jcc/education/postgraduate-programmes/interdisciplinary-phd-programme-veterinary-medicine

Interested students should contact Dr Wenlong (Colin) Cai, Assistant Professor of Aquatic Animal Health, Department of Infectious Diseases and Public Health , Jockey Club College of Veterinary Medicine and Life Sciences, 31 To Yuen Street, City University of Hong Kong, Kowloon, Hong Kong https://www.cityu.edu.hk/ph/staff/dr-cai-wenlong

Assistant Professor of Fish Pathology or Immunology
Department of Rangeland, Wildlife and Fisheries Management College of Agriculture and Life Sciences
Texas A&M University
College Station, TX
Link: https://apply.interfolio.com/96875

GENERAL DUTIES AND RESPONSIBILITIES: The successful candidate will be expected to enhance a faculty addressing various aspects of aquaculture and fisheries management in the Department of Rangeland, Wildlife and Fisheries Management (RWFM). They also will be expected to collaborate with and enhance existing faculty with expertise in the areas of fish nutrition, physiology, and fisheries management as it pertains to fish health and diseases. This position will be an integral component of the Department's research and teaching programs in aquaculture and fisheries management. Included duties will be the development and leadership of an independent, extramurally-funded, internationally recognized applied research program with a focus on warm water finfish that directly addresses disease management or diagnostic issues facing aquaculture producers and private pond owners within Texas and the U.S. The appointee will be expected to teach courses

related to their expertise in the recently established undergraduate curriculum. Courses (or a portion of the course) may include Fish Health and Diseases, Principles and Practices of Wildlife/Fisheries Management, and a summer Aquatic Field Experience course. Graduate courses in the candidate's area of expertise that may include Fish Immunology, Pathology, or Health and Diseases. RWFM is an applied management focused department and as such the successful candidate is expected to work with aquaculture producers, private fisheries owners, pond management companies, as well as support state and federal agencies such as the Texas Parks and Wildlife Department.

See attached .pdf for more information.

Zebrafish Related Job Announcements
https://wiki.zfin.org/display/jobs/Zebrafish-Related+Job+Announcements

RESOURCES/NEWS

Call for Papers
Guest editors for microorganisms propose a special issue on "Microbiomes of Aquatic Organisms". See attached .pdf for details.

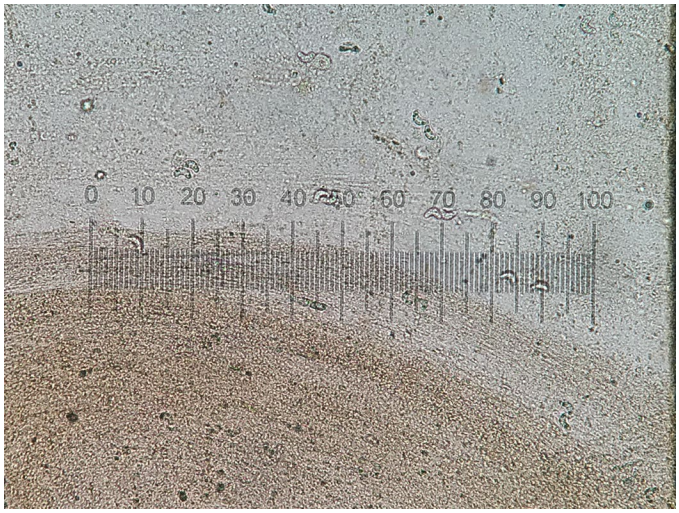
Publication of articles from the 3rd Sea Lamprey International Symposium (SLIS III)
The full supplemental volume from SLIS III has been fully published in the Journal of Great Lakes Research 47[Suppl.1]:S1-S814. The final articles are now available on-line at:
https://www.sciencedirect.com/journal/journal-of-great-lakes-research/vol/47/suppl/S1

All of the articles are open access and free to download. Please circulate this link to interested individuals, including co-authors and colleagues who may not have been able to attend SLIS III.

Aquatic Animal Drug Approval Partnership (AADAP) Updates are now available online:
https://www.fws.gov/fisheries/AADAP/aadap_update.html

AFS Job Board changes
Check out the new AFS Career Center with new and improved features for both job seekers and employers. Job hunters now benefit from improved search functions and email alerts. While employers can peruse candidate applications and submit jobs more quickly and easily through an online submission form with a credit card payment system. Individual AFS members can still advertise for assistants and internship positions at no charge. See the AFS member employer pricing options.

EDITOR'S RANDOM PICS



Myxospores of *Ceratonova shasta* from the lower intestine of a juvenile summer steelhead, 200x objective, Lake Billy Chinook, OR, 2017.

TEXAS A&M AGRILIFE RESEARCH | EXTENSION

TEXAS A&M UNIVERSITY
AGRICULTURE & LIFE SCIENCES
DEPARTMENT OF RANGELAND, WILDLIFE, AND FISHERIES MANAGEMENT

Assistant Professor of Fish Pathology or Immunology
Department of Rangeland, Wildlife and Fisheries Management
College of Agriculture and Life Sciences
Texas A&M University

POSITION: The Department of Rangeland, Wildlife, and Fisheries Management, in the College of Agriculture and Life Science at Texas A&M University in College Station, Texas invites applications for an Assistant Professor position with an emphasis in fish pathology or immunology. This is a 9-month tenure-track academic appointment with (60%) research, (30%) teaching, (10%) services with an anticipated start date of no earlier than 1 August 2022.

GENERAL DUTIES AND RESPONSIBILITIES: The successful candidate will be expected to enhance a faculty addressing various aspects of aquaculture and fisheries management in the Department of Rangeland, Wildlife and Fisheries Management (RWFM). They also will be expected to collaborate with and enhance existing faculty with expertise in the areas of fish nutrition, physiology, and fisheries management as it pertains to fish health and diseases. This position will be an integral component of the Department's research and teaching programs in aquaculture and fisheries management. Included duties will be the development and leadership of an independent, extramurally-funded, internationally recognized applied research program with a focus on warm water finfish that directly addresses disease management or diagnostic issues facing aquaculture producers and private pond owners within Texas and the U.S. The appointee will be expected to teach courses related to their expertise in the recently established undergraduate curriculum. Courses (or a portion of the course) may include Fish Health and Diseases, Principles and Practices of Wildlife/Fisheries Management, and a summer Aquatic Field Experience course. Graduate courses in the candidate's area of expertise that may include Fish Immunology, Pathology, or Health and Diseases. RWFM is an applied management focused department and as such the successful candidate is expected to work with aquaculture producers, private fisheries owners, pond management companies, as well as support state and federal agencies such as the Texas Parks and Wildlife Department.

REQUIREMENTS: Ph.D. in Aquaculture, Fisheries Science, Veterinary Medicine, or related field is required. A Doctorate of Veterinary Medicine (DVM) with aquatic animal health coursework or experience preferred, but not required. Postdoctoral or clinical veterinary experience is preferred. Evidence of outstanding research and publishing capacities is required, along with the potential to establish and maintain strong teaching and graduate education programs.

RESOURCES: Texas A&M University (TAMU) is a public, land-grant institution with many high-quality academic units conducting research in areas of natural resource conservation and management. The successful candidate will be offered a competitive salary, startup package and laboratory space and/or field equipment, as well as access to research facilities at the Aquacultural Research and Teaching Facility (ARTF) associated with the Department of Rangeland, Wildlife and Fisheries Management, Texas A&M AgriLife Research, and the College of Agriculture and Life Sciences. The candidate will have opportunities to collaborate with a broad range of TAMU System researchers around the state including the Aquatic Diagnostics Laboratory within RWFM, the Texas A&M Veterinary Medical Diagnostic Laboratory, and the Texas A&M College of Veterinary Medicine and Biomedical Sciences.

APPLICATION PROCESS: Applications will only be accepted through *Interfolio* **apply.interfolio.com/96875**. Applicants must submit: (1) a cover letter of interest, (2) detailed curriculum vitae, (3) a two-page statement of teaching and research plans, and 4) contact information for three professional references who can provide a critical evaluation of the applicant's qualifications for the position. Review of applications will begin January 1, 2022, and continue until the position is filled.

For questions, email inquiries to the attention of Dr. Del Gatlin, Search Committee Chair, Department of Ecology and Conservation Biology, Texas A&M University, College Station, TX 77843, via Ms. Theresa Nemec e-mail: tnemec@tamu.edu.

Texas A&M University is committed to enriching the learning and working environment for all visitors, students, faculty, and staff by promoting a culture that embraces inclusion, diversity, equity, and accountability. Diverse perspectives, talents, and identities are vital to accomplishing our **mission** and living our **core values**.

Texas A&M University is an Equal Opportunity/Affirmative Action/Veterans/Disability Employer committed to diversity. Texas A&M University is aware that attracting and retaining exceptional faculty often depends on meeting the needs of two careers and having policies that contribute to work-life balance. For more information, visit https://employees.tamu.edu/ocrm/eeo or http://dof.tamu.edu/Faculty-Resources/Faculty-WorkLife

microorganisms

IMPACT FACTOR 4.128

Covered in: PubMed

an Open Access Journal by MDPI

Microbiomes of Aquatic Organisms

Guest Editors:

Prof. Dr. Terence L. Marsh
Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA

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Laboratoire Microorganismes : Génome Environnement, Université Blaise Pascal, BP 8002, 6, 63171 Aubière Cedex, France

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Deadline for manuscript submissions:
31 May 2022

Message from the Guest Editors

Dear Colleagues,

To better understand the potentially disastrous effects of the environmental factors on aquatic ecosystems, we propose a Special Issue "Microbiomes of Aquatic Organisms" focusing on the microbiome of aquatic animals and plants, including:

- The phylogenetic structure and function of the microbiomes of aquatic animals and plants in health and disease;
- The assembly of these microbiomes;
- The identification and characterization of pathogenic and probiotic strains and consortia;
- Mechanisms of attachment of the microbiome;
- Approaches that would enhance survival and recruitment of aquatic species through greater understanding of microbial communities in aquatic ecosystems.

Prof. Dr. Terence L. Marsh
Dr. Télesphore Sime-Ngando
Guest Editors

microorganisms

IMPACT FACTOR 4.128

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an Open Access Journal by MDPI

Editor-in-Chief

Prof. Dr. Martin Von Bergen
Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research—UFZ, Permoserstr. 15, 04318 Leipzig, Germany

Message from the Editor-in-Chief

"Microorganism" merges the idea of the very small with the idea of the evolving reproducing organism is a unifying principle for the discipline of microbiology. Our journal recognizes the broadly diverse yet connected nature of microorganisms and provides an advanced publishing forum for original articles from scientists involved in high-quality basic and applied research on any prokaryotic or eukaryotic microorganism, and for research on the ecology, genomics and evolution of microbial communities as well as that exploring cultured microorganisms in the laboratory.

