Journal of Fish Diseases 2013, **36**, 447–451 doi:10.1111/jfd.12005

Short Communication

First record of the fish pathogen *Flavobacterium columnare* genomovar II from bluegill, *Lepomis macrochirus* (Rafinesque), with observations on associated lesions

S A Bullard, H Mohammed and C R Arias

Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL, USA

Keywords: bluegill, *Flavobacterium columnare*, genomovar II, *Lepomis macrochirus*.

Flavobacterium columnare is the causative agent of 'columnaris disease' (Davis 1922), a disease that kills or debilitates a wide ecological and phylogenetic spectrum of temperate and tropical freshwater fish (Bernardet 1989; Bernardet & Grimont 1989; Bernardet *et al.* 1996; Olivares-Fuster *et al.* 2007; Bullard, McElwain & Arias 2011). It is regarded as a predominant pathogen of fish maintained in both food production aquaculture systems and the ornamental fish trade (Decostere *et al.* 1999a,b; Schneck & Caslake 2006), and it is of special interest in warm-water aquaculture ponds in the southeastern US, where it is regarded as one of the primary bacterial pathogens that constrains culture of channel catfish, *Ictalurus punctatus* (Rafinesque) (see Thune, Stanley & Cooper 1993; Plumb 1999). Much of the biological information about this bacterium derives from aquaculture epizootics (Olivares-Fuster *et al.* 2007, 2011), wherein columnaris disease is marked by epidermal sloughing and scale loss concomitant with a symmetrical body discolouration that has been called 'saddleback' lesion (Cone, Miller & Austin 1980; Bullard *et al.* 2011). The 3 genomovars (Ursing *et al.* 1995) of *F. columnare* (see Triyanto & Wakabayashi 1999) exhibit differential virulence (Arias *et al.* 2004; Thomas-Jinu & Goodwin 2004; Darwish & Ismaiel 2005; Shoemaker *et al.* 2008), but information on their prevalence, host distribution and pathobiology is largely indeterminate.

Bluegill, *Lepomis macrochirus* Rafinesque, is a widely introduced centrarchid that ranges throughout the Mississippi River and adjacent basins in North America. This fish was co-introduced as a forage fish for the recreationally prized largemouth bass, *Micropterus salmoides* (Lacépède), and its populations thrive along with those of largemouth bass in lakes, ponds and creek pools as well as densely vegetated shorelines of impoundments (Boschung & Mayden 2004). Bluegill is a generalist omnivore that tolerates a wide range of salinities and thermal regimes, which has contributed to its high level of invasiveness: now a documented invasive species in >20 countries (Kawamura *et al.* 2006; Fuller, Nico & Williams 1999).

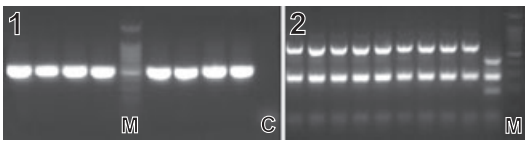
During 19–23 April 2012, a significant bluegill kill occurred throughout a series of hydrologically linked earthen ponds located in east-central Alabama (32°40′51.89″N; 85°30′39.73″W). Moribund bluegill were sampled for bacterial analysis with a sterile loop and cultured on Shieh agar supplemented with tobramycin at a concentration of 1 μg mL^{-1} (Decostere, Haesebrouck & Devriese 1997) at 28 °C for 48 h. Rhizoid, yellow-pigmented colonies were considered putative *F. columnare* cultures and were re-isolated on Shieh agar lacking antibiotic. Pure cultures were incubated in Shieh broth at 28 °C with shaking

Correspondence *S A Bullard Department of Fisheries and Allied Aquacultures, 203 Swingle Hall, Auburn University, Auburn, Alabama 36849, USA (e-mail: ash.bullard@auburn.edu)*

