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Genetic and serological diversity of *Flavobacterium psychrophilum* isolates from salmonids in United Kingdom

Thao P. H. Ngo[1], Kerry L. Bartie[1], Kim D. Thompson[1, 2], David W. Verner-Jeffreys[3], Rowena Hoare[1], Alexandra Adams[1]

[1] Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK

[2] Moredun Research Institute, Pentlands Science Park, Penicuik, EH26 0PZ, UK

[3] The Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Barrack Road, Weymouth, DT4 8UB, UK

Corresponding author: Thao Phuong Huynh Ngo, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA. E-mail: tpn1@stir.ac.uk

Abstract

Flavobacterium psychrophilum is one of the most important bacterial pathogens affecting cultured rainbow trout (*Oncorhynchus mykiss*) and is increasingly causing problems in Atlantic salmon (*Salmo salar* L.) hatcheries. Little is known about the heterogeneity of *F. psychrophilum* isolates on UK salmonid farms. A total of 315 *F. psychrophilum* isolates, 293 of which were collected from 27 sites within the UK, were characterised using four genotyping methods and a serotyping scheme. A high strain diversity was identified among the isolates with 54 pulsotypes, ten $(GTG)_5$-PCR types, two 16S rRNA allele lineages, seven plasmid profiles and three serotypes. Seven PFGE groups and 27 singletons were formed at a band similarity of 80%. PFGE group P (n= 75) was found to be numerically predominant in eight sites within the UK. Two major PFGE clusters and 13 outliers were found at the band similarity of 40%. The predominant profile observed within the UK *F. psychrophilum* isolates examined was PFGE cluster II – $(GTG)_5$-PCR type r1 – 16S rRNA lineage II – serotype Th (70/156 isolates examined, 45 %). Co-existence of genetically and serologically heterogeneous isolates within each farm was detected, confounding the ability to control RTFS outbreaks. The occurrence over time (up to 11 years) of *F. psychrophilum* pulsotypes in three representative sites (Scot I, Scot III and Scot V) within Scotland was examined, potentially providing important epidemiological data for farm management and the development of site-specific vaccines.

Keywords: Rainbow trout fry syndrome, bacterial cold water disease, *Flavobacterium psychrophilum*, genotyping, serotyping, plasmids

1. Introduction

Flavobacterium psychrophilum, a Gram-negative, filamentous, psychrotrophic bacterium first isolated in 1948, is an important bacterial pathogen in salmonid culture industry worldwide (Borg, 1948). It has been described as the aetiological agent of rainbow trout fry syndrome

(RTFS) and bacterial cold water disease (BCWD) (Bernardet et al. 1996; Faruk, 2002; Starliper, 2011). Its presence on fish farms requires close surveillance and the use of antimicrobial agents to control the disease. Although many attempts have been made to develop a broad spectrum (as opposed to an autogenous) commercial vaccine against RTFS during the last 20 years, new strategies and initiatives are still needed for vaccine development, which is hindered by the prevalence of a wide range of *F. psychrophilum* strains (Gómez et al. 2014). Hence, the analysis of genetic diversity and population structure of this bacterium using molecular typing methods is essential to improve our understanding of this pathogen and, in turn, disease control at farm level.

Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) can be used to investigate the genetic variation of a bacterial population. With its high discriminatory ability and reproducibility, PFGE has been used successfully for molecular epidemiological characterisation of *F. psychrophilum* isolates in Japan (Arai et al. 2007), USA (Chen et al. 2008), Chile (Avendaño-Herrera et al. 2009), Spain (Del Cerro et al. 2010) and Finland (Sundell et al. 2013). These studies have shown that the genetic diversity of *F. psychrophilum* in these areas was associated with both geographical origin and the fish host from which the isolates were collected. Furthermore, PFGE analysis has been used to determine the source of BCWD infections (Arai et al. 2007) and to investigate the horizontal and vertical transmission of *F. psychrophilum* within and between facilities (Chen et al. 2008).

Serotyping is useful for both diagnosis and seroepidemiological studies. Three serotypes (Th, Fd, Fp^T) established by Lorenzen and Olesen (1997) and three O-antigen groups (O-1, O-2 and O-3) established by Izumi and Wakabayashi (1999) are most commonly used for serotyping *F. psychrophilum* (Madsen and Dalsgaard, 2000). Some serotypes were clearly associated with the fish species from which the isolates were collected. Serotypes Th and O-3 are proposed to be broadly similar and predominant among *F. psychrophilum* strains isolated from rainbow trout in

RTFS/BCWD outbreaks (Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999; Mata et al. 2002).

In the UK, *F. psychrophilum* was first reported in diseased rainbow trout in 1992 (Santos et al. 1992). However, to date, no studies on the epidemiology of *F. psychrophilum* strains recovered in the UK have been published. Understanding the population structure of this fish pathogen is important for predicting outbreaks and setting up effective RTFS/BCWD control strategies, such as vaccination programs. The aim of this study was to describe the diversity of *F. psychrophilum* isolates from affected farms within the UK using a combination of molecular and serotyping methods.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

A total of 315 *F. psychrophilum* isolates were included in this study of which the majority of the isolates (214) were collected from diseased fish, five isolates from apparently healthy fish and the remaining 96 isolates from fish with unknown health status. From 63 sampling points, 293 *F. psychrophilum* isolates were collected from 27 sites within the UK, two of which were unknown, between 2005 – 2015 (20 sites in Scotland, six in England and one in Northern Ireland), and ten isolates were from three farms within France and Ireland (Table 1). Forty-two *F. psychrophilum* isolates collected from 29 sampling points and 12 reference strains (Table 2) in this collection were supplied by nine sources in the UK, Ireland, Denmark, Finland, France, Chile and USA. On 24 sampling occasions, multiple colonies were selected from the primary isolation plate of an infected fish sample in order to monitor the genetic variation within the *F. psychrophilum* strain population.

The presence of *F. psychrophilum* was confirmed using a nested PCR method targeting the 16S ribosomal RNA gene, as described by Toyama et al. (1994). For all the experiments, the *F.*

psychrophilum isolates were routinely grown in modified Veggietone (MV) medium [veggitones GMO-free soya peptone (Oxoid, UK), 5 g l^{-1}; yeast extract (Oxoid, UK), 0.5 g l^{-1}; magnesium sulphate heptahydrate (Fisher chemicals, UK), 0.5 g l^{-1}; anhydrous calcium chloride (BHD), 0.2 g l^{-1}; dextrose (Oxoid, UK), 2 g l^{-1}; agar (solid medium; Oxoid, UK), 15 g l^{-1}; pH 7.3] at 15 ^{o}C for 72 – 96 h. The broth culture was shaken at 140 rpm. Stock cultures were maintained at -70 ^{o}C in tryptone–yeast extract–salts medium supplemented with glucose [FLP – tryptone (Oxoid, UK), 4.0 g L^{-1}; yeast extract, 0.4 g L^{-1}; anhydrous calcium chloride, 0.2 g L^{-1}; magnesium sulphate heptahydrate, 0.5 g L^{-1}; D(+)-glucose (Sigma, UK), 0.5 g L^{-1}; Cepeda et al. 2004] with 10% glycerol and on Protect-Multi-purpose cryobeads (Technical Service Consultants Ltd, UK).