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Journal Rank: JCR - Q2 (*Microbiology*)

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The fish pathogen *Flavobacterium columnare* represents four distinct species: *Flavobacterium columnare*, *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov. and *Flavobacterium oreochromis* sp. nov., and emended description of *Flavobacterium columnare*

Benjamin R. LaFrentz [a,*,1], Stanislava Králová [b,c,1], Claire R. Burbick [d,e], Trevor L. Alexander [d], Conner W. Phillips [d], Matt J. Griffin [f], Geoffrey C. Waldbieser [g], Julio C. García [a], Fernanda de Alexandre Sebastião [h], Esteban Soto [h], Thomas P. Loch [i], Mark R. Liles [c], Kevin R. Snekvik [d,e]

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ABSTRACT

Flavobacterium columnare is the causative agent of columnaris disease in freshwater fish and four discrete genetic groups exist within the species, suggesting that the species designation requires revision. The present study determined the taxonomic status of the four genetic groups of *F. columnare* using polyphasic and phylogenomic approaches and included five representative isolates from each genetic group (including type strain ATCC 23463^T; genetic group 1). 16S rRNA gene sequence analysis revealed genetic group 2 isolate AL-02-36^T, genetic group 3 isolate 90-106^T, and genetic group 4 isolate Costa Rica 04-02-TN^T shared less than <98.8 % sequence identity to *F. columnare* ATCC 23463^T. Phylogenetic analyses of 16S rRNA and *gyrB* genes using different methodologies demonstrated the four genetic groups formed well-supported and distinct clades within the genus *Flavobacterium*. The average nucleotide identity (ANI) and digital DNA-DNA hybridization (GGDC) values between *F. columnare* ATCC 23463^T, genetic group 2 isolate AL-02-36^T, genetic group 3 isolate 90-106^T, and genetic group 4 isolate Costa Rica 04-02-TN^T were less than 90.84% and 42.7%, respectively. Biochemical and physiological characteristics were similar among the four genetic groups; however, quantitative differences in fatty acid profiles were detected and MALDI-TOF analyses demonstrated numerous distinguishing peaks unique to each genetic group. Chemotaxonomic, MALDI-TOF characterization and ANI/GGDC calculations afforded differentiation between the genetic groups, indicating each group is a discrete species. Herein, the names *F. covae* sp. nov. (AL-02-36^T), *F. davisii* sp. nov. (90-106^T), and *F. oreochromis* sp. nov. (Costa Rica 04-02-TN^T) are proposed to represent genetic groups 2, 3, and 4, respectively.

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Introduction

The genus *Flavobacterium* consists of species typically inhabiting fresh [1,10,11], glacial [84] and sea waters [51,77], sediments [44,59], compost and soils [9,30,76] or polar regions [33,34]. Many *Flavobacterium* spp. also colonize unique environments, such as

Abbreviations: RFLP, restriction fragment length polymorphism; ANI, average nucleotide identity; GGDC, genome-to-genome distance calculation; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; FAME, fatty acid methyl ester.

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[1] These authors contributed equally to this work.

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plant rhizospheres [82,83] and Antarctic microbial mats [72,73]. Since the establishment of the genus in the early 1920 s [3], some members of *Flavobacterium* have had significant importance in wild and captive fish populations, as several flavobacterial species have been confirmed as important fish pathogens.

Flavobacterial diseases are a serious threat to fish farming and fish stocks worldwide. Numerous *Flavobacterium* spp. have been found in association with fish and implicated and/or confirmed as disease causing agents, including *F. johnsoniae* [64], *F. hydatis* [66], *F. succinicans* [24], *F. spartansi* [45], *F. inkyongense* [16], *F. chilense* [28], *F. araucananum* [28], *F. oncorhynchi* [79], *F. plurextorum* [78], *F. tructae* [81], *F. piscis* [81], *F. collinsii* [80], *F. branchiarum* [80], and *F. branchiicola* [80]. However, the three species that are most recognized as freshwater fish pathogens and thus have been most extensively studied are *F. branchiophilum* (bacterial gill disease), *F. psychrophilum* (bacterial cold water disease, rainbow trout fry syndrome), and *F. columnare* (columnaris disease) [46].

Flavobacterium columnare is a devastating fish pathogen and the causative agent of columnaris disease in wild and cultured fish populations worldwide [18,20,49,62]. In aquaculture, high mortality rates are common and result in significant economic losses [57,74] due to fish mortality, reduced feeding activity during epizootics, and increased treatment expenditures. Columnaris disease was first described in 1917 by Herbert Spencer Davis, who proposed the name *Bacillus columnaris* given the tendency for the bacteria to form column-like masses upon examination of material scraped from lesions of infected fish [15]. Although Davis was unable to culture the bacterium, he performed a full characterization of the disease and the agent, including reports of the occurrence and cause of columnaris disease, pathogenesis, method of infection, as well as treatment and control measures [15]. Twenty-two years later the bacterium was successfully isolated *in vitro* and renamed *Chondrococcus columnaris* [53]. Subsequently the bacterium was reclassified as *Cytophaga columnaris* [22], *Flexibacter columnaris* [5], and finally, *Flavobacterium columnare* [7].

A large degree of genetic diversity among isolates of *F. columnare* has been known since the late 1980 s. Song et al. [65] examined the phenotypic, biochemical, and DNA relatedness of a panel of 26 isolates collected from various species of fish with columnaris disease. The results indicated the phenotypic and biochemical characteristics of the isolates were homogenous, apart from a few differences in colony morphology. At the DNA level, most of the isolates shared > 70 % homology, while two of the isolates exhibited low DNA homology (<29 %), which suggests a lack of conspecificity [65]. As a result, the authors indicated these two isolates may represent new species of bacteria. Triyanto & Wakabayashi [71] developed a restriction fragment length polymorphism (RFLP) assay using partial amplicons of the 16S rRNA gene of *F. columnare*. Their subsequent analysis delineated their isolates into three groups. The groups corresponded with phylogenetic analysis of 16S rRNA gene sequences and DNA hybridization results, indicating the groups represented three different species. However, apart from some differences in nitrate reduction and growth at different temperatures, the isolates were phenotypically and biochemically homogenous [71]. Therefore, they proposed to divide the isolates into three genomovars (I, II, and III) until a larger collection of isolates could be investigated.

The 16S-RFLP technique became the standard for typing *F. columnare* isolates until recently, when LaFrentz et al. [41] performed a thorough multilocus phylogenetic analysis (MLPA) of fifty *F. columnare* isolates. The results demonstrated four phylogenetically distinct genetic groups, similar to the results of Kayansamruaj et al. [29]. LaFrentz et al. [41] also demonstrated 16S-RFLP does not accurately reflect this genetic diversity and proposed that isolates be assigned to genetic group rather than genomovar. DNA hybridization comparisons further support the previous suggestions that multiple species of bacteria exist within *F. columnare* [65,71] that correspond to the four genetic groups (1, 2, 3, and 4). Large-scale surveys of prokaryotic genomes have deemed ANI values of 95 % as the standard for species demarcation, with values >95 % representing intraspecific variability, and values <94 % representing discrete species [27]. Kumru et al. [37] reported an average nucleotide identity (ANI) value of 90.71 % following the comparison of a genetic group 1 and 2 genome. Criscuolo et al. [14] reported ANI values between a genetic group 3 isolate and representative isolates of genetic groups 1 and 2 vary from 85.09 % to 85.6 %. Kayansamruaj et al. [29] reported digital DNA-DNA hybridization values of less than 44 % when comparing genomes of genetic groups 1, 2, 3, and 4, and suggested the species designation of *F. columnare* may need revision.

There are biological implications of this genetic diversity. An association between genetic group 1 isolates and salmonids has been documented [41] and laboratory virulence experiments confirmed a higher virulence of this genetic group in rainbow trout, *Oncorhynchus mykiss* [19]. Genetic group 3 isolates have recently been recovered from columnaris cases in rainbow and steelhead trout [17] and previous research demonstrated moderate virulence of this genetic group in rainbow trout [19]. Genetic group 2 isolates appear to be more virulent in channel catfish [63], and an association between genetic group 4 isolates and tilapia, *Oreochromis* spp., has been documented [41].

With a solid understanding of the genetic diversity of *F. columnare* and availability of numerous isolates, the present study was designed to determine the phylogeny of the four genetic groups of *F. columnare*. A polyphasic approach was taken to further confirm the phylogenetic relationships and compare phenotypic, biochemical, and chemotaxonomic properties of representative isolates. Isolates affiliated with genetic group 1 are maintained as *F. columnare*. The remaining genetic groups are proposed as new species in the genus *Flavobacterium*. Herein, the names *F. covae* sp. nov. (AL-02-36^T), *F. davisii* sp. nov. (90-106^T), and *F. oreochromis* sp. nov. (Costa Rica 04-02-TN^T) are proposed to represent genetic groups 2, 3, and 4, respectively.

Material and methods

Isolation, preservation and culture conditions

The present study includes a thorough characterization of 19 isolates previously identified as *F. columnare* and assigned to each of the four recognized genetic groups [41]. Temporally and geographically diverse isolates were included and were collected from a variety of fish species with columnaris disease (Table 1). In addition, the *F. columnare* type strain (ATCC 23463^T; genetic group 1) and seven type strains from the closest phylogenetic neighbours within the genus *Flavobacterium* were obtained from culture collections. These included *F. amniphilum* LMG 29727^T, *F. brevivitae* LMG 29004^T, *F. inkyongense* JCM 31385^T, *F. lacunae* LMG 28710^T, *F. terrae* LMG 28895^T, *F. verecundum* LMG 29005^T and *F. vireti* CCTCC AB2014312^T. The isolates were routinely cultured on Difco™ R2A agar (Becton, Dickinson and Company) or in modified Shieh broth [42] at 28 °C, and were cryopreserved in 20 % (v/v) glycerol at −80 °C.

For MALDI-TOF analyses, all isolates were stored in 50 % buffered glycerol and maintained at −80 °C until use. Isolates were cultured on tryptone yeast extract agar supplemented with 1 % milk (TYEM) (University of California-Davis Biological Media Services, Davis, California). All isolates were incubated aerobically at 25 °C for 24–48 h, depending on growth characteristics of the isolates, prior to preparation and analysis.

Table 1
Isolates, previously identified as *Flavobacterium columnare*, used in the present study, including the year and fish host of isolation, geographic origin, and genetic group.

Isolate	Year	Fish host	Origin	Genetic Group
ATCC 23463 ^{T}	Not known	Chinook salmon	Washington (USA)	1
Israel	Not known	Common carp	Israel	1
IA-S-4	2011	Walleye	Iowa (USA)	1
ALG-03-063	2003	Channel catfish	Alabama (USA)	1
CSF-298-10	2010	Rainbow trout	Idaho (USA)	1
AL-02-36	2002	Largemouth bass	Alabama (USA)	2
ALG-00-530	2000	Channel catfish	Alabama (USA)	2
C#2	Not known	Not known	Not known	2
PT-14-00-151	2000	Channel catfish	Mississippi (USA)	2
94-081	1994	Channel catfish	Mississippi (USA)	2
90-106	1990	Channel catfish	Mississippi (USA)	3
GA-02-14	2002	Rainbow trout	Georgia (USA)	3
ARS-1	1996	Channel catfish	Alabama (USA)	3
TI2063	2007	Tilapia	Africa	3
ARS-15-12	2015	Nile tilapia	Florida (USA)	3
Costa Rica 04-02-TN	2004	Tilapia	Costa Rica	4
BZ-1-02	2002	Nile tilapia	Brazil	4
TI1690	2005	Tilapia	Honduras	4
TI2056	2007	Tilapia	China	4
TI1371	2004	Tilapia	Indonesia	4

Phylogenetic analyses

Publicly available 16S rRNA and *gyrB* gene sequences for the panel isolates, *F. columnare* ATCC 23463^{T}, and 7 closely related *Flavobacterium* species were downloaded from the National Center for Biotechnology Information (NCBI) nucleotide and EzBioCloud database [31]. Isolates for which sequence data was not available were cultured as above and genomic DNA was extracted from cell pellets using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol for Gram-negative bacteria. Genomic DNA was quantified by Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) and partial 16S rRNA or *gyrB* gene targets were amplified and sequenced following previously established protocols [39,56]. Sequence similarity with other bacterial species was initially assessed using BLASTn searches of the non-redundant nucleotide database from NCBI. Newly obtained 16S rRNA and *gyrB* gene sequences were deposited into GenBank under accession numbers MW352986 - MW353014.