Journal of Fish Diseases

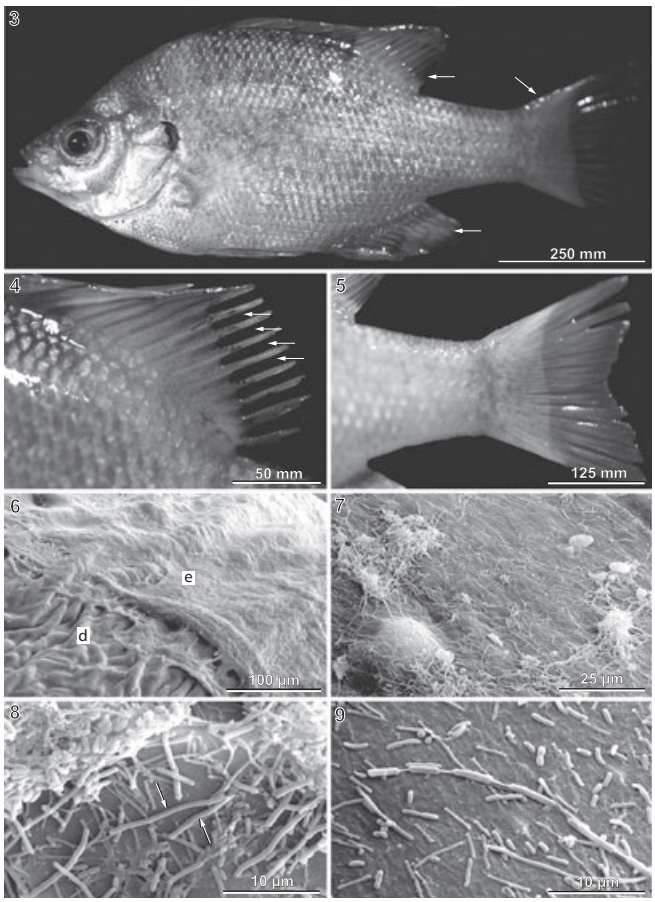
for 24 h, and DNA extraction was conducted using the DNeasy Tissue kit (Qiagen) following the manufacturer's instructions. Definitive identification of putative isolates used specific *F. columnare*-PCR as per Welker *et al.* (2005). Confirmed isolates were ascribed to genomovars by restriction fragment length polymorphism (RFLP) of the 16S rRNA gene according to Triyanto & Wakabayashi (1999). After bacterial samples were taken, lesioned fish ($n = 7$) were photographed and routinely processed for scanning electron microscopy (SEM) and histopathology. For SEM, gill arches (including filaments and lamellae) plus skin samples approximately 5–10 mm^2 were excised from the margin of the saddleback lesion, from within the lesioned area, and from a site adjacent to the lesion. Tissues were dehydrated in a graded ethanol (EtOH) series, transferred from 100% EtOH to a 50:50 (v:v) of 100% EtOH and hexamethyldisilazane (HMDS) for 1 h, immersed in HMDS for 1 h, air-dried for 3 h, mounted on metal stubs and sputter coated with 15 nm of gold palladium (Bullard *et al.* 2011). For histopathology, portions of lesioned and non-lesioned skin, gill and kidney were immersed in 10% neutral buffered formalin for at least 48 h, dehydrated in a graded series of EtOHs, embedded in paraffin, sectioned at 4 μm, routinely stained with haematoxylin and eosin and mounted on glass slides.

All sampled bluegill yielded rhizoid, yellow colonies were confirmed as *F. columnare* by specific PCR (Fig. 1), and 16S-RFLP analyses ascribed all of those isolates to genomovar II (Fig. 2). Grossly, moribund bluegills (Fig. 3) lacked the characteristic body colouration of unaffected bluegill gathered from the same pond. Lesions in these bluegills comprised a pale, discoloured region of the body surface immediately beneath the spinous portion of the dorsal fin (Figs 3 & 4), about the caudal peduncle (Fig. 5) and on the anal fin (Fig. 3). The lesioned area at the level of the spinous dorsal fin was bilaterally symmetrical, and the membranous tissue between the dorsal fin spines was partially absent, seemingly eroded (Fig. 4). No scale loss was observed grossly. SEM revealed regions of discontinuous epidermis (Fig. 6). The underlying dermis was highly rugose (Fig. 6), and the epidermis was covered by a dense field of bacteria showing the typical morphology of *F. columnare* cells (extremely elongate bacilli) (Figs 7 & 8). In some areas, where the presumptive *F. columnare* cells were less abundant, we observed other variously shaped bacterial cells, including streptobacilli, short rods and cocci (Fig. 9). Histopathology (Figs 10–15) revealed that the lesioned tegument of the moribund bluegill was extensively altered (epidermal hyperplasia), and the underlying dermis was infiltrated by lymphocytes (Fig. 12). Numerous gill lamellae of moribund bluegill were fused and, typically, interlamellar water channels were obstructed by hyperplastic gill epithelial cells (Figs 13 & 14) and cellular debris. Numerous proximal tubules of the kidney in moribund bluegill harboured abundant rodlet cells (Fig. 15). No metazoan parasite was observed in any tissue section.