2.2. Macrorestriction analysis by pulsed-field gel electrophoresis (PFGE)

The PFGE protocol was performed as described previously (Bartie et al. 2012) on the 315 *F. psychrophilum* isolates using restriction enzyme *Sac*I (New England BioLabs, UK) (Chen et al. 2008). The electrophoresis conditions comprised switch times of 2 - 6 s at 200 V at 15 ^{o}C for 37 h. Following electrophoresis, the gel was stained for 30 min in 1 µg mL^{-1} ethidium bromide (Sigma, UK), and then destained in Milli-Q distilled water (DW) for at least 1 h. A low range PFG marker (New England BioLabs, UK) was used as a molecular size marker.

Table 1. Details of 303 *F. psychrophilum* isolates collected from 27 fish farm sites within the UK and three farms in Europe during 2005 – 2015

Site	Year of isolation	No. of sampling times	Host source	No. of individual fish sampled	No. of isolates
Scot I	2005 - 2015	16	RT	35	87
Scot II	2013	2	RT	2	2
Scot III	2011 - 2015	5	RT	13	44
Scot IV	2013	1	RT	3	5
Scot V	2013 - 2015	4	RT	25	55
Scot VI	2009	1	RT	1	1
Scot VII	2007	1	RT	1	1
Scot VIII	2005	1	RT	1	1
Scot IX	2006	1	AS	1	1
Scot X	2011 - 2013	3	AS	3	3
Scot XI	2015	1	AS	4	4
Scot XII	2010	1	AS	1	1
Scot XIII	2005	1	AS	1	1
Scot XIV	2013	2	AS	2	2
Scot XV	2013	2	AS	8	8
Scot XVI	2014 - 2015	4	RT	8	14
Scot XVII	2007	1	RT	1	1
Scot XVIII	2009	1	RT	1	1
Unknown	2009-2012	2	RT	2	2
Sub-total Scotland	*2005 - 2015*	*50*	*RT/AS*	*113*	*234*
Eng I	2013	3	RT	8	24
Eng II	2015	1	RT	4	13

Eng III	2015	1	RT	1	2
Eng IV	2015	1	RT	1	1
Eng V	2007	1	RT	1	1
Eng VI	2007	1	RT	1	1
N Ire I	2013	2	RT	9	17
Sub-total UK	*2005 - 2015*	*10*	*RT/AS*	*138*	*293*
Ireland	2006	1	AS	1	1
France	Unknown - 2013	2	RT	5	9
	Total	63		144	303

RT, rainbow trout; AS, Atlantic salmon.

Table 2. Origins and typing summary of the twelve reference strains of *F. psychrophilum* used in this study

Strains	Isolation source	Year	Host source	Pulsotype	Serotype	rep-PCR profile	16S rRNA allele	Plasmid profile	MLST based sequence type[4]
1/3 Th 1994 [1]	Denmark	1994	RT	T3	Th	r1a	CSF	p1	
NCIMB 13383 [1]	Denmark	1990	RT	T3	Th	r1a	CSF	p1	ST2
NCIMB 13384 [1]	Denmark	1990	RT	U1	Fd	r1a	CSF	p1	
P13 3/96 Th [2]	Finland	1996	RT	T3	Th	r1a	-	p1	
P5 10/96 Th [2]	Finland	1996	RT	T3	Th	r1a	CSF	p1	
P8 3/96 Fd [2]	Finland	1996	RT	T3	Fd	r1a	-	p1	
32/97 chile [3]	Chile	1997	RT	H	Fd	r2	CSF	p1	
59/95 chile [3]	Chile	1995	RT	T6	Fd	r1a	CSF	p8	
NCIMB 1947^T	USA	unknown	CS	Y	Fp^T	r1b	Both	p2	ST13
CSF 259-93	USA	1993	RT	N	Fd	r1a	CSF	p1	ST10
046-04 Idaho	USA	2004	RT	K	Th	r1b	CSF	p1	
302-95, Idaho	USA	1995	RT	L1	Fd	r1a	CSF	p1	

RT, rainbow trout; CS, coho salmon;

Reference: [(1)] Lorenzen and Olesen, 1997; [(2)] Madetoja et al. 2001; [(3)] Faruk et al. 2002; [(4)] Online multilocus sequence typing database for *F. psychrophilum*: http://pubmlst.org/fpsychrophilum

2.3. Repetitive PCR (rep-PCR) genomic fingerprinting

All the 315 *F. psychrophilum* isolates were typed using rep-PCR based on the single $(GTG)_5$ repetitive primer as previously described (Bartie et al. 2012). Ten microlitres of each PCR product was separated on a 1.5 % UltraPure and trade Agarose-1000 (Invitrogen, Fisher Scientific, UK) in chilled 0.5 X TAE buffer [20 mM Tris, 10 mM acetic acid (Fisher chemicals, UK), and 0.5 mM ethylenediaminetetraacetic acid (EDTA; Sigma, UK)]. A GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK) was used as molecular size marker. Following electrophoresis, the gels were stained and destained as described above.

2.4. 16S rRNA allele-specific PCR assay

To investigate the presence of one or both 16S rRNA alleles, a PCR assay was performed as described by Ramsrud et al. (2007) on 169 representative *F. psychrophilum* isolates from different genotypes (54 pulsotypes, ten $(GTG)_5$-PCR profiles) and different origins (i.e. 30 sites, 63 sampling points and 12 reference strains) contained with the strain collection. Five microlitres of each PCR product was electrophoresed on 1.0 % (w/v) agarose gel (Bioline, UK) containing 0.1 $\mu g\ mL^{-1}$ ethidium bromide in 0.5 X TAE buffer. The GeneRuler Express DNA Ladder was used as molecular size marker to confirm the presence or absence of the coho salmon-derived ATCC 49418 (298 bp) or rainbow trout-derived CSF 259-93 (600 bp) alleles.

2.5. Plasmid profiling

Plasmid profiles of 169 isolates from representative genotypes and origins were investigated. The extraction of plasmid DNA from *F. psychrophilum* isolates was performed as previously described (Bartie et al. 2012). The approximate molecular size of the plasmids was estimated using a Supercoiled DNA ladder (New England BioLabs, UK) and the known plasmid contents of

23 two reference strains *Escherichia coli* V517 and 39R861 (Macrina et al. 1978). Plasmid profiles

24 were identified by differences in size and number of resulting DNA bands.