The 16S rRNA gene sequences from the 19 panel isolates, *F. columnare* ATCC 23463^{T}, 7 closely related *Flavobacterium* species, and other select *Flavobacterium* species retrieved from the NCBI and the EzBioCloud databases were aligned and trimmed using the Molecular Evolutionary Genetics Analysis (MEGA7) software [35]. The evolutionary relatedness based on 16S rRNA gene sequences was inferred by Neighbor-Joining (NJ) [60] and maximum likelihood (ML) methods using MEGA7. For the NJ method, evolutionary distances were computed according to the Tamura-Nei model [67]. For the ML method, the General Time Reversible model [GTR + G + I; 50] was determined as the best fit model based on Bayesian Information Criterion score in MEGA7. Initial trees for the heuristic search were obtained by applying the neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach. For both methods, all positions containing gaps and missing data were eliminated, leaving a total 1330 positions in the final dataset. The final trees were constructed from 1000 bootstrap replicates and were rooted with the reference sequence (U41350) from *Capnocytophaga ochracea* ATCC 27872^{TT}.

The *gyrB* gene sequences of the 19 isolates and 8 closely related *Flavobacterium* species were aligned, trimmed, and evolutionary relatedness was inferred by the ML method as described above using the GTR + G + I model [50]. There was a total of 1,189 positions in the final dataset, the final tree was constructed from 1,000 bootstrap replicates, and was rooted with *F. psychrophilum* JIP 02/86 and *F. johnsoniae* UW101^{T}.

Whole genome sequencing

Isolates AL-02-36^{T} (genetic group 2), 90-106^{T} (genetic group 3), and Costa Rica 04-02-TN^{T} (genetic group 4) were revived from cryopreservation on G media agar plates [21] and swabs of pure colonies were used to inoculate 9 mL of G media broth [21]. Cultures were expanded at 28 °C with gentle shaking (150 rpm). Aliquots (3 mL) of expanded culture were concentrated by centrifugation (20,000 × *g* for 5 min) in a Sorvall RC 6 Plus centrifuge (Thermo Fisher Scientific). High molecular weight (HWM) bacterial genomic DNA (gDNA) was isolated from stock pellets using Gentra Puregene DNA Isolation Kit (Qiagen Bioinformatics, Germantown, MD) following the manufacturer's suggested protocol for Gram-negative bacteria scaled up 3X. Precipitated gDNA was resuspended in 195 μL of EB Buffer (Qiagen) and further purified by the addition of 5 μL of PureLink™ RNAse A (Invitrogen), 50 μL of 5 N NaCl, and 75 μL of 100 % ethanol (ETOH). Samples were placed on a rotomixer (approximately 20 rpm) for 10 min. Impurities were pelleted for 20 min at 20,000 × *g* and the supernatant decanted into a clean 1.5 mL Lo-Bind Eppendorf tube®. To precipitate gDNA, 425 μL of 100 % ETOH was added, mixed by gentle inversion, incubated at −80 °C for 1 h and gDNA pelleted by centrifugation at 20,000 × *g* for 3 min. The supernatant was removed, and the pellet was washed with 300 μL of 70 % ETOH and subjected to centrifugation at 20,000 × *g* for 3 min. The supernatant was removed with a micropipette and the gDNA pellet air dried for 15 min. The precipitated gDNA was resuspended in 50 μL EB Buffer and template purity was evaluated spectrophotometrically (NanoDrop 2000) targeting a 260/280 ratio ranging from 1.8-2.0 and 260/230 ratios from 1.6-2.2.

Multiplexed genomic DNA libraries were produced from HMW gDNA using the rapid transposon based RBK004 kit and sequenced on v9.4.1 flow cells using a GridION instrument (Oxford Nanopore Technologies, Oxford, UK). Sequencing data was demultiplexed by MinKNOW software (currently GridION Release 19.12.6) and each genome was assembled using Canu v1.8 [32], with consensus sequence correction by Medaka v0.11.5 (Oxford Nanopore).

The assembled genome sequences were submitted to NCBI for annotation using the Prokaryotic Genome Annotation Pipeline (PGAP, version 4.12). Average nucleotide identities (ANI) and digital DNA-DNA hybridization values between *F. columnare* ATCC

23463^T (genetic group 1), AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-02-TN^T (genetic group 4), and *F. terrae* DSM18829^T, were calculated using the OrthoANI algorithm in the Orthologous Average Nucleotide Identity Tool (OAT) [43] and DMSZ GGDC2.1 [48], respectively. Lastly, G + C content (%) was estimated from genome sequences.

Morphology

Colony morphology was assessed on Difco™ R2A agar (Becton, Dickinson and Company). Motility was assessed using phase contrast microscopy and a "tunnel slide" as previously described [47]. The presence of flexirubin-type pigments was investigated using a 20 % (w/v) KOH solution (Hardy Diagnostics) and Congo red adsorption was tested [6]. Cellular morphology was described by light microscopy, Gram-staining and by transmission electron microscopy (TEM). For TEM, *F. columnare* ATCC 23463^T, AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-02-TN^T (genetic group 4) were grown for 17 h in modified Shieh broth. Following growth, 10 μL of culture was placed onto formvar/carbon coated copper grids (300 mesh; Electron Microscopy Sciences) for 5 min, wicked away with filter paper, negative stained with 2 % phosphotungstic acid, and cells were visualized with a Zeiss EM10 transmission electron microscope (Zeiss).

Temperature, NaCl and pH tolerance

Temperature range and NaCl tolerance of all panel isolates, including closely related *Flavobacterium* species, were determined on R2A agar plates. The pH tolerance was resolved on R2A agar plates where the pH values were adjusted with hydrochloric acid/-sodium hydroxide and were checked and confirmed after autoclaving. Inoculated plates were incubated for 48 h at 28 °C. Growth was tested at different temperatures (10, 12, 15, 30, 35, 36, 37, 38, 39 and 40 °C). Salt tolerance was assessed at 0.5, 0.75, 1.0 and 1.2 % of NaCl, and pH tolerance was assessed at pH 5 to 9.5 in intervals of 0.5 pH unit at 28 °C.

Biochemical and physiological characterization

All 19 panel isolates, the *F. columnare* type strain ATCC 23463^T and seven most related reference strains were characterized by a set of biochemical and physiological tests relevant for the genus *Flavobacterium* according to the minimal standards description protocols [6].

The following tests were performed: catalase activity was based on production of bubbles after the addition of a drop of 3 % (v/v) H_2O_2 (BD BBL™ Catalase Reagent Droppers; Becton, Dickinson and Company); oxidase presence was tested with BactiDrop™ OXIDASE (Remel) according to manufactureŕs instructions; oxidation-fermentation test [26]; production of urease [12] and with urea agar slants (Hardy Diagnostics) according to manufactureŕs instructions; arginine dihydrolase, ornithine and lysine decarboxylase production (Becton, Dickinson and Company); egg-yolk reaction [54]; utilization of citrate on Simmoń s citrate agar slants (Hardy Diagnostics); ONPG via rapid test broth cultivation (Hardy Diagnostics); production of H_2S on triple sugar iron (TSI) agar slants and sulfide, indole, motility (SIM) tubes (Hardy Diagnostics); utilization of sodium malonate in BBL™ malonate broth (Becton, Dickinson and Company); utilization of acetamide on acetamide agar [52]; reduction of nitrates and nitrites using a nitrate reduction test (Sigma-Aldrich); production of indole using SIM tubes and Kovac's reagent (Hardy Diagnostics); hydrolysis of DNA on the DNAse test agar with methyl green (HiMedia); hydrolysis of Tween 80 and gelatin [55]; hydrolysis of esculin and starch [2]; hydrolysis of casein and L-tyrosine [38]; cellulose (R2A broth with strip of Whatman paper No.1) [1]. Aerobic growth was tested on Difco™ R2A agar (Becton, Dickinson and Company), modified Shieh agar [42], Difco™ Nutrient Agar (Becton, Dickinson and Company), Difco™ Tryptic Soy Agar (Becton, Dickinson and Company) and Tryptic Soy Agar + 5% sheep blood (Remel), Difco™ MacConkey agar (Becton, Dickinson and Company), Endo agar (HiMedia), and Difco™ Marine agar (Becton, Dickinson and Company). Anaerobic growth was tested on R2A agar using an anaerobic jar and AnaeroPack®-Anaero system (Mitsubishi Gas Chemical Company, Inc.) and microaerophilic growth was performed on R2A agar using CampyGen Compact system (Oxoid) according to manufacturer's directions. All listed biochemical and physiological tests were inoculated using cells grown on R2A agar at 28 °C and read daily for up to 10 d, except for L-tyrosine hydrolysis test (read daily for up to 15 d). Enzymatic activity and carbohydrate fermentation were determined using API ZYM and API 50 CH strips (bioMérieux) according to the manufacturer's instructions, which enabled a comprehensive biochemical characterization of isolates.

Additional phenotypic tests were performed using the identification test kit GEN III MicroPlate™ (Biolog) with Protocol A. All 19 panel isolates and the *F. columnare* type strain ATCC 23463^T were grown in modified Shieh broth for 17 h at 28 °C with shaking (175 rpm), cells were pelleted by centrifugation for 10 min at 3,260 × *g*, and supernatant was removed. The cells were resuspended into inoculating fluid (IF-A) to a transmittance of 95%, then inoculated into the Gen III plates according to manufacturer's directions. Plates were incubated for 7 d at 28 °C and read using a Biolog MicroStation and Biolog's Microbial Identification Systems software.

Antimicrobial susceptibility

The 19 panel isolates representing genetic groups 1–4 (Table 1), *F. columnare* ATCC 23463^T (genetic group 1) and seven reference strains were tested for antibiotic susceptibility. Bacterial isolates were plated onto modified Shieh agar, incubated at 25 °C for 48 h, then one colony was transferred to 5 mL of modified Shieh broth and incubated for another 24 – 48 h (O.D. ∼ 1), at 25 °C, 150 rpm. Sensititre Avian 1F plates (Thermo Scientific) were used to investigate antimicrobial susceptibility using the broth microdilution method to determine minimal inhibitory concentrations (MIC). Cation-adjusted Mueller-Hinton II broth (CAMHB; Difco) at a concentration of 4 g L^{-1} in sterile double distilled water was used following publish protocols [23]. Bacteria were diluted 1:1000 in the diluted CAMHB and then 50 μL was pipetted in each well of the Avian plate. Plates were incubated at 25 °C for 24–72 h prior to MIC determination.