Figures 1–2 Molecular typing. Fig. 1. Identification of *Flavobacterium columnare* genomovar II isolates (lanes 1–4 and 6–9) from bluegill, *Lepomis macrochirus*; lane 5 is the 100-bp molecular marker (M) and lane 9 is the non-template PCR control (C). Fig. 2. 16S-RFLP analysis of selected *F. columnare* genomovar II isolates (lanes 1–8) from bluegill; lane 9 is strain BGFS-27 (control for *F. columnare* genomovar II); lane 10 is strain ARS-1 (control for *F. columnare* genomovar I); and lane 11 is the 1-kb molecular marker (M).

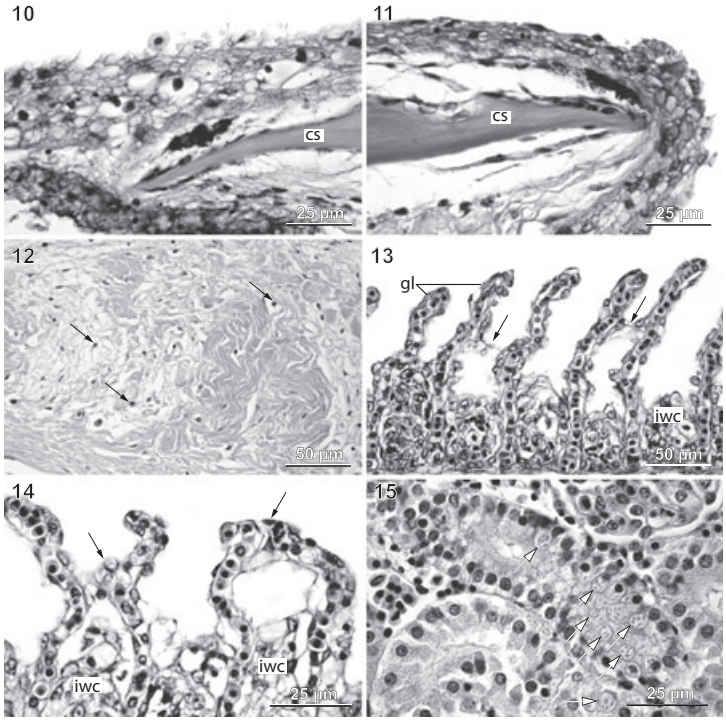
This study represents the first published record of *F. columnare* genomovar II from bluegill. A review of the peer-reviewed literature plus a perusal of accession data from the American Type Culture Collection (Manassas, VA, USA), Spanish Type Culture Collection (Valencia, Spain), Japanese Type Culture Collection (Saitama, Japan) and NCBI GenBank as well as personal correspondence with J. F. Bernardet (French National Institute for Agricultural Research, Paris, France) failed to identify any previous documentation of this bacterial genomovar–fish association. In 1986, several years before Triyanto & Wakabayashi (1999) first isolated genomovar II, a putative infection by *F. columnare* (as *Flexibacter*) in bluegill was detailed in a leaflet authored by Bullock and colleagues (US Fish & Wildlife Service, Fish Disease Leaflet 72). This report could have represented *F. columnare* genomovar II because it detailed an acute lesion that was similar to the one we report herein, that is, skin lesions involving outer margins of fins. The present study provides further pathological details of infection by this particular



Figures 3–9 Light and scanning electron microscopy of bluegill, *Lepomis macrochirus*, from an earthen pond in east-central Alabama and harbouring *Flavobacterium columnare* genomovar II. Scale bar values beside each bar. Fig. 3. Skin discolouration (arrows) consistent with infection by *F. columnare* at base and proximal portion of spinous dorsal fin, base and proximal portion of caudal fin and distal portion of anal fin. Fig. 4. Higher magnification view of Fig. 3 showing skin discolouration about dorsal fin. Note that the membranous portion of the spinous dorsal fin is absent (arrows) and likely eroded. Fig. 5. Higher magnification view of Fig. 3 showing skin discolouration of caudal fin. Fig. 6. Sloughing epidermis (e) and rugose, exposed dermis (d) along lesion margin beneath spinous dorsal fin. Fig. 7. Bacterial cells dispersed across surface of epidermis beneath soft-ray portion of dorsal fin adjacent to lesion margin. Fig. 8. Higher magnification view of Fig. 7, showing probable cells of *F. columnare* (arrows). Fig. 9. Streptobacilli, short rod-shaped bacterial cells as well as cocci.

genomovar on bluegill, and the alterations to skin and gill seemed consistent with, although perhaps more qualitatively severe than, previously documented infections of *F. columnare* in other fish (Cone *et al.* 1980; Bullard *et al.* 2011).