25 *2.6. Serological characterisation*

26 Titres of the absorbed polyclonal antisera (kindly provided by Dr Tom Wiklund from Laboratory

27 of Aquatic Pathobiology, Åbo Akademi University, Finland) against three *F. psychrophilum*

28 strains NCIMB 1947^T, NCIMB 13384 and NCIMB 13383 were determined using an enzyme

29 linked immunosorbent assay (ELISA) as previously described (Faruk et al. 2002). Two replicates

30 of dilutions at 1:20,000, 1:50,000, 1:100,000, 1:200,000, 1:500,000, 1:1,000,000 and 1:2,000,000

31 of each antiserum made in phosphate buffered saline (PBS) buffer [0.02 M sodium dihydrogen

32 phosphate (Fisher chemicals, UK), 0.02 M disodium hydrogen phosphate (Fisher chemicals, UK),

33 0.15 M sodium chloride (Oxoid, UK), pH 7.2] supplemented with 1 % bovine serum albumin

34 (BSA; Fisher chemicals, UK) were used. The titre was defined as the reciprocal value of the

35 highest dilution of a serum sample showing three times the mean absorbance value of the negative

36 control.

37 Serotypes of 169 *F. psychrophilum* strains representing different genotypes and origins were

38 examined. A bacterial suspension adjusted to a concentration of 1 x 10^8 colony forming units

39 (CFU) per millilitre and heat treated at 55 ^oC for 15 min (Lorenzen and Olesen, 1997) was used to

40 coat the plates for the ELISAs as previously described (Faruk et al. 2002).

41 *2.7. DNA fingerprinting data analysis*

42 DNA profiles resulting from the PFGE and rep-PCR methods were visualised and the gel images

43 were captured using Bioimaging INGENIUS system (Syngene, UK). Numerical analysis of the

44 DNA fingerprints was performed using Gel Compar II software (Applied Maths, Belgium).

45 Dendrograms were constructed using the unweighted average pair group method of arithmetic
46 averages (UPGMA), with the Jaccard similarity coefficient and a 0.8 % tolerance and a 0.3 %
47 optimisation (PFGE) or a 0.4 % tolerance (rep-PCR). $(GTG)_5$-PCR type was defined as a group of
48 isolates with a banding pattern similarity of ≥95 %.

49 According to the guidelines for interpreting chromosomal DNA restriction patterns produced by
50 PFGE (Tenover et al. 1995), a banding pattern similarity of ≥80 % (fewer than seven band
51 difference) was used to define a group of possibly related isolates, termed a PFGE group when
52 including more than one pulsotype, or a singleton when represented by a single pulsotype. A cut-
53 off at 95 % similarity of the Jaccard's coefficient (fewer than three bands of difference) was used
54 to indicate PFGE pulsotypes containing closely related isolates.

55 **3. Results**

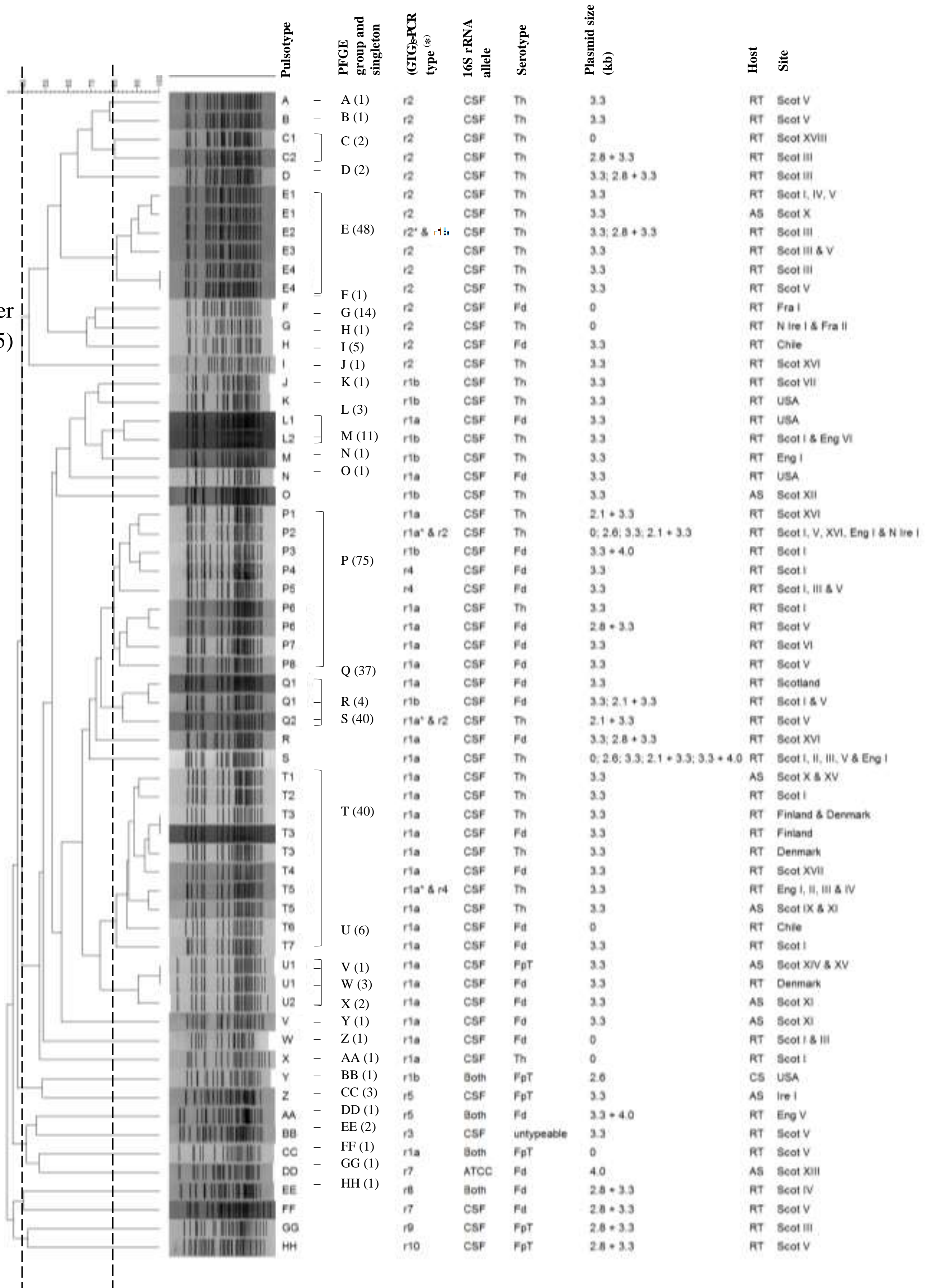
56 *3.1. Macrorestriction analysis by PFGE*

57 Three hundred and fourteen *F. psychrophilum* isolates were successfully typed using *Sac*I PFGE.
58 A total of 54 pulsotypes, each containing approximately 22 fragments ranging in size from 20 kb
59 to 200 kb were identified (Fig. 1). Seven PFGE groups (≥80 % similarity) and 27 singletons were
60 identified.