Chemotaxonomic analyses

All 19 panel isolates, the *F. columnare* type strain ATCC 23463^T and seven most related reference strains were cultivated on R2A agar for 24 h at 28 °C and 35 mg of wet biomass (in duplicate) was collected from plates for the analysis of fatty acid methyl esters (FAME). Saponification of bacteria, methylation, FAME extraction, and gas chromatography (Agilent Technologies 6850 network gas chromatography system; Agilent Technologies, Inc.) were performed as previously described [58]. Chromatography results were analyzed using the Sherlock Microbial Identification System RCLIN6 6.2 library (version 6.2; MIDI, Inc.). The duplicate FAME results were averaged for each isolate.

For additional chemotaxonomic analysis, freeze-dried biomass (∼0.5 g) was prepared from bacterial cells grown in modified Shieh broth cultivated at 28 °C for 17 h with shaking at 80 rpm. The polar lipids and respiratory quinones of *F. columnare* ATCC 23463^T, AL-02-36^T (genetic group 2), 90-106^{TT} (genetic group 3), and Costa

Rica 04-02-TN^T (genetic group 4) as representatives for each group were determined by the identification service of the DSMZ (Braunschweig, Germany) following standard protocols [8,68–70].

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass Spectrometry (MALDI-TOF MS)

Cultures were streaked for isolation on TYEM medium and determined to be pure before proceeding with MALDI-TOF MS analyses. Multiple colonies were used from each agar plate to ensure enough bacterial mass was obtained for detection and formic acid/ethanol tube extraction was performed according to the manufacturer's instruction as this produced more consistent spectra. 96-position steel MALDI-TOF MS target plates (Bruker Daltonics, Bremen, Germany) were inoculated with 1 μL supernatant, overlaid with 1 μL of HCCA matrix solution [(α-cyano-4-hydroxy cinnamic acid matrix (Bruker Daltonic)] reconstituted to 10 mg HCCA/mL with standard organic solvent of 50 % acetonitrile, 47.5 % water, and 2.5 % trifluoroacetic acid [Sigma-Aldrich, St. Louis, Missouri]), and allowed to dry at room temperature.

Microflex MALDI-TOF MS (Bruker Daltonics) was performed within 4 h of applying bacterial isolates to the target to obtain peak spectra. Prior to testing, the instrument was calibrated using 1 μL Bacterial Test Standard (Bruker Daltonics) applied to the target and overlaid with HCCA matrix solution as above.

A main spectral profile (MSP) was generated for the *F. columnare* type strain ATCC 23463^T and strains AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-02-TN^T (genetic group 4). The MSPs were created following the manufacturer's recommendation (Bruker Daltonics). Briefly, three biological replicates were used to create 10 technical replicates for a total of 30 spots per strain. Spectra were then obtained by MALDI-TOF MS (Bruker Daltonics). The spectra were processed using the flexControl and flexAnalysis software (Bruker Daltonics) which included baseline subtraction, smoothing and elimination of substandard spectra. The MALDI BioTyper software was used to create each MSP with a minimum of 20 acceptable spectra per entry. Quality control parameters were applied to each MSP per manufacturer's instruction which included verification of a score of greater than 2.7 when compared to itself and a peak frequency greater than 75 %. The BDAL library (Bruker Daltonics) was then added to the MSP hierarchy with the custom MSP to ensure no erroneous identifications occurred. The spectra of the closely related *Flavobacterium* spp. and remaining isolates from each genetic group of *F. columnare* were compared to both the custom MSPs and the 80 BDAL library *Flavobacterium* genus entries (Bruker Daltonics), and scores recorded. Bacterial species identification was performed using the manufacturer's instructions and scoring criteria; a score of at least 2.3 was considered a confident species-level identification. A score falling between 2.0 and 2.3 was considered a confident genus-level identification, with a probable species identification. A score between 1.7 and 1.99 was considered a probable genus-level identification. A score falling below 1.7 was considered a non-identification.

To generate a model for differentiating isolates of the four genetic groups, an initial training set analysis was performed to determine if differences in peak spectra could be observed between the genetic groups using ClinProTools Software (Bruker Daltonics). The spectra used for MSP generation of *F. columnare* ATCC 23463^T, AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-02-TN^T (genetic group 4) were used in the model development. Multiple peak differences were observed on manual inspection of spectra between 2,000 and 11,000 *m/z* (mass to charge ratio) where spectra were most robust. A peak statistic table was generated, and statistical analysis performed using a One-way ANOVA test. Multiple significant peak differences were observed between the four genetic groups indicating a differentiating biomarker model was possible. A Supervised Neural Network (SNN) algorithm model, a prototype-based classification algorithm, was then developed. Peak selection for the model was adjusted until the model passed external validation with 100 % of spectra identified correctly for each genetic group. The accuracy of the SNN model was tested against the other isolates from each genetic group using two biological replicates with three technical replicates and visual inspection of spectra was performed to confirm model identification.

Results and discussion

Phylogenetic analyses

Comparative 16S rRNA gene analysis identified the 19 panel isolates as members of the genus *Flavobacterium*. Within their respective groups, isolates of genetic groups 1 and 2 exhibited sequence identities of 99.9 to 100 %. Genetic group 1 isolates exhibited the highest 16S rRNA gene sequence identities with *F. columnare* ATCC 23463^T (average of 99.3 %), genetic group 2 isolates (range of 98.7 to 98.8 %), genetic group 4 isolates (range of 97.5 to 98.1 %), and *F. inkyongense* IMCC27201^T (97.8 %). Isolate AL-02-36^T, representing genetic group 2, exhibited the highest sequence identity with *F. columnare* ATCC 23463^T (98.8 %), Costa Rica 04-02-TN^T (genetic group 4; 98.4 %), *F. inkyongense* IMCC27201^T (98.1 %), and *F. terrae* R2A1-13^T (97.7 %). The isolates of genetic group 3 exhibited sequence identities ranging from 98.8 to 99.9 %, with isolate TI2063 being an outlier with lower percent identity to the other isolates (98.8 to 99.0 %). Isolate 90-106^T, representing genetic group 3, exhibited the highest sequence identity with AL-02-36^T (genetic group 2; 97.5 %), *F. columnare* ATCC 23463^T (97.2 %), Costa Rica 04-02-TN^T (genetic group 4; 96.9 %), and *F. verecundum* TTM-46^T (96.5 %). The isolates of genetic group 4 exhibited sequence identities ranging from 98.6 to 100 %, with isolate BZ-1-02 being an outlier with lower percent similarity to the other isolates (98.6 %). Isolate Costa Rica 04-02-TN^T, representing genetic group 4, exhibited the highest sequence identity with AL-02-36^T (genetic group 2; 98.4 %), *F. inkyongense* IMCC27201^T (98.3 %), *F. columnare* ATCC 23463^T (98.1 %), and *F. terrae* R2A1-13^T (97.2 %).

Phylogenetic analysis of the 16S rRNA genes of the 19 panel isolates and closely related *Flavobacterium* species using the ML method revealed isolates of genetic groups 1, 2, and 3 each formed well-supported, genetic group-specific monophyletic clades with bootstrap values of 97 to 100 (Fig. 1). While isolates of genetic group 4 also formed a separate clade (bootstrap value of 41), the BZ-1-02 isolate was an outlier, with the other four genetic group 4 isolates forming a well-supported clade (bootstrap value 100). Together with *F. inkyongense* IMCC27201^T, members of the genetic groups 1, 2 and 4 formed a well-supported monophyletic clade with a bootstrap value of 99, which was distinct from genetic group 3 isolates. Similar results were obtained using the NJ method (Supplementary Fig. S1) as well as previously published 16S rRNA phylogenies comprised of isolates from the four genetic groups [29,41]. Phylogenetic analysis of the partial *gyrB* gene sequences (Fig. 2) of the 19 panel isolates, *F. columnare* ATCC 23463^T and eight closely related *Flavobacterium* species demonstrated the four genetic groups formed extremely well-supported clades (bootstrap values 99 to 100), consistent with previous reports of *gyrB* phylogenies, which were in agreement with MLPA [41]. The phylogeny based on *gyrB* more clearly placed strain BZ-1-02 within the clade of genetic group 4 isolates, with strong bootstrap support (value of 100). Similar to the 16S analysis, *gyrB* data supports assertions that each respective genetic group is distinct from other closely related

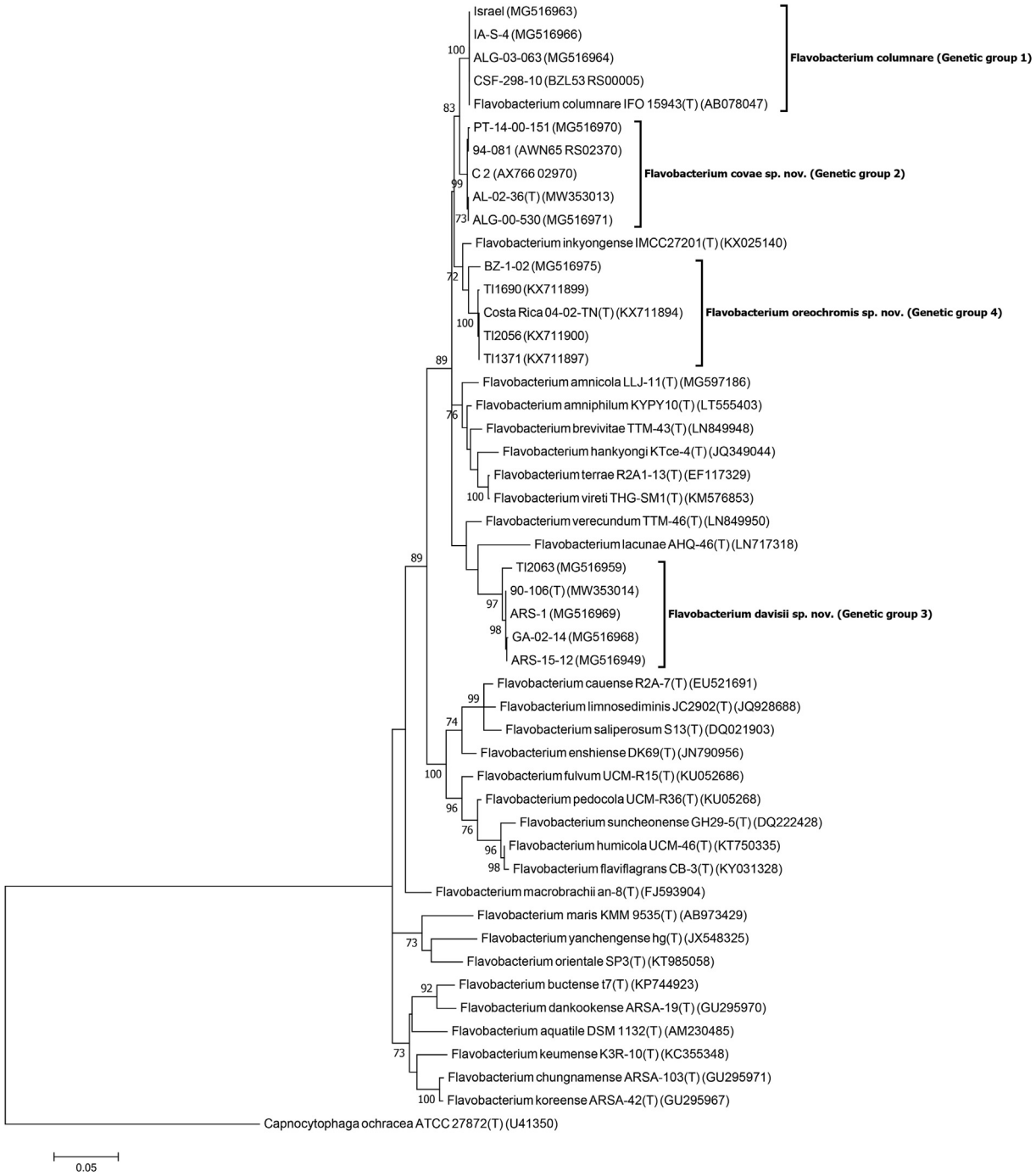

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic positions of genetic group 1, 2, 3, and 4 isolates among the closest relatives within the genus *Flavobacterium*. Relatedness was inferred using the maximum likelihood method based upon the General Time Reversible model [GTR + G + I; 50] and rooted with *Capnocytophaga ochracea* ATCC 27872^T. The percentage of trees in which the associated sequences clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 49 nucleotide sequences, all positions containing gaps and missing data were eliminated, and there was a total of 1330 positions in the final dataset.

members of the genus *Flavobacterium* and that isolates in genetic groups 1, 2 and 4 share a common ancestry that is distinct from isolates in genetic group 3.