Information on both the fish–host distribution and geographical distribution of *F. columnare* genomovar II, the most virulent genomovar among warm-water fish (Triyanto & Wakabayashi 1999; Arias *et al.* 2004; Olivares-Fuster *et al.* 2007, 2011; Shoemaker *et al.* 2008), is critically important to those attempting to control columnaris disease in warm-water aquaculture settings. Our results indicate that bluegills are susceptible to morbidity and mortality associated with infection by *F. columnare* genomovar II (Figs 3–15). Hence, fish aquaculturists should avoid polyculture including bluegill because this fish may function as a source population for the pathogen. That bluegill harbour infections of *F. columnare* genomovar II is likewise also significant to freshwater fish conservation biology because bluegill is a widely introduced, highly invasive species that ranges worldwide and throughout temperate freshwater ecosystems (Fuller *et al.* 1999; Kawamura *et al.* 2006). If carrying this genomovar, bluegill may vector it to naive, endemic fish populations. The acute nature of the columnaris disease we observed herein seemingly precludes



Figures 10–15 Histology of bluegill, *Lepomis macrochirus*, from an earthen pond in east-central Alabama and harbouring *Flavobacterium columnare* genomovar II. Scale bar values beside each bar. Fig. 10. Skin about insertion of ctenoid scale (cs), showing hyperplastic epithelium. Fig. 11. Skin about distal edge of ctenoid scale (cs), showing similar features as Fig. 10 along with evidence of pronounced epidermal separation from scale. Fig. 12. Stratum compactum showing lymphocytic infiltrates (arrows) and probable focus of water-logging. Fig. 13. Fusion (arrows) of gill lamellae (gl) and obstruction of interlamellar water channels (iwc). Fig. 14. Lamellar fusion (arrows) and obstructed interlamellar water channels (iwc). Fig. 15. Rodlet cells (arrows) in wall of a kidney proximal tubule.

bluegill as a subclinical carrier of this pathogen, but bacteriological screening of hatchery-raised bluegill should nevertheless precede introducing them into waters within their native range.

Abundant rodlet cells were in the kidney (Fig. 15) of moribund bluegill with clinical signs of acute columnaris disease. Although sometimes associated with parasitic infections (Blaylock, Bullard & Whipps 2004; Leino 1995), these cells contain the antimicrobial peptide piscidin (Reite & Evensen 2006; Silphaduang, Colorni & Noga 2006; Corrales *et al.* 2010) and could have a relationship with bacterial disease. Our present data cannot strongly support that notion, but bluegill and *F. columnare* may comprise an accessible and cost-effective model to conduct future studies testing this association.

Acknowledgements

We thank Dr Russell Wright for alerting us to the bluegill epizootic. This is a contribution of the Southeastern Cooperative Fish Parasite and Disease Project and was supported by NSF-DEB 1112729, 1051106 and 1048523 and an Alabama Agricultural Experiment Station HATCH award.

References

Arias C.R., Wheeler T.L., Shoemaker C.A., Abernathy J.W. & Klesius P.H. (2004) Genetic fingerprinting of *Flavobacterium columnare* isolates form cultured fish. *Journal of Applied and Environmental Microbiology* **97**, 421–428.

Bernardet J.F. (1989) '*Flexibacter columnaris*': first description in France and comparison with bacterial strains from other origins. *Diseases of Aquatic Organisms* **6**, 37–44.

Bernardet J.F. & Grimont P.A.D. (1989) Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. no., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida, and Masumura 1986. *International Journal of Systematic Bacteriology* **39**, 346–354.

Bernardet J.F., Sergers P., Vancanneyt M., Berthe F., Kersters K. & Vandamme P. (1996) Cutting a Gordian knot:

emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym *Cytophaga aquatilis* Strohl and Tait 1978). *International Journal of Systematic Bacteriology* **46**, 128–148.

Blaylock R.B., Bullard S.A. & Whipps C.M. (2004) *Kudoa hypoepicardialis* n. sp. (Myxozoa: Kudoidae) and associated lesions from the heart of seven perciform fishes in the northern Gulf of Mexico. *Journal of Parasitology* **90**, 584–593.

Boschung H.T. & Mayden R.L. (2004) *Fishes of Alabama*. Smithsonian Books, Washington, DC.

Bullard S.A., McElwain A. & Arias C.R. (2011) Scanning electron microscopy of "saddleback" lesions associated with experimental infections of *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus* (Siluriformes: Ictaluridae), and zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae). *Journal of the World Aquaculture Society* **42**, 906–913.