61 Two major PFGE clusters, I (n = 75) and II (n = 226), formed at a similarity of 40 % and their
62 outliers (n= 13) were revealed in the dendrogram. Cluster I was composed of two PFGE groups
63 and seven singletons (A – I) and 75 *F. psychrophilum* isolates retrieved from 11 sites in Scotland,
64 Northern Ireland, Chile and France. Cluster I was found to be associated with the large
65 predominant cluster II formed at the 47 % similarity level. Cluster II with five PFGE groups and
66 ten singletons (J – X) contained the majority of the study isolates (226/314, 72 %), 216 of which

67 **Fig. 1.** Dendrogram of 314 *F. psychrophilum* isolates constructed using the UPGMA
68 method, based on 62 PFGE banding patterns obtained using *Sac*I. For each banding
69 pattern; *Sac*I-PFGE pulsotype, pulsotype group and singleton (number of isolates),
70 $(GTG)_5$-PCR type, 16S rRNA allele, serotype, plasmid size, fish host and site code are
71 shown. A vertical line at 40% similarity that classified the isolates into three major *Sac*I-
72 PFGE clusters I and II (number of isolates are shown as well). The asterisk (*) represents
73 the dominant $(GTG)_5$-PCR type in each *Sac*I-PFGE subtype. RT, rainbow trout; AS,
74 Atlantic salmon; CS, coho salmon.

75


Cluster I (75)
Pulsotype
PFGE group and singleton
(GTG)5-PCR type (*)
16S rRNA allele
Serotype
Plasmid size (kb)
Host
Site
A (1)
B (1)
C (2)
D (2)
E (48)
F (1)
G (14)
H (1)
I (5)
J (1)
K (1)
L (3)
M (11)
N (1)
O (1)
P (75)
Q (37)
R (4)
S (40)
T (40)
U (6)
V (1)
W (3)
X (2)
Y (1)
Z (1)
AA (1)
BB (1)
CC (3)
DD (1)
EE (2)
FF (1)
GG (1)
HH (1)
A r2 CSF Th 3.3 RT Scot V
B r2 CSF Th 3.3 RT Scot V
C1 r2 CSF Th 0 RT Scot XVIII
C2 r2 CSF Th 2.8 + 3.3 RT Scot III
D r2 CSF Th 3.3; 2.8 + 3.3 RT Scot III
E1 r2 CSF Th 3.3 RT Scot I, IV, V
E1 r2 CSF Th 3.3 AS Scot X
E2 r2* & r1b CSF Th 3.3; 2.8 + 3.3 RT Scot III
E3 r2 CSF Th 3.3 RT Scot III & V
E4 r2 CSF Th 3.3 RT Scot III
E4 r2 CSF Th 3.3 RT Scot V
F r2 CSF Fd 0 RT Fra I
G r2 CSF Th 0 RT N Ire I & Fra II
H r2 CSF Fd 3.3 RT Chile
I r2 CSF Th 3.3 RT Scot XVI
J r1b CSF Th 3.3 RT Scot VII
K r1b CSF Th 3.3 RT USA
L1 r1a CSF Fd 3.3 RT USA
L2 r1b CSF Th 3.3 RT Scot I & Eng VI
M r1b CSF Th 3.3 RT Eng I
N r1a CSF Fd 3.3 RT USA
O r1b CSF Th 3.3 AS Scot XII
P1 r1a CSF Th 2.1 + 3.3 RT Scot XVI
P2 r1a* & r2 CSF Th 0; 2.6; 3.3; 2.1 + 3.3 RT Scot I, V, XVI, Eng I & N Ire I
P3 r1b CSF Fd 3.3 + 4.0 RT Scot I
P4 r4 CSF Fd 3.3 RT Scot I
P5 r4 CSF Fd 3.3 RT Scot I, III & V
P6 r1a CSF Th 3.3 RT Scot I
P6 r1a CSF Fd 2.8 + 3.3 RT Scot V
P7 r1a CSF Fd 3.3 RT Scot VI
P8 r1a CSF Fd 3.3 RT Scot V
Q1 r1a CSF Fd 3.3 RT Scotland
Q1 r1b CSF Fd 3.3; 2.1 + 3.3 RT Scot I & V
Q2 r1a* & r2 CSF Th 2.1 + 3.3 RT Scot V
R r1a CSF Fd 3.3; 2.8 + 3.3 RT Scot XVI
S r1a CSF Th 0; 2.6; 3.3; 2.1 + 3.3; 3.3 + 4.0 RT Scot I, II, III, V & Eng I
T1 r1a CSF Th 3.3 AS Scot X & XV
T2 r1a CSF Th 3.3 RT Scot I
T3 r1a CSF Th 3.3 RT Finland & Denmark
T3 r1a CSF Fd 3.3 RT Finland
T3 r1a CSF Th 3.3 RT Denmark
T4 r1a CSF Fd 3.3 RT Scot XVII
T5 r1a* & r4 CSF Th 3.3 RT Eng I, II, III & IV
T5 r1a CSF Th 3.3 AS Scot IX & XI
T6 r1a CSF Fd 0 RT Chile
T7 r1a CSF Fd 3.3 RT Scot I
U1 r1a CSF FpT 3.3 AS Scot XIV & XV
U1 r1a CSF Fd 3.3 RT Denmark
U2 r1a CSF Fd 3.3 AS Scot XI
V r1a CSF Fd 3.3 AS Scot XI
W r1a CSF Fd 0 RT Scot I & III
X r1a CSF Th 0 RT Scot I
Y r1b Both FpT 2.6 CS USA
Z r5 CSF FpT 3.3 AS Ire I
AA r5 Both Fd 3.3 + 4.0 RT Eng V
BB r3 CSF untypeable 3.3 RT Scot V
CC r1a Both FpT 0 RT Scot V
DD r7 ATCC Fd 4.0 AS Scot XIII
EE r8 Both Fd 2.8 + 3.3 RT Scot IV
FF r7 CSF Fd 2.8 + 3.3 RT Scot V
GG r9 CSF FpT 2.8 + 3.3 RT Scot III
HH r10 CSF FpT 2.8 + 3.3 RT Scot V

77 were collected from 20 sites within the UK and the remaining ten were composed of reference
78 strains from the USA, Denmark, Chile and Finland. The reference strain CSF 259-93 (pulsotype
79 N) was found to belong to cluster II. A collection of outliers to clusters I and II composed of ten
80 remaining singletons Y – HH. The *F. psychrophilum* type strain NCIMB 1947^T (pulsotype Y) was
81 located in this collection of outliers together with 12 other isolates obtained within the UK and
82 Ireland.