Genome sequencing results

Analysis of the genome sequences of *F. columnare* ATCC 23463^T (genetic group 1), AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-04-TN^T (genetic group 4) revealed genome sizes ranged from 3.12 to 3.54 Mbp, while GC content ranged from 30.4 to 31.4 % (Table 2). Genome distances calculated by ANI revealed the isolate with the highest genomic similarity to *F. columnare* ATCC 23463^T (genetic group 1) was AL-02-36^T (genetic group 2) with a value of 90.8 %, while comparisons to the other isolates were less than 85.3 % (Fig. 3). The ANI values obtained for comparisons of all other genomes were less than 85.5 % (Fig. 3), well below the 95–96 % threshold used to distinguish between members of closely related species [13,25]. Similarly, digital

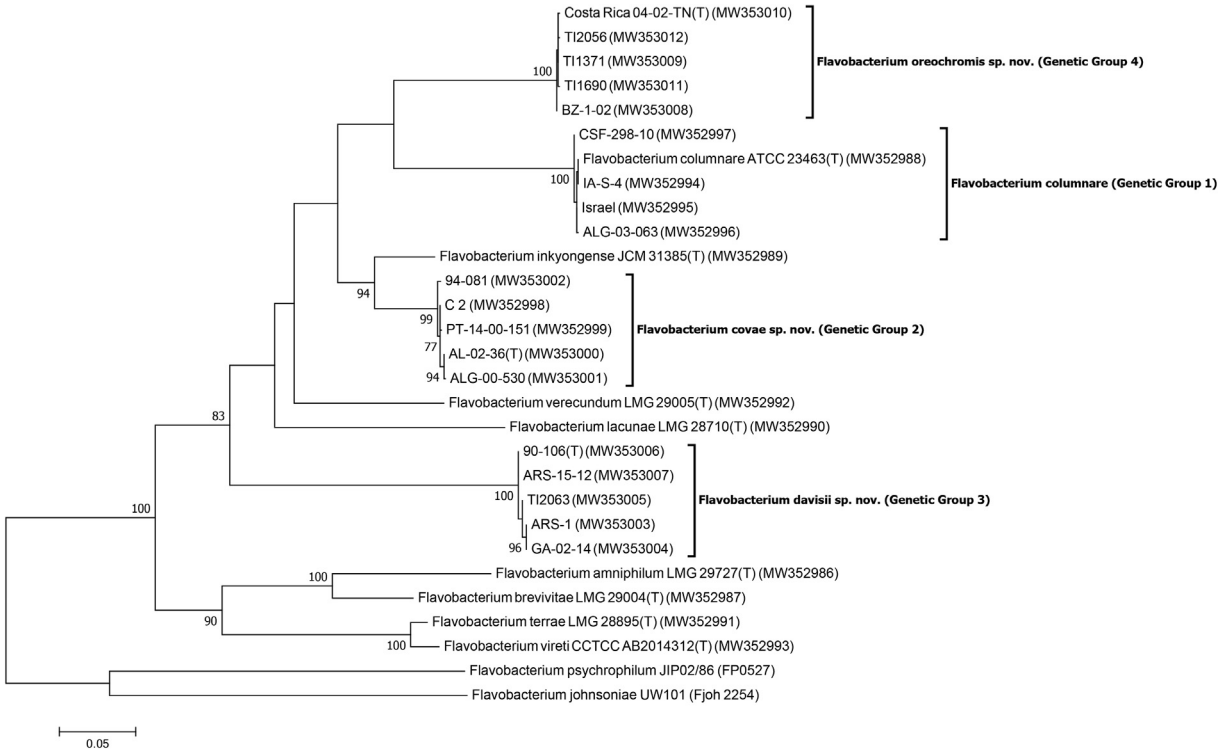

Fig. 2. Phylogenetic tree based on *gyrB* gene sequences showing the phylogenetic positions of genetic group 1, 2, 3, and 4 isolates among the closest relatives within the genus *Flavobacterium*. Relatedness was inferred using the maximum likelihood method based upon the General Time Reversible model [GTR + G + I; 50] and rooted with *F. psychrophilum* JIP 02/86 and *F. johnsoniae* UW101^T. The percentage of trees in which the associated sequences clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The analysis involved 29 nucleotide sequences, all positions containing gaps and missing data were eliminated, and there was a total of 1,189 positions in the final dataset.

Table 2
Genome characteristics of *Flavobacterium columnare* ATCC 23464 ^{T} (genetic group 1), *F. covae* sp. nov. AL-02-36 ^{T} (genetic group 2), *F. davisii* sp. nov. 90-106 ^{T} (genetic group 3), *F. oreochromis* sp. nov. Costa Rica 04–02-TN^T (genetic group 4), and *F. terrae* DSM 18829 ^{T}.

Isolate	*F. columnare* ATCC 23463 ^{T} (genetic group 1)	*F. covae* sp. nov. AL-02-36 ^{T} (genetic group 2)	*F. davisii* sp. nov. 90-106 ^{T} (genetic group 3)	*F. oreochromis* sp. nov. Costa Rica 04-02-TN^T (genetic group 4)	*F. terrae* DSM 18829 ^{T}
Genome size (Mbp)	3.12	3.40	3.40	3.54	3.16
No. contigs	181	1	1	1	19
Coverage for consensus correction	-	143X	306X	138X	-
Coverage for assembly	-	36X	40X	40X	-
GC content (%)	31.4	31.0	31.2	30.4	31.8
Total genes	2838	3360	3241	3401	2915
Coding genes	2702	2356	2327	2599	2845
Total CDSs	2776	3249	3109	3274	2860
Pseudo genes	74	893	782	675	15
rRNAs	3	26	36	30	3
tRNAs	56	82	93	94	49
ncRNAs	3	3	3	3	3
Accession No.	PCMX01000000	CP067379	CP067378	CP067377	FQZI00000000

DNA-DNA hybridization estimations between all genomes were less than 42.7 % (Fig. 3), well below the suggested cutoff of 70 % [27,48,75] for delineating bacterial species. The data set was expanded by including publicly available genomes of isolates assigned to the four genetic groups. Genome distances calculated by digital DNA-DNA hybridization within groups ranged from 92.5 to 98 %, 88.0 to 100 %, 87.4 to 93.7 %, and 93.1 to 100 % for genetic groups 1, 2, 3, and 4, respectively (Supplementary Fig. S2). Digital DNA-DNA hybridization values for all comparisons of genomes of isolates belonging to different genetic groups were less than 42 %, (Supplementary Fig. S2), confirming isolates of genetic groups 2, 3, and 4 represent new species in the genus *Flavobacterium*. These reported genome distances are consistent with those reported in similar studies [14,29,36,37].

Morphology, cultural, biochemical and physiological characteristics

Isolates among all four genetic groups were aerobic, Gram-stain-negative rods with rounded ends, occurring singly, with gliding motility. The cells of representative isolates from each genetic group were approximately 0.3 to 0.4 μm wide, while lengths ranged from 5 to 10 μm, 9 to 14 μm, 4 to 8 μm, and 5 to 6 μm for *F. columnare* ATCC 23463^T (genetic group 1), AL-02-36^T (genetic group 2), 90-106^T (genetic group 3), and Costa Rica 04-02-TN^T

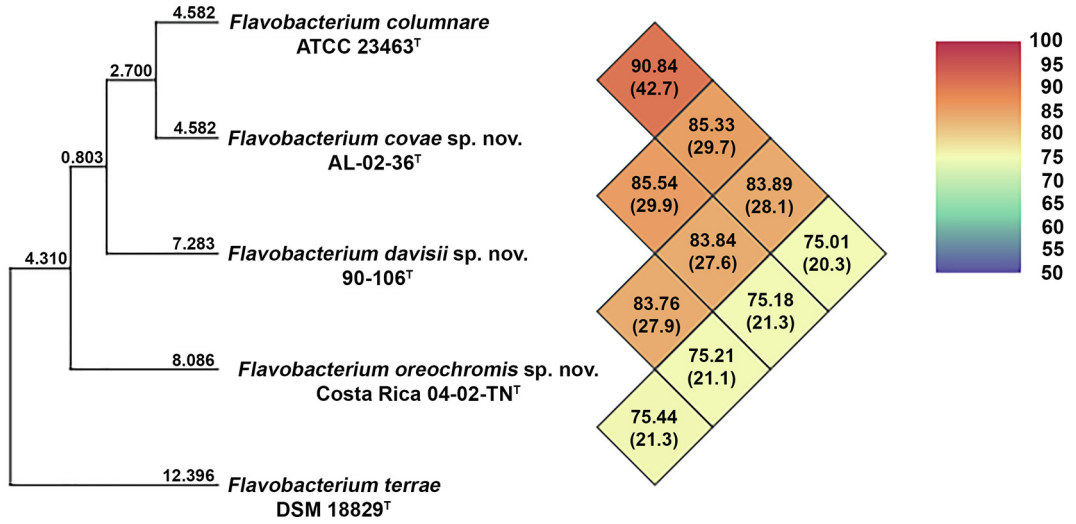

Fig. 3. Average nucleotide identity [OrthoANI calculated with Orthologous Average Nucleotide Identity Tool [OAT; 43] and digital DNA-DNA hybridization (in parentheses) values obtained by comparing whole genome sequences of *Flavobacterium columnare* ATCC 23463^T (genetic group 1), *F. covae* sp. nov. AL-02-36^T (genetic group 2), *F. davisii* sp. nov. 90-106^T (genetic group 3), *F. oreochromis* sp. nov. Costa Rica 04-02-TN^T (genetic group 4), and *F. terrae* DSM 18829^T.

(genetic group 4), respectively (Supplementary Figs. S3-S6). All isolates formed relatively flat, yellowish pigmented colonies on R2A agar with adherent and spreading rhizoid growth. Colony size was variable regardless of genetic group, whereby most isolates formed colonies with diameters of 6 to 11 mm. Three isolates consistently produced smaller colonies (1 to 3 mm), namely *F. columnare* ATCC 23463^T, genetic group 1 isolate ALG-03-063, and genetic group 4 isolate BZ-1-02. All isolates of the four genetic groups were positive for flexirubin-type pigments and absorbed Congo red.

