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Genetic diversity of *Flavobacterium psychrophilum* isolated from rainbow trout in France: Predominance of a clonal complex

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ABSTRACT

Flavobacterium psychrophilum is the causative agent of "bacterial cold water disease" and "rainbow trout fry syndrome" in salmonid farming worldwide. These diseases, especially rainbow trout fry syndrome, are among the main hazards for French aquaculture. In this study, a multilocus sequence typing approach (MLST) was used to evaluate