83 *3.2. Rep-PCR using the (GTG)$_5$ primer*

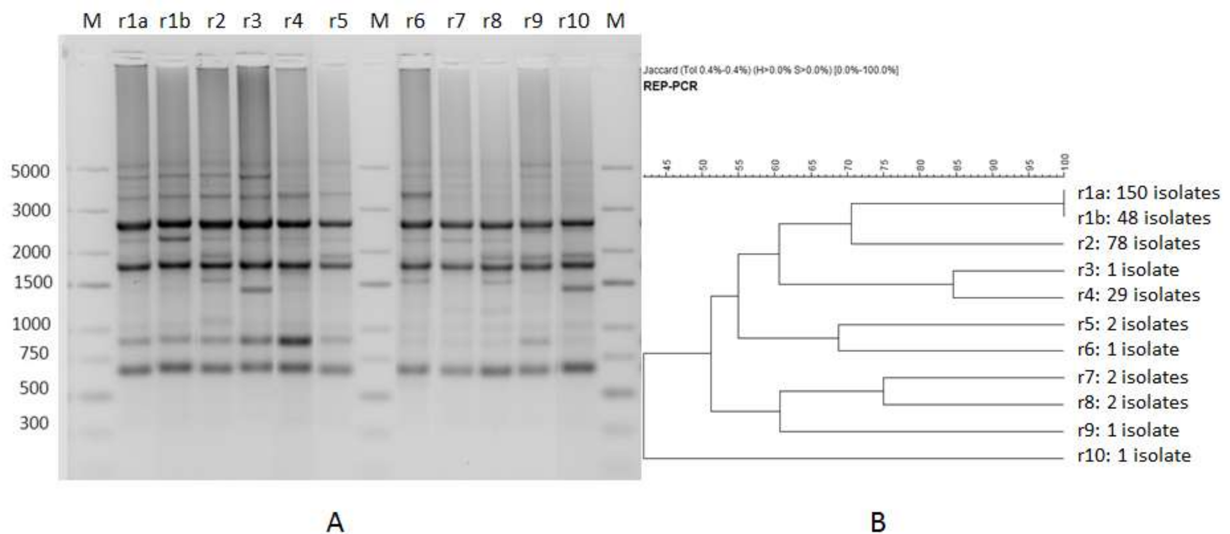
84 Analysis of the 315 *F. psychrophilum* isolates by rep-PCR using the (GTG)$_5$ primer revealed 12-
85 14 fragments ranging in size from 600 to 5000 bp (Fig. 2A), predominated by two intense bands
86 of *c.* 1800 bp and *c.* 2600 bp. Ten (GTG)$_5$-PCR types, r1 to r10, were identified based on subtle
87 differences on band position and intensity (Fig. 2B). The majority of the isolates (198/315, 63%)
88 were classified into the (GTG)$_5$-PCR type r1, which possessed two subtypes (r1a and r1b) defined
89 by a band intensity difference at 2300 bp. Isolates belonging to this major (GTG)$_5$-DNA type r1
90 were widely distributed within the UK and worldwide and recovered from three different host
91 species (rainbow trout, Atlantic salmon and coho salmon).

92 *3.3. 16S rRNA allele-specific PCR assay*

93 Only six of the 169 tested *F. psychrophilum* isolates, including the type strain NCIMB 1947^T were
94 positive for both the ATCC 49418 and the CSF 259-93 allele (five from rainbow trout and one
95 from coho salmon); one isolate obtained from rainbow trout possessed the ATCC 49418 allele
96 only; whereas all the remaining study isolates (142 from rainbow trout and 20 from Atlantic
97 salmon) contained the CSF 259-93 allele only (Table 3).

98

Fig. 2. (A) Representative ten $(GTG)_5$-PCR types obtained for 315 *F. psychrophilum* isolates tested. Lane M is the GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK). Numbers on the left indicate the size of the bands of the molecular size marker in bp. (B) Dendrogram created by the GelCompar II software package (Applied Maths, Belgium) using UPGMA with the Jaccard similarity coefficient and a 0.4% tolerance on the basis of the $(GTG)_5$-PCR profiles.



106 **Table 3.** Distribution of 16S rRNA alleles among tested *F. psychrophilum* isolates

107

Host	Lineage I		Lineage II	Total number of isolates
	ATCC allele	ATCC and CSF alleles	CSF allele	
Rainbow trout	1	5	142	148
Salmon	0	1	20	21
Total number of isolates	1	6	162	169

108

109 *3.4. Plasmid profiling*

110 Plasmid profiling detected seven distinct profiles among 169 *F. psychrophilum* isolates (p1 – p7;
111 Fig. 3). These profiles were composed of one 3.3 kb plasmid (profile p1, n= 105), one 2.6 kb
112 plasmid (p2, n= 3), one 4.0 kb plasmid (p3, n= 1), two plasmids of 3.3 and 2.1 kb (p4, n= 32), two
113 plasmids of 4.0 and 3.3 kb (p5, n= 3), two plasmids of 3.3 and 2.8 kb (p6, n= 10) or no plasmid at
114 all (p7, n= 15).

115 One hundred and fifty four isolates (91 %) possessed plasmids, of which 71 % had a single
116 plasmid and the remainder harboured a combination of two plasmids. *F. psychrophilum* type
117 strain NCIMB 1947^T possessed one plasmid of 2.6 kb. The plasmid profile (p1) was found to be
118 most widespread and detected in *F. psychrophilum* isolates retrieved from the UK (five sites in
119 England and 14 sites in Scotland), Denmark, Finland, Ireland, Chile and the USA. Profiles p3 and
120 p6 were found only in Scotland (six sites), whereas profile p4 was identified in seven sites within
121 the UK (England, one site; Scotland, five sites; and Northern Ireland, one site).

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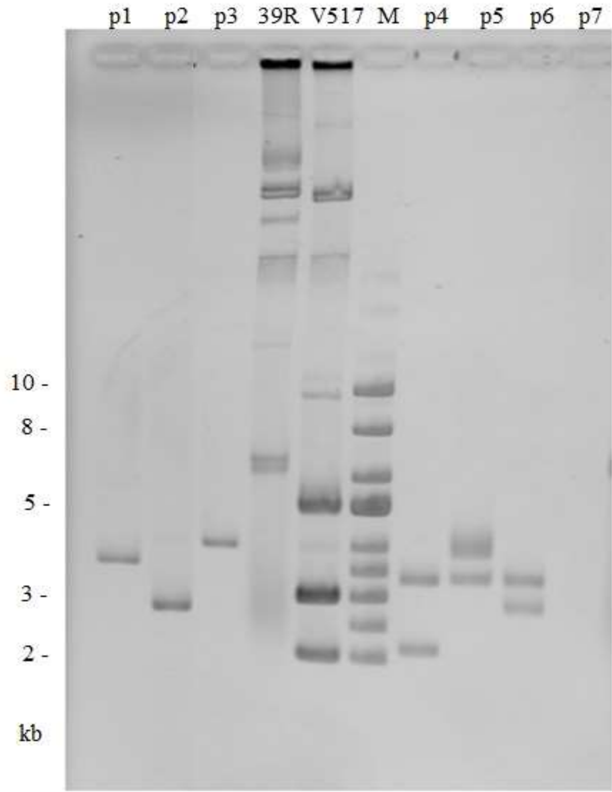

134 **Fig. 3.** Seven plasmid profiles (p – p7) identified among 185 *F. psychrophilum* isolates
135 using a 0.7 % agarose in 0.5X TAE. Lane 39R: *E. coli* 39R816 (7.0, 36-63-147 kb); Lane
136 V517: *E. coli* V517 (2.1, 2.7-3.0, 3.9, 5.1-5.5, 7.2, 54 kb); Lane M: Supercoiled DNA
137 ladder (New England BioLabs, UK). Profile p1: one 3.3 kb plasmid; p2: one 2.6 kb
138 plasmid; p3: one 4.0 kb plasmid; p4: two plasmids of 3.3 and 2.1 kb; p5: two plasmids of
139 4.0 and 3.3 kb; p6: two plasmids of 3.3 and 2.8 kb; and p7: or no plasmid.