Isolates among all genetic groups grew effectively at temperatures ranging from 15 to 36 °C; however, subtle differences were observed (Table 3). Genetic group 1 and 3 isolates were capable of growth at 12 °C (4/5 and 2/5 isolates, respectively), and only isolates of genetic group 1 grew at 10 °C (1/5 isolates). Genetic group 4 isolates were capable of growth at 40 °C (3/5 isolates). All isolates grew effectively in the presence of 0.5 % NaCl, with variable results at greater salt concentrations (Table 3). All isolates were capable of growth at pH of 6.3 to 8.0, with variable results at higher and lower pH. All isolates grew well in aerobic and microaerophilic conditions (except for *F. columnare* ATCC 23463^T not capable of microaerophilic growth). Anaerobic growth was negative for all isolates. Growth was positive for all isolates on R2A agar, NA, and negative for all isolates on TSA, MA, Endo and MacConkey agar. Some members of genetic groups 1 and 2 were able to grow weakly on TSA supplemented with sheep blood. Results are tabulated in Table 3.

All tested isolates were catalase and oxidase positive (Table 3). The spectrum of other tests relevant for delineation of novel *Flavobacterium* species [6] is listed in Table 3. Isolates from each genetic group were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activity, and negative for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activity (Supplementary Table S1). All isolates from genetic group 1 were negative for cystine arylamidase and α-chymotrypsin activity, whereas reactions for isolates of genetic groups 2, 3, and 4 were strain dependent. Isolates from each genetic group were negative for the fermentation of carbohydrates on the API 50 CH. Similarly, the closely related *Flavobacterium* species were also negative for the fermentation of carbohydrates. All panel isolates, along with reference *Flavobacterium* spp., were further positive for hydrolysis of gelatin, starch and casein and negative for utilization of cellulose (Table 3). Differences between the four genetic groups and reference strains were found in the ability to hydrolyse DNA, Tween 80 and L-tyrosine. *Flavobacterium amniphilum*, *F. verecundum*, and the panel isolates from the four genetic groups were unable to hydrolyse esculin, whereas the other reference strains were positive. A list of tests that can be used for distinguishing members of all four genetic groups from closely related *Flavobacterium* spp. is tabulated in Table 4.

All isolates among the four genetic groups utilized glucuronamide and were resistant to vancomycin, tetrazolium violet, and tetrazolium blue using Biolog Gen III microplates (Supplementary Table S2). Isolates belonging to genetic groups 2 and 3 tended to utilize more carbon sources compared to isolates of genetic groups 1 and 4. However, due to the variability observed for isolates within and between the four genetic groups, none of the biochemical characteristics were found to be helpful for distinguishing between the proposed species.

Antimicrobial susceptibility

Two closely related species, *F. amniphilum* and *F. verecundum*, were unable to grow in the diluted CAMHB, preventing determination of the MIC for the antimicrobials tested (Supplementary Table S3). There was variability in the antimicrobial susceptibilities of isolates within genetic groups, but no relevant differences were detected among the four genetic groups and the closely related *Flavobacterium* species in terms of inherent susceptibility/sensitivity to the tested antimicrobial agents (Supplementary Table S3).

Chemotaxonomic analyses

The predominant fatty acids of *F. columnare* (genetic group 1) and genetic groups 2, 3, and 4 were $C_{15:1}$ iso G (15.4 – 17.6 %), $C_{15:0}$ iso (34.9 – 41.0 %), $C_{16:0}$ iso (6.1 – 8.7 %), $C_{17:0}$ iso 3OH (6.0 – 7.0 %) and Summed Feature 9 ($C_{16:0}$ 10-methyl / $C_{17:1}$ iso $\omega 9c$) (7.4 – 8.9 %) (Supplementary Table S4). Fatty acid profiles were

Table 3
Phenotypic characteristics of *F. columnare* (n = 5 isolates including type strain ATCC 23463 ^{T}), *F. covae* sp. nov. (n = 5 isolates), *F. davisii* sp. nov. (n = 5 isolates), *F. oreochromis* sp. nov. (n = 5 isolates) and their closest relative *Flavobacterium* spp. strains: *Flavobacterium amniphilum* LMG 29727 ^{T}, *Flavobacterium brevivitae* LMG 29004 ^{T}, *Flavobacterium columnare* ATCC 23463 ^{T}, *Flavobacterium inkyongense* JCM 31385 ^{T}, *Flavobacterium lacunae* LMG 28710 ^{T}, *Flavobacterium terrae* LMG 28895 ^{T}, *Flavobacterium verecundum* LMG 29005 ^{T} and *Flavobacterium vireti* CCTCC AB2014312^{T}. All data are from this study. (+): positive; (w): weakly positive; (-): negative; (v): variable results among isolates.

Characteristic	*F. columnare* (genetic group 1)	*F. covae* sp. nov. (genetic group 2)	*F. davisii* sp. nov. (genetic group 3)	*F. oreochromis* sp. nov. (genetic group 4)	*F. amniphilum* LMG 29727 ^{T}	*F. brevivitae* LMG 29004 ^{T}	*F. inkyongense* JCM 31385 ^{T}	*F.* lacunae LMG 28710 ^{T}	*F. terrae* LMG 28895 ^{T}	*F. verecundum* LMG 29005 ^{T}	*F. vireti* CCTCC AB2014312^{T}
Growth at different temperatures											
10 °C	v	-	-	-	+	-	w	-	+	-	+
12 °C	v	-	v	-	+	+	+	+	+	w	+
15–36 °C	+	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	v	+	-	+	+	+	+	-	-
38 °C	v	+	v	+	-	-	+	-	+	-	-
39 °C	v	v	-	v	-	-	+	-	+	-	-
40 °C	-	-	-	v	-	-	+	-	-	-	-
Growth in % NaCl											
0.5% NaCl	+	+	+	+	w	+	+	-	+	-	+
0.75% NaCl	v	+	+	+	-	+	-	-	+	-	+
1.0 % NaCl	v	v	+	v	-	-	-	-	+	-	+
1.2% NaCl	v	-	-	-	-	-	-	-	+	-	+
Growth at different pH											
pH 5.5	-	-	-	-	-	-	-	-	-	-	-
pH 5.8	-	-	v	+	-	-	-	-	-	w	-
pH 5.9	v	v	v	+	-	+	-	-	w	w	-
pH 6.0	v	v	v	+	-	+	+	-	+	+	-
pH 6.3	+	+	+	+	-	+	+	-	+	+	+
pH 6.5	+	+	+	+	+	+	+	-	+	+	+
pH 8.0	+	+	+	+	+	+	+	+	+	+	+
pH 9.0	+	+	v	-	+	+	+	+	+	+	+
pH 9.5	v	v	-	-	+	+	+	+	+	-	+
Growth on:											
TSA	-	-	-	-	-	-	-	-	+	-	+
TSA + blood	-	v	v	-	-	-	-	-	+	-	+
NA	+	+	+	+	+	+	+	+	+	+	+
MA	-	-	-	-	-	-	-	-	-	-	-
Endo agar	-	-	-	-	-	-	-	-	-	-	-
MacConkey	-	-	-	-	-	-	-	-	-	-	-
Growth condition:											
Anaerobic growth	-	-	-	-	-	-	-	-	-	-	-
Microaerophilic growth	v	+	+	+	+	+	+	+	+	+	+
Production of:											
Catalase	+	+	+	+	+	+	+	+	+	+	+
Flexirubin-type pigments	+	+	+	+	-	-	+	+	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Activity of:											
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-
Lysine		decarboxylase	-	-	-	-	-	-	-	-	-
-	-										
Ornithine		decarboxylase	-	-	-	-	-	-	-	-	-
-	-										
Urease	-	-	-	-	-	-	-	-	-	-	-
Reduction of:											
Nitrates	+	v	v	v	-	-	-	-	-	-	-

(*continued on next page*)

Table 3 (*continued*)

Characteristic	*F. columnare* (genetic group 1)	*F. covae* sp. nov. (genetic group 2)	*F. davisii* sp. nov. (genetic group 3)	*F. oreochromis* sp. nov. (genetic group 4)	*F. amniphilum* LMG 29727 ^{T}	*F. brevivitae* LMG 29004 ^{T}	*F. inkyongense* JCM 31385 ^{T}	*F.* lacunae LMG 28710 ^{T}	*F. terrae* LMG 28895 ^{T}	*F. verecundum* LMG 29005 ^{T}	*F. vireti* CCTCC AB2014312^{T}
Nitrites	-	-	-	-	-	-	-	-	-	-	-
Utilization of:											
Acetamide	-	-	-	-	-	-	-	-	-	-	-
Simmon's citrate	-	-	-	-	-	-	-	-	-	-	-
Sodium malonate	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of:											
Casein	+	+	+	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-	-	-	-
DNA	+	+	+	+	-	-	-	-	+	w	+
Esculin	-	-	-	-	-	+	+	+	+	-	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+
Lecithin (egg-yolk)	+	+	+	+	+	+	+	+	+	+	+
L-tyrosine	-	-	-	-	-	-	-	-	+	-	+
ONPG	-	-	v	-	-	-	+	-	+	-	+
Starch	+	+	+	+	+	+	+	+	+	+	+
Tween 80	-	-	v	-	-	-	-	-	+	-	+
Other:											
Congo red absorption	+	+	+	+	-	+	+	+	+	-	-
OF test	-	-	-	-	-	-	-	-	-	-	-

Table 4
Phenotypic characteristics that differentiate *Flavobacterium columnare* (n = 5 isolates including type strain ATCC 23463 ^{T}), *Flavobacterium covae* sp. nov. (n = 5 isolates), *Flavobacterium davisii* sp. nov. (n = 5 isolates), and *Flavobacterium oreochromis* sp. nov. (n = 5 isolates) from closely related *Flavobacterium* spp. All data are from this study. (+): positive; (w): weakly positive; (-): negative; (v): variable results among isolates.

Characteristic	*F. columnare* (genetic group 1)	*F. covae* sp. nov. (genetic group 2)	*F. davisii* sp. nov. (genetic group 3)	*F. oreochromis* sp. nov. (genetic group 4)	*F. amniphilum* LMG 29727 ^{T}	*F. brevivitae* LMG 29004 ^{T}	*F. inkyongense* JCM 31385 ^{T}	*F.* lacunae LMG 28710 ^{T}	*F. terrae* LMG 28895 ^{T}	*F. verecundum* LMG 29005 ^{T}	*F. vireti* CCTCC AB2014312^{T}
Temperature range °C	15–37	15–38	15–36	15–38	12–36	12–37	12–40	12–37	12–39	15–36	12–36
% NaCl range	0–0.5	0–0.75	0–1.0	0–0.75	< 0.5	0.5–0.75+	≤0.5+	0.0	0–1.2	0.0-	0–1.2
pH range	6.3–9.0	6.3–9.0	6.3–8.0	5.8–8.0	6.5–9.5	5.9–9.5	6.0–9.5	8.0–9.5	6.0–9.5	6.0–9.0	6.3–9.5
Growth on:											
TSA	-	-	-	-	-	-	-	-	+	-	+
TSA + blood	-	v	v	-	-	-	-	-	+	-	+
Characteristic:											
Flexirubin-type pigments	+	+	+	+	-	-	+	+	-	-	-
Congo red	+	+	+	+	-	+	+	+	+	-	-
Nitrate production	+	v	v	v	-	-	-	-	-	-	-
DNA	+	+	+	+	-	-	-	-	+	w	+
Tween 80	-	-	v	-	-	-	-	-	+	-	+
L-tyrosine	-	-	-	-	-	-	-	-	+	-	+
Esculin	-	-	-	-	-	+	+	+	+	-	+
ONPG	-	-	v	-	-	-	+	-	+	-	+
Cystine arylamidase	-	v	v	v	w	w	w	w	w	+	w
Trypsin	v	+	+	+	w	-	+	+	w	+	w
α-chymotrypsin	-	+	v	v	-	-	w	w	-	+	-

Table 5
Size (*m/z*, mass to charge ratio) and intensity (a.u., arbitrary units) of unique peaks (greater than 2000 a.u.) identified for *F. columnare* ATCC 23463 ^{T} and proposed type strains of *F. covae* sp. nov., *F. davisii* sp. nov., and *F. oreochromis* sp. nov. Peaks in bold face were used in the SNN model to accurately assign all isolates to *F. columnare* and proposed species.