Cone D.K., Miller J.D. & Austin W.K. (1980) The pathology of "saddleback" disease of underyearling Atlantic salmon (*Salmo salar*). *Canadian Journal of Zoology* **58**, 1283–1287.

Corrales J., Mulero I., Mulero V. & Noga E.J. (2010) Detection of antimicrobial peptides related to piscidin 4 in important aquacultured fish. *Developmental and Comparative Immunology* **34**, 331–343.

Darwish A.M. & Ismaiel A.A. (2005) Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S-23S rDNA spacer. *Molecular and Cellular Probes* **19**, 267–274.

Davis H.S. (1922) A new bacterial disease of fresh-water fishes. *US Bureau of Fisheries Bulletin* **38**, 261–280.

Decostere A., Haesebrouck F. & Devriese L.A. (1997) Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. *Journal of Clinical Microbiology* **35**, 322–324.

Decostere A., Haesebrouck F., Charlier G. & Ducatelle R. (1999a) The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). *Veterinary Microbiology* **67**, 287–298.

Decostere A., Haesebrouck F., Van Driessche E., Charlier G. & Ducatelle R. (1999b) Characterization of the adhesion of *Flavobacterium columnare* (*Flexibacter columnare*) to gill tissue. *Journal of Fish Diseases* **22**, 465–474.

Fuller P.L., Nico L.G. & Williams J.D. (1999) *Nonindigenous Fishes Introduced Into Inland Waters of the United States*. Special Publication 27. American Fisheries Society, Bethesda, MD.

Kawamura K., Yonekura R., Katano O., Taniguchi Y. & Saitoh K. (2006) Origin and dispersal of bluegill sunfish, *Lepomis macrochirus*, in Japan and Korea. *Molecular Ecology* **15**, 613–621.

Leino R.L. (1995) Reaction of rodlet cells to a myxosporean infection in kidney of the bluegill, *Lepomis macrochirus*. *Canadian Journal of Zoology* **74**, 217–225.

Olivares-Fuster O., Baker J.L., Terhune J.S., Shoemaker C.A., Klesius P.H. & Arias C.R. (2007) Host-specific association between *Flavobacterium columnare* genomovars and fish species. *Systematic and Applied Microbiology* **30**, 624–633.

Olivares-Fuster O., Bullard S.A., McElwain A., Jose Llosa M. & Arias C.R. (2011) Kinetics of *Flavobacterium columnare* adhesion to channel catfish (*Ictalurus punctatus*) and zebra danio (*Danio rerio*) tissues after immersion challenge. *Diseases of Aquatic Organisms* **96**, 221–227.

Plumb J.A. (1999) *Health Maintenance and Principal Microbial Diseases of Cultured Fish*. Iowa State University Press, Ames, Iowa.

Reite O.B. & Evensen O. (2006) Inflammatory cells of teleostean fish: a review focusing on mast cell/eosinophilic granule cells and rodlet cells. *Fish and Shellfish Immunology* **20**, 192–208.

Schneck J.L. & Caslake L.F. (2006) Genetic diversity of *Flavobacterium columnare* isolated from fish collected from warm and cold water. *Journal of Fish Diseases* **29**, 245–248.

Shoemaker C.A., Olivares-Fuster O., Arias C.R. & Klesius P. H. (2008) *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*). *Veterinary Microbiology* **127**, 353–359.

Silphaduang U., Colorni A. & Noga E.J. (2006) Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. *Diseases of Aquatic Organisms* **72**, 241–252.

Thomas-Jinu S. & Goodwin A.E. (2004) Acute columnaris infection in channel catfish, *Ictalurus punctatus* (Rafinesque): efficacy of practical treatments for warmwater aquaculture ponds. *Journal of Fish Diseases* **27**, 23–28.

Thune R.L., Stanley L.A. & Cooper R.K. (1993) Pathogenesis of Gram negative bacterial infections in warmwater fish. *Annual Review of Fish Diseases* **3**, 37–68.

Triyanto & Wakabayashi H. (1999) Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes. *Fish Pathology* **34**, 65–71.

Ursing J.B., Rossello-Mora R., Garcia-Valdes E. & Lalucat J. (1995) Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. *International Journal of Systematic Bacteriology* **45**, 604.

Welker T.L., Shoemaker C.A., Arias C.R. & Klesius P.H. (2005) Transmission and detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus*. *Diseases of Aquatic Organisms* **63**, 129–138.

Received: 20 June 2012
Revision received: 31 July 2012
Accepted: 8 August 2012