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146 *3.5. Serotyping*

147 Antisera titres were determined by ELISA with homologous antigens (Table 4). One hundred and
148 sixty eight of the 169 isolates examined could be serotyped, with 109, 48 and 11 isolates
149 belonging to serotype Th, Fd and Fp^T, respectively. One Scottish isolate did not react with any of
150 the antisera used. No isolates cross-reacting with more than one antisera were observed. The
151 majority of isolates from rainbow trout (98/149, 66 %) and Atlantic salmon (11/19, 58 %)
152 possessed serotype Th.

153 **Table 4.** Antigens and rabbit antisera titres included in this study

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Antigen	Antiserum	Titre
F. psychrophilum NCIMB 13383	anti – Th	1,000,000
F. psychrophilum NCIMB 13384	anti – Fd	100,000
F. psychrophilum NCIMB 1947^T	anti – Fp^T	200,000

155

156 *3.6. Population analyses of the UK F. psychrophilum isolates*

157 PFGE groups E (cluster I), P, Q, T and the singleton S (cluster II) were numerically dominant and
158 accounted for 234 of the 292 study isolates (80 %). *F. pychrophilum* isolates belonging to PFGE
159 group P (n = 75) including eight pulsotypes were found to predominate within the UK originating
160 from eight sites in Scotland, England and Northern Ireland. Singleton (pulsotype) S possessed 40
161 isolates originating from four Scottish sites and one English site. Meanwhile, PFGE group E with
162 four pulsotypes (n = 48, five sites) and PFGE group Q with two pulsotypes (n = 37, three sites)
163 contained isolates exclusive to Scottish sites. PFGE group T (n = 40) comprised of seven

164 pulsotypes and appeared to be the predominant genotype of European *F. psychrophilum* isolates
165 used in this study, originating within the UK (34 isolates, six sites in Scotland and four in
166 England), Finland, Denmark as well as elsewhere in Chile. In addition to the pulsotypes detected
167 at multiple sites, most of PFGE singletons (24/27) were unique to 15 sites, e.g. singletons A, B,
168 BB, CC, FF and HH at site Scot V.

169 The majority of UK *F. psychrophilum* isolates (186/292, 64 %) were collected from three Scottish
170 sites (Scot I, Scot III and Scot V). The genetic heterogeneity over 3 – 11 years of *F.*
171 *psychrophilum* isolates within each site is summarised in Figure 4. Site Scot I included 87 isolates
172 that were collected from 35 fish samples over 16 sampling points during ten years (2005 – 2015),
173 and were categorised into five PFGE groups (E, L, Q, P, T) and three singletons (S, X, W)
174 belonging to the two major clusters. The co-infection of isolates of distinct PFGE groups in an
175 individual fish was noted in two fish.

176 In site Scot III, three PFGE groups (C, E, P) and four singletons (D, W, GG) were identified
177 among 44 isolates retrieved from 13 fish and five sampling points during 2011 – 2015. The mixed
178 infection of heterogeneous isolates was recorded in June 2015. *F. psychrophilum* isolates
179 recovered from site Scot III in this study belonged to the two major PFGE clusters and their
180 outliers.

181 Three PFGE groups (E, P, Q) and seven singletons (A, B, BB, CC, FF, HH) were recognised
182 among 55 *F. psychrophilum* isolates from site Scot V from June 2013 to June 2015 over four
183 sampling points. Six individual fish showed the co-infection of *F. psychrophilum* isolates
184 belonging to two distinct pulsotypes. Similar to site Scot III, *F. psychrophilum* isolates from site
185 Scot V were also distributed in the two major PFGE clusters and their outliers.

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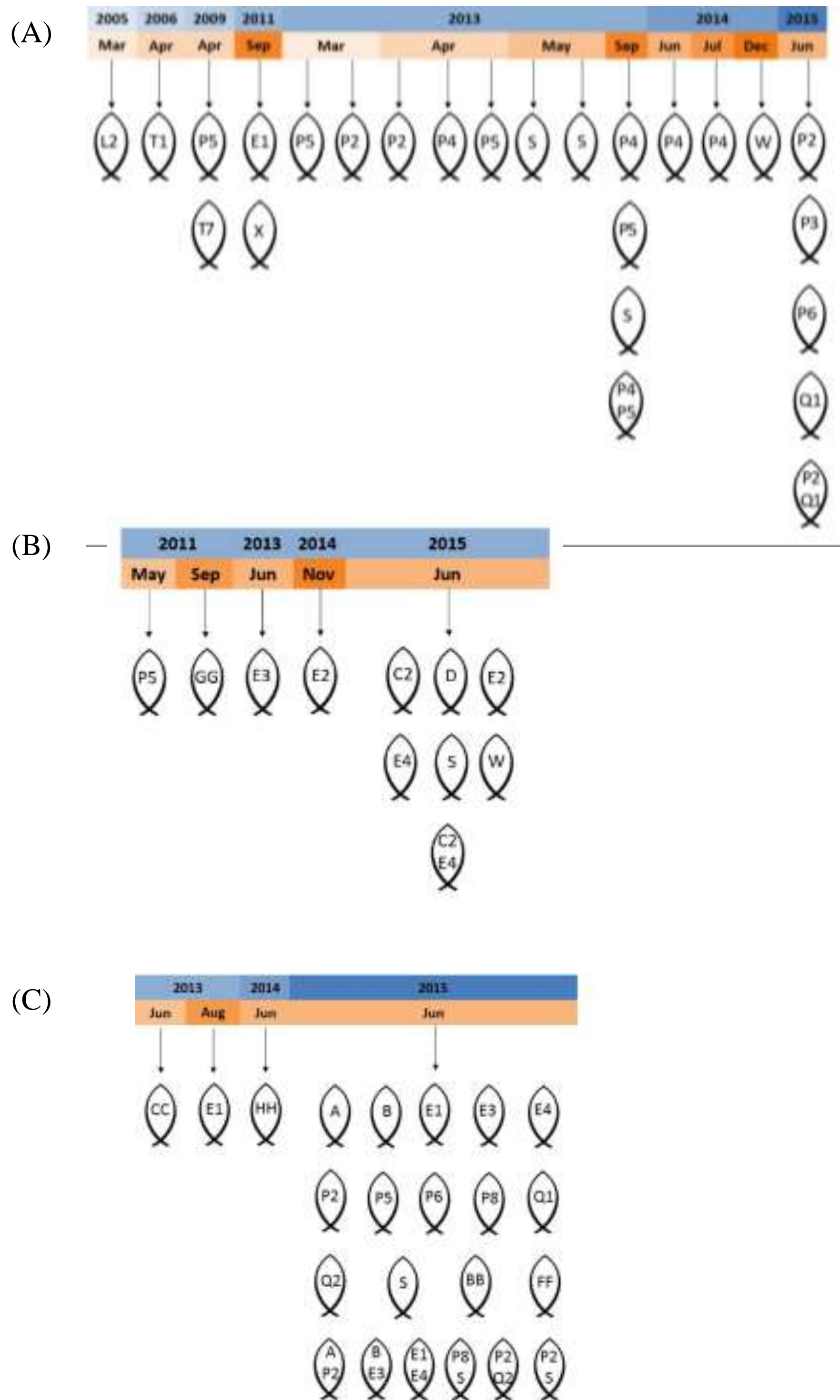

209 **Fig. 4.** The occurrence over 3-11 years of *F. psychrophilum* pulsotypes at three Scottish
210 sites. (A) site Scot I, (B) site Scot III, (C) site Scot V.