Isolate	Unique Peaks *m/z* (a.u.)			
F. columnare ATCC 23463 ^{T} (genetic group 1)	2054.73 (2071)	2333.36 (2366)	2732.17 (2778)	2983.17 (5735)
	3105.41 (3936)	4642.03 (2292)	5243.03 (2194)	6214.53 (4475)
	6599.58 (2324)	7546.04 (2375)		
F. covae sp. nov. AL-02–36 ^{T} (genetic group 2)	3122.43 (4438)	3408.75 (2068)	**3606.08 (6101)**	3685.06 (3347)
	4599.83 (4402)	4620.97 (5415)	4699.26 (3920)	4730.04 (2545)
	5088.00 (2778)	5250.70 (5302)	5507.68 (6789)	6247.84 (7470)
	6310.84 (2313)	6327.94 (2838)	6564.53 (2818)	6864.74 (2938)
	7213.3 (10077)	7276.33 (3107)	7371.33 (5213)	9396.32 (2860)
	10107.50 (4298)	10172.59 (2017)	10498.55 (3627)	
F. davisii sp. nov. 90–106 ^{T}(genetic group 3)	3194.08 (2363)	3261.18 (3643)	3375.83 (2046)	3585.96 (4562)
	3816.89 (7052)	4229.44 (6061)	5273.00 (3761)	5459.92 (13856)
	5478.13 (2248)	5569.07 (2354)	5619.6 (2782)	**6525.36 (7326)**
	7173.83 (6569)	9251.96 (5555)	10543.77 (2020)	
F. oreochromis sp. nov. Costa Rica 04–02-TN^{T} (genetic group 4)	3268.67 (4722)	3283.09 (1470)	3779.85 (4968)	5040.12 (3605)
	5257.94 (4358)	**6540.2 (10079)**	6555.96 (2080)	7560.35 (6245)
	9254.6 (4871)	10078.05 (2412)	10513.25 (2774)	

Table 6
Identification of isolates using MALDI-TOF and the SSN Model generated from custom MSPs derived from *F. columnare* ATCC 23463 ^{T}, *F. covae* sp. nov. AL-02-36 ^{T}, *F. davisii* sp. nov. 90-106 ^{T}, and *F. oreochromis* sp. nov. Costa Rica 04-02-TN^{T}.

Isolate	MALDI-TOF score ± SD	MALDI-TOF Identification	SSN Model Identification
Israel	2.348 ± 0.046	*F. columnare*[1]	*F. columnare*
IA-S-4	2.312 ± 0.041	*F. columnare*[1]	*F. columnare*
ALG-03-063	2.391 ± 0.069	*F. columnare*[1]	*F. columnare*
CSF-298-10	2.327 ± 0.042	*F. columnare*[1]	*F. columnare*
ALG-00-530	2.551 ± 0.013	*F. covae* sp. nov.[2]	*F. covae* sp. nov.
C#2	2.668 ± 0.044	*F. covae* sp. nov.[2]	*F. covae* sp. nov.
PT-14-00-151	2.311 ± 0.294	*F. covae* sp. nov.[2]	*F. covae* sp. nov.
94-081	1.913 ± 0.121	*F. covae* sp. nov.[2]/*F. columnare*[1]	*F. covae* sp. nov.
GA-02-14	2.500 ± 0.111	*F. davisii* sp. nov.[3]	*F. davisii* sp. nov.
ARS-1	2.603 ± 0.143	*F. davisii* sp. nov.[3]	*F. davisii* sp. nov.
TI2063	2.604 ± 0.106	*F. davisii* sp. nov.[3]	*F. davisii* sp. nov.
ARS-15-12	2.563 ± 0.156	*F. davisii* sp. nov.[3]	*F. davisii* sp. nov.
BZ-1-02	2.419 ± 0.091	*F. oreochromis* sp. nov.[4]	*F. oreochromis* sp. nov.
TI1690	2.447 ± 0.110	*F. oreochromis* sp. nov.[4]	*F. oreochromis* sp. nov.
TI2056	2.310 ± 0.150	*F. oreochromis* sp. nov.[4]	*F. oreochromis* sp. nov.
TI1371	2.424 ± 0.155	*F. oreochromis* sp. nov.[4]	*F. oreochromis* sp. nov.
F. amniphilum LMG 29727^{T}	1.300 ± 0.081	Multiple IDs	No identification
F. brevivitae LMG 29004^{T}	0.993 ± 0.088	Multiple IDs	No identification
F. inkyongense JCM 31385^{T}	1.312 ± 0.083	Multiple IDs	No identification
F. lacunae LMG 28710^{T}	1.544 ± 0.106	*F. davisii* sp. nov.[3]	No identification
F. terrae LMG 28895^{T}	1.282 ± 0.096	Multiple IDs	No identification
F. verecundum LMG 29005^{T}	1.232 ± 0.283	Multiple IDs	No identification
F. vireti CCTCC AB2014312^{T}	1.172 ± 0.132	Multiple IDs	No identification

[1] Custom MSP from *F. columnare* ATCC 23463 ^{T} (genetic group 1).
[2] Custom MSP from *F. covae* sp. nov. AL-02–36 ^{T} (genetic group 2).
[3] Custom MSP from *F. davisii* sp. nov. 90–106 ^{T} (genetic group 3).
[4] Custom MSP from *F. oreochromis* sp. nov. Costa Rica 04-02-TN^{T} (genetic group 4).

qualitatively similar; however, quantitative differences could be useful for differentiating *F. columnare* (genetic group 1) and the three newly proposed species, especially higher percentages of $C_{15:0}$ iso in genetic group 2 and 3 isolates, and higher percentages of $C_{16:0}$ iso in genetic group 2 isolates. Additionally, trace levels of $C_{15:0}$ anteiso were detected in genetic group 3 isolates and trace levels of $C_{17:1}$ *ω5c* were detected in genetic groups 2 and 3 compared to higher percentages in the other groups. *Flavobacterium columnare* (genetic group 1) can be differentiated from the three other suggested species by higher amounts of $C_{16:0}$ iso 3-OH and $C_{13:0}$ iso. Quantitative differences in fatty acids with less than 1 % of the total were identified between *F. columnare* (genetic group 1) and genetic groups 2, 3, and 4 (Supplementary Table S4).

Further chemotaxonomic analyses revealed the presence of the major polar lipid phosphatidylethanolamine (PE), an unidentified aminolipid (AL), and two unidentified lipids, L1 and L2 (Supplementary Fig. S7) in *F. columnare* ATCC 23463^{T}, AL-02-36^{T} (genetic group 2), 90-106^{T} (genetic group 3), and Costa Rica 04-02-TN^{T} (genetic group 4). In the respiratory quinone analysis, only the major menaquinone, MK-6, was identified in each strain. These results confirm that each genetic group represents species within the genus *Flavobacterium* and are closely related based on polar lipid profiles.

MALDI-TOF analyses

MSPs (main spectral profiles) were generated for *F. columnare* ATCC 23463^{T} (genetic group 1), AL-02-36^{T} (genetic group 2), 90-106^{T} (genetic group 3), and Costa Rica 04-02-TN^{T} (genetic group 4). Analysis of the spectra identified numerous peaks (*m/z*) unique

Table 7
Protologue descriptions of *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov., and *Flavobacterium oreochromis* sp. nov.