211 By combining genotypic and serotypic methods, the predominant profile within the UK *F.*
212 *psychrophilum* population, PFGE cluster II – $(GTG)_5$-PCR type r1 – 16S rRNA lineage II –
213 serotype Th (70/156 isolates studied, 45%) was noted. The simultaneous infection of isolates
214 harboring distinct pulsotypes, $(GTG)_5$-PCR types, serotypes or plasmids profiles was observed in
215 eight, seven, six and seven of the 27 UK sites sampled respectively.

216 **4. Discussion**

217 Macrorestriction analysis by PFGE is able to detect fine-scale genetic differences between
218 isolates, suitable for epidemiological investigation within a particular region or even smaller
219 geographical scale (a farm for example). A total of 45 different pulsotypes were able to be
220 resolved from the 292 UK *F. psychrophilum* isolates examined. PFGE-based analyses have also
221 been successfully deployed to investigate the genetic diversity of *F. psychrophilum* in other
222 regions. Arai et al. (2007) described 20 PFGE groups for 81 Japanese isolates from 19 prefectures;
223 Chen et al. (2008), 32 pulsotypes for 139 USA isolates from four sites; and Del Cerro et al.
224 (2010), 10 PFGE groups for 25 Spanish isolates from 12 fish farms. A high genetic relatedness
225 was found in Chilean isolates (12 isolates from six sites in the study of Avendaňo-Herrera et al.
226 2009) and Finnish isolates (42 isolates from three sites, Sundell et al. 2013) when the majority of
227 them had the band similarity exceeding 87.5 % and 94 % respectively. Comparison of our results
228 with those of the two other studies in which the *Sac*I enzyme was used in PFGE analysis (Chen et
229 al. 2008; Avendaňo-Herrera et al. 2009) reveals a difference in the minimum degrees of
230 homology, i.e. 72 % and 54 % in the studies by Avendaňo-Herrera et al. and Chen et al.
231 respectively and 32 % in our work. Although it is difficult to compare the results between studies
232 due to the variation of the protocols and interpretive criteria used, these findings indicated a

233 higher overall genetic diversity of UK *F. psychrophilum* isolates, observed by PFGE using *Sac*I
234 enzyme.

235 Despite a large number of pulsotypes identified, most of the 292 UK isolates (80 %) could be
236 resolved into four genetically distinct PFGE groups (P, E, Q, T) and singleton S, that were
237 consistently recovered from farms across the UK over the six years sampled (2009 – 2015). This
238 is suggestive that particular pulsotypes possess selective advantages, over the wide range of other
239 pulsotypes recovered that favour their transmission and colonisation of salmonids within UK
240 farms. The recovery of similar PFGE groups from multiple sites over an extended period also
241 likely reflects the structure of the UK rainbow trout industry, where the movement of live fish
242 between sites is widespread, encouraging the spread of associated pathogens. In addition, it is
243 possible to closely align the main PFGE profiles in this work and previous studies: PFGE group T
244 (this work) = PFGE genotype 2 (Avendaňo-Herrera et al. 2009); PFGE group L (this work) =
245 PFGE profiles ID05 and ID06 (Chen et al. 2008). It is indicated that *F. psychrophilum* isolates
246 belonging to three subpopulations (F – H, J – O and T – U) may have been spread among different
247 countries by trade of rainbow trout and associated products. These results broadly support the
248 contention of Nilsen et al. (2014) that *F. psychrophilum* displays an epidemic population
249 structure. A similar pattern has been observed in a whole genome-sequencing (WGS) study of the
250 salmonid pathogen *Renibacterium salmoninarum*, where it was demonstrated the isolates
251 circulating in UK salmonids could be resolved into a limited number of subgroups, with the
252 original founders likely introduced into the UK via trade in salmonid eggs some 40-50 years
253 previously (Brynildsrud et al. 2014).

254 The occurrence of 24 unique PFGE singletons in 15 sites possibly represents local adaption of
255 particular strains to sites or environmental niches (Arai et al. 2007; Del Cerro et al. 2010). These

256 singletons could be representative of a wider group of environmental isolates that may represent
257 less virulent endemic isolates and act more like opportunistic pathogens (Nilsen et al. 2014), so
258 are infrequently recovered. It is also possible that they are illustrative of repeated introductions of
259 *F psychrophilum* strains from other sources, with only some of these variants become established.
260 The persistence of isolates belonging to a certain predominant pulsotype within a site (e.g. PFGE
261 group P in site Scot I from March 2013 to June 2015) could increase the risk of epizootic episodes
262 in consecutive years, as suggested by Madetoja et al. (2002).

263 A concurrent mixture of isolates belonging to distinct pulsotypes, serotypes or plasmid profiles is
264 in keeping with the observations of Madetoja et al. (2002), Chen et al. (2008), Del Cerro et al.
265 (2010), Kim et al. (2010) and Sundell et al. (2013). These findings stress the importance of
266 examining more than a single colony from an infected fish in monitoring the genetic variation of
267 *F. psychrophilum* strain population. Although the role of these heterogeneous isolates within
268 mixed infection remains unknown, the co-existence of these isolates does afford an opportunity
269 for genetic acquisition and recombination among *F. psychrophilum* isolates (Nilsen et al. 2014).

270 There have been limited studies on linking specific pulsotypes of *F. psychrophilum* with
271 RTFS/BCWD outbreaks in fish farms, especially those involving the coinfection of heterogeneous
272 isolates (Nilsen et al. 2014). In the current study, the co-existence of more than one virulent
273 isolate (e.g. pulsotypes G and P2 in site N Ire I, pulsotypes T1 and U1 in site Scot XV) were
274 recorded but only one virulent isolate was found predominant, suggesting this isolate tends to be
275 associated with a disease outbreak. The selection of virulent strains may depend on some
276 underlying factors related to fish husbandry of the farm (Madetoja et al. 2002; Sundell et al.
277 2013). Although the contribution of these multiple strains within an outbreak, as well as the
278 variation in virulence, is still unclear, these authors suggested that together with the majority of

279 isolates within a farm belonging to a homogeneous group, a minority of isolates showing different
280 genetic and serological characteristics was always present and might be required in the pathogenic
281 process, resulting in a systemic infection. In addition, apparently healthy fish carrying *F.*
282 *psychrophilum* isolates might act as reservoirs for shedding pathogens into surrounding water and
283 be responsible for the wide dissemination of diverse isolates within and between sites (Chen et al.
284 2008).

285 Due to the limited numbers of *F. psychrophilum* isolates derived from salmon hosts in this study
286 compared to rainbow trout (22 vs 293 isolates), a host-specific association between pulsotypes, as
287 reported previously (Arai et al. 2007; Chen et al. 2008; Avendaño-Herrera et al. 2009), was not
288 observed. Similarly, the distribution of the CSF 259-93 and ATCC 49418 16S rRNA gene
289 polymorphisms among *F. psychrophilum* isolates did not reflect host-specificity, in accordance
290 with the observations of Hesami et al. (2008) and Valdebenito and Avendaño-Herrera (2009).

291 A combination of genetic and phenotypic typing methods is useful in evaluating strain population
292 diversity. In this study, one *F. psychrophilum* isolate was unable to be typed by PFGE due to the
293 DNA degradation but this isolate was amendable to the $(GTG)_5$-PCR method and the $(GTG)_5$-
294 PCR type r6 of this isolate was exclusive. Similarly, one non-serotypeable isolate possessed
295 unique pulsotype BB and $(GTG)_5$-PCR type r3. The $(GTG)_5$- PCR types r1 and r2 were found to
296 be associated with PFGE cluster II and PFGE cluster I respectively, suggesting rep-PCR could be
297 utilised as a rapid diagnostic marker to assess genetic variation within the *F. psychrophilum*
298 species. Consistent with the findings of Lorenzen and Olesen (1997), isolates obtained from
299 RTFS/BCWD outbreaks in the UK mainly belonged to serotype Th. Isolates of serotype Fp^T,
300 which were previously evidenced to be less pathogenic to rainbow trout in comparison with Th
301 and Fd serotypes (Madsen and Dalsgaard 1999, 2000; Madetoja et al. 2002), were found in four

302 diseased Atlantic salmon, one apparently healthy rainbow trout and one diseased rainbow trout
303 collected from four UK sites. Thus, the lower pathogenicity of this serotype in rainbow trout
304 needs to be confirmed by further fish challenge trials. One untypeable isolate retrieved from
305 rainbow trout may represent a new serotype of *F. psychrophilum* (Lorenzen and Olesen, 1997;
306 Izumi and Wakabayashi, 1999; Madetoja et al. 2002). Low molecular weight plasmids of 3.3 kb
307 were detected in the majority (150/169, 89 %) of isolates, consistent with earlier observations
308 (Chakroun et al. 1998; Madsen and Dalsgaard, 2000).

309 Further work to confirm the population structure of UK *F. psychrophilum* isolates could be
310 accomplished by applying multilocus sequence typing (MLST) and whole genome sequencing to
311 enable inter-laboratory comparison and international surveillance. A large globally spread clonal
312 complex (CC) of the species *F. psychrophilum* almost exclusive to the rainbow trout has been
313 identified by MLST and is shown to have the sequence type ST2 or ST10 as the predicted
314 common ancestor (Nicolas et al., 2008; Siekoula-Nguedia et al., 2012; Nilsen et al., 2014). The
315 fact that the PFGE cluster II from this study included two reference strains CSF 259-93 (ST10)
316 and NCIMB 13383 (ST2) suggests that the isolates belonging to this PFGE cluster may be
317 associated with CC-ST2 or CC-ST10 by MLST analysis. The reference strain NCIMB 1947^T
318 (ST13) was represented as a singleton by both methods. These results indicate a likely overlap
319 between the relationships inferred by PFGE and MLST in investigating the population structure of
320 *F. psychrophilum*.

321 **5. Conclusion**

322 PFGE data on the UK *F. psychrophilum* diversity broadly supports the hypothesis of an epidemic
323 population structure of this bacterium. The country-wide distribution of several PFGE groups or
324 singletons may reflect local fish farming practices, where there is widespread movement of live

325 fish between sites. The simultaneous presence of genetically and serologically divergent clones
326 additionally confound RTFS prevention and control. Understanding the genetic diversity present
327 at particular sites or regions is crucial in preventing the spread of epidemic clones and allowing
328 in-depth studies to assess the differences between environmental isolates and clinical isolates, and
329 the significance of the *F. psychrophilum* strain variation in vaccine development against
330 RTFS/BCWD.

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441 **Table 1.** Details of 303 *F. psychrophilum* isolates collected from 27 sites within the UK and three
442 farms in Europe during 2005 – 2015

443 **Table 2.** Origins and typing summary of the twelve reference strains of *F. psychrophilum* used in
444 this study

445 **Table 3.** Distribution of 16S rRNA alleles among tested *F. psychrophilum* isolates

446 **Table 4.** Antigens and rabbit antisera titers included in this study

447 **Fig. 1.** Dendrogram of 314 *F. psychrophilum* isolates constructed using the UPGMA method,
448 based on 62 PFGE banding patterns obtained using *Sac*I. For each banding pattern; *Sac*I-PFGE
449 pulsotype, pulsotype group and singleton (number of isolates), $(GTG)_5$-PCR type, 16S rRNA
450 allele, serotype, plasmid size, fish host and site code are shown. A vertical line at 40% similarity
451 that classified the isolates into three major *Sac*I-PFGE clusters I and II (number of isolates are
452 shown as well). The asterisk (*) represents the dominant $(GTG)_5$-PCR type in each *Sac*I-PFGE
453 subtype. RT, rainbow trout; AS, Atlantic salmon; CS, coho salmon.

454 **Fig. 2.** (A) Representative ten $(GTG)_5$-PCR types obtained for 315 *F. psychrophilum* isolates
455 tested. Lane M is the GeneRuler Express DNA Ladder (Fermentas, Fisher Scientific, UK).
456 Numbers on the left indicate the size of the bands of the molecular size marker in bp. (B)

457 Dendrogram created by the GelCompar II software package (Applied Maths, Belgium) using
458 UPGMA with the Jaccard similarity coefficient and a 0.4% tolerance on the basis of the $(GTG)_5$-
459 PCR profiles.

460 **Fig. 3.** Seven plasmid profiles (p – p7) identified among 185 *F. psychrophilum* isolates using a 0.7
461 % agarose in 0.5X TAE. Lane 39R: *E. coli* 39R816 (7.0, 36-63-147 kb); Lane V517: *E. coli* V517
462 (2.1, 2.7-3.0, 3.9, 5.1-5.5, 7.2, 54 kb); Lane M: Supercoiled DNA ladder (New England BioLabs,
463 UK). Profile p1: one 3.3 kb plasmid; p2: one 2.6 kb plasmid; p3: one 4.0 kb plasmid; p4: two
464 plasmids of 3.3 and 2.1 kb; p5: two plasmids of 4.0 and 3.3 kb; p6: two plasmids of 3.3 and 2.8
465 kb; and p7: or no plasmid.

466 **Fig. 4.** The occurrence over 3-11 years of *F. psychrophilum* pulsotypes at three Scottish sites. (A)
467 site Scot I, (B) site Scot III, (C) site Scot V.