Genus name	*Flavobacterium*	*Flavobacterium*	*Flavobacterium*
Species name	*Flavobacterium covae*	*Flavobacterium davisii*	*Flavobacterium oreochromis*
Specific epithet	*covae*	*davisii*	*oreochromis*
Species status	sp. nov.	sp. nov.	sp. nov.
Species etymology	co'vae. N.L. gen. n. *covae*, of Cova, named in honour of the late Dr. Covadonga Rodriguez Arias alias "Cova", for her passion and research on columnaris disease	da.vi'si.i. N.L. gen. n. *davisii*, of Davis, named in honour of Dr. Herbert Spencer Davis, who first described columnaris disease in freshwater fish	o.re.o.chro'mis. N.L. gen. n. *oreochromis*, of the tilapia genus *Oreochromis*, referring to the host association with tilapia
Description of the new taxon and diagnostic traits	• Rods with rounded ends, predominantly filamentous forms 9.0–14.0 μm × 0.3–0.5 μm • occurrence – singly • no endospores • motile by gliding • flexirubin type of pigment (yellow) • absorbs Congo Red • growth on R2A and Nutrient agar • no growth on TSA, Marine agar, Endo agar, MacConkey agar • microaerophilic growth • no anaerobic growth • temperature range of 15–38 °C • pH range of 6.3–9.0 • NaCl range of 0–0.75% • non-halophile (1.2% inhibits growth) • catalase positive • oxidase positive • glucose not fermented in OF medium • arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase negative • does not utilize citrate, acetamide, malonate • does not produce indole, urease, H_2S • positive for lecithinase activity • hydrolyses DNA, gelatin, starch, casein • does not hydrolyse Tween 80, esculin, ONPG, cellulose • positive by API ZYM for Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase • negative by API ZYM for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase • positive by Gen III microplates (Biolog) for D-galactose, L-fucose, gelatin, glycyl-L-proline, glucuronamide, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, aztreonam • The major fatty acids are $C_{15:0}$ iso, $C_{15:1}$ iso G, $C_{16:0}$ iso, Summed Feature 9 ($C_{16:0}$ 10-methyl / $C_{17:1}$ iso *ω9c*) and $C_{17:0}$ iso-3OH • The major menaquinone: MK-6 • The polar lipid profile contains: phosphatidylethanolamine, one unidentified aminolipid, two unidentified lipids • the whole cell protein profile contains unique 3606 *m/z* peak • the G + C content of type strain: 31.0 mol% • variable tests are: growth at 39 °C, growth in the presence of 1% NaCl, growth at pH 5.9–6.0 and 9.5, growth on TSA supplemented with blood, production of nitrate and positivity for cystine arylamidase	• Rods with rounded ends, 4.0–8.0 μm × 0.3–0.4 μm • occurrence-singly • no endospores • motile by gliding • flexirubin type of pigment (yellow) • absorbs Congo Red • growth on R2A and Nutrient agar • no growth on TSA, Marine agar, Endo agar, MacConkey agar • microaerophilic growth • no anaerobic growth • temperature range of 15–36 °C • pH range of 6.3–8.0 • NaCl range of 0–1.0% • non-halophile (1.2% inhibits growth) • catalase positive • oxidase positive • glucose not fermented in OF medium • arginine dihydrolase, ornithine decarboxylase, lysine decarboxylate negative • does not utilize citrate, acetamide, malonate • does not produce indole, urease, H_2S • positive for lecithinase activity • hydrolyses DNA, gelatin, starch, casein • does not hydrolyse esculin, cellulose • positive by API ZYM for Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase • negative by API ZYM for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase • positive by Gen III microplates (Biolog) for glucuronamide, acetoacetic acid, vancomycin, tetrazolium violet, tetrazolium blue • The major fatty acids are $C_{15:0}$ iso, $C_{15:1}$ iso G, $C_{16:0}$ iso, Summed Feature 9 ($C_{16:0}$ 10-methyl / $C_{17:1}$ iso *ω9c*) and $C_{17:0}$ iso-3OH • The major menaquinone: MK-6 • The polar lipid profile contains: phosphatidylethanolamine, one unidentified aminolipid, two unidentified lipids • the whole cell protein profile contains unique 6525 *m/z* peak • the G + C content of type strain: 31.2 mol% • variable tests are: growth at 12 °C, 37–38 °C, growth at pH 5.8–6.0 and 9.0, growth on TSA supplemented with blood, production of nitrate, hydrolysis of Tween 80 and ONPG, positivity for cystine arylamidase and α-chymotrypsin	• Rods with rounded ends, 5.0–6.0 μm × 0.3–0.4 μm • occurrence-singly • no endospores • motile by gliding • flexirubin type of pigment (yellow) • absorbs Congo Red • growth on R2A and Nutrient agar • no growth on TSA, TSA supplemented with blood, Marine agar, Endo agar, MacConkey agar • microaerophilic growth • no anaerobic growth • temperature range of 15–38 °C • pH range of 5.8–8.0 • NaCl range of 0–0.75% • non-halophile (1.2% inhibits growth) • catalase positive • oxidase positive • glucose not fermented in OF medium • arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase negative • does not utilize citrate, acetamide, malonate • does not produce indole, urease, H_2S • positive for lecithinase activity • hydrolyses DNA, gelatin, starch, casein • does not hydrolyse Tween 80, esculin, ONPG, cellulose • positive by API ZYM for Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase • negative by API ZYM for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase • positive by Gen III microplates (Biolog) for D-fructose-6-PO4, glucuronamide, acetoacetic acid, vancomycin, tetrazolium violet, tetrazolium blue • The major fatty acids are $C_{15:0}$ iso, $C_{15:1}$ iso G, $C_{16:0}$ iso, Summed Feature 9 ($C_{16:0}$ 10-methyl / $C_{17:1}$ iso *ω9c*) and $C_{17:0}$ iso-3OH • The major menaquinone: MK-6 • The polar lipid profile contains: phosphatidylethanolamine, one unidentified aminolipid, two unidentified lipids • the whole cell protein profile contains unique 6540 *m/z* peak • the G + C content of type strain: 30.4 mol% • variable tests are: growth at 39–40 °C, growth in presence of 1.0% NaCl, production of nitrate, positivity for cystine arylamidase and α-chymotrypsin

Table 7 (*continued*)

Genus name	*Flavobacterium*	*Flavobacterium*	*Flavobacterium*
Country of origin (type strain)	United States of America	United States of America	Costa Rica
Region of origin (type strain)	state of Alabama	state of Mississippi	unknown
Date of isolation (type strain)	2002	1990	2004
Source of isolation (type strain)	largemouth bass (*Micropterus salmoides*)	channel catfish (*Ictalurus punctatus*)	tilapia (*Oreochromis sp.*)
16S rRNA gene accession numbers	MW353013 (type) MG516971; AX766_02970; AWN_RS02370; MG516970	MW353014 (type) MG516959; MG516969; MG516968; MG516949	KX711894 (type) MG516975; KX711899; KX711900; KX711897
gyrB gene accession numbers	MW353000 (type) MW352998; MW352999; MW353001; MW353002	MW353006 (type) MW353003-MW353005; MW353007	MW353010 (type) MW353008; MW353009; MW353011; MW353012
Genome accession number	CP067379	CP067378	CP067377
Genome status	draft	draft	draft
Genome size	3.40	3.40	3.54
GC mol%	31.0	31.2	30.4
Number of strains in study	5	5	5
Source of isolation of non-type strains	diseased fish	diseased fish	diseased fish
Designation of the Type Strain	AL-02-36 ^{T}	90-106 ^{T}	Costa Rica 04-02-TN^{T}
Strain Collection Numbers	ATCC TSD-246 ^{T} = CCM 9171 ^{T}	ATCC TSD-247 ^{T} = CCM 9172 ^{T}	ATCC TSD-248 ^{T} = CCM 9173 ^{T}

to each genetic group (Table 5; Supplementary Fig. S8). The remaining four isolates from each genetic group and the closely related *Flavobacterium* spp. were analyzed and all spectra were compared to both the custom MSPs derived from the four genetic groups and the 80 *Flavobacterium* spp. entries in the BDAL library (Table 6). All genetic group 1 isolates were identified as *F. columnare* ATCC 23463^{T} with scores > 2.3. Three of the four genetic group 2 isolates were identified as AL-02-36^{T} with scores > 2.3, while the fourth isolate 94-081 exhibited a score of 1.9. All genetic group 3 and 4 isolates were identified as 90-106^{T} (genetic group 3) and Costa Rica 04-02-TN^{T} (genetic group 4), respectively, with scores > 2.3. No identifications were made for the 7 closely related *Flavobacterium* spp. with scores < 1.7 (Table 6), suggesting a lack of congruence among spectral profiles.

The SNN model developed was able to accurately assign all isolates to their respective genetic groups (Table 6). The peaks used in the model were located at 3606, 6525, and 6540 *m/z*. The model was based on the 3606 *m/z* peak unique to genetic group 2 isolates, 6525 *m/z* peak unique to genetic group 3 isolates, 6540 *m/z* peak unique to genetic group 4 isolates, and absence of any of the three peaks for genetic group 1 isolates. Importantly, use of the SNN model allowed for the accurate identification of 94-081 (genetic group 2), which gave a lower MALDI score based on the custom MSP from AL-02-36^{T} (genetic group 2). Some isolates such as 94-081 (genetic group 2) gave variable results and the lower score obtained may be due to technical issues associated with these fastidious organisms. Future research on the use of MALDI-TOF for identification of *F. columnare* and the three proposed species should examine growth conditions and their impact on reproducibility, and the functional significance of these unique peaks. These results confirm the use of whole cell proteins as a phenotypic marker for accurate discrimination between the four genetic groups of *F. columnare* and enable identification of the four proposed species in routine laboratories via MALDI-TOF without genetic sequencing.

Conclusion

The results of the present study are consistent with previous research demonstrating phenotypic variation within and between the genetic groups of *F. columnare* [4,5,7,61,71], and suggest the lack of robust biochemical markers for distinguishing between the groups. However, the polyphasic and phylogenomic characterization of the present study conclusively demonstrates that the four genetic groups of *F. columnare* represent four different species. Quantitative differences in fatty acid profiles and MALDI-TOF were useful for discriminating between the four genetic groups. *In silico* DNA-DNA hybridization and ANI value calculations fully support these groups as unique species with values well below the thresholds used to distinguish between members of closely related species. These genomic differences have been exploited to develop a multiplex PCR to accurately assign isolates to the proposed species [40].

The importance of segregating *F. columnare* into four species is illustrated by host and virulence differences. Laboratory infection studies demonstrated that genetic group 1 isolates are highly virulent in rainbow trout, while genetic group 3 and 2 isolates exhibited moderate and low virulence, respectively [19]. These data support the observation that most isolates recovered from columnaris disease cases in rainbow trout and other salmonids belong to genetic group 1, and genetic group 3 isolates may be problematic for the industry [17,41]. It is of interest that isolates in these groups were capable of growth at colder temperatures, possibly providing a basis for this association. Genetic group 2 isolates appear to be more virulent than isolates belonging to genetic groups 1 and 3

in channel catfish [63]. Finally, an association between tilapia and genetic group 4 isolates has been documented and this genetic group has only been recovered from Asia and South/Central America [41]. Since these pathogens are globally distributed and have significant impacts on wild and cultured fish species, recognition of the four species will advance and improve research to define host-pathogen-environment relationships, epidemiology, and develop effective control and prevention measures in aquaculture. Such research needs to target the correct bacterial species and research findings can be properly interpreted by correct and consistent taxonomic assignment.

Based on these findings, it is proposed that genetic group 1 isolates remain recognized as *F. columnare* while isolates of genetic groups 2, 3, and 4 be recognized as novel *Flavobacterium* species for which the names *Flavobacterium covae* sp. nov. (type strain AL-02-36^T = ATCC TSD-246^T = CCM 9171^T), *Flavobacterium davisii* sp. nov. (type strain 90-106^T = ATCC TSD-247^T = CCM 9172^T), and *Flavobacterium oreochromis* sp. nov. (type strain Costa Rica 04-02-TN^T = ATCC TSD-248^T = CCM 9173^T) are respectively proposed. Formal description of *Flavobacterium covae* sp. nov., *Flavobacterium davisii* sp. nov. and *Flavobacterium oreochromis* sp. nov. is given in Table 7.

Emended description of *Flavobacterium columnare* (Bernardet and Grimont 1989) Bernardet et al. 1996

The description is as before [5,7] with the additions given below. Hydrogen sulfide (H_2S) is not produced, Tween 80 is not hydrolysed, starch is hydrolysed, some isolates grow in the presence of 1.2 % NaCl, some isolates grow at temperatures of 10–14 °C and up to 38–39 °C. Grows from pH 6.3–9.0, with some isolates exhibiting growth at pH of 5.9–6.0 and pH 9.5. All isolates grow in microaerophilic atmosphere except for type strain ATCC 23463^T. All isolates grow on nutrient agar. Positive by Gen III microplates (Biolog) for D-fructose-6-PO4, glucuronamide, acetoacetic acid, vancomycin, tetrazolium violet, tetrazolium blue, and aztreonam. Major respiratory quinone is MK-6. Polar lipid profile consists of the major polar lipid phosphatidylethanolamine (PE), an unidentified aminolipid (AL), and two unidentified lipids, L1 and L2. Main spectrum profile of whole cell proteins is characteristic with absence of peaks 3606, 6525, and 6540 *m/z*.

The GenBank/EMBL/DDBJ accession numbers for the near full length 16S rRNA gene sequences and whole genome sequences of *Flavobacterium covae* sp. nov. AL-02-36^T (=ATCC TSD-246^T = CCM 9171^T), *Flavobacterium davisii* sp. nov. 90-106^T (=ATCC TSD-247^T = CCM 9172^T) and *Flavobacterium oreochromis* sp. nov. Costa Rica 04-02-TN^T (=ATCC TSD-248^T = CCM 9173^T) are MW353013, MW353014, KX711894 and CP067379, CP067378, CP067377, respectively. Eight supplementary figures and four supplementary tables are available with the online version of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2021.126293.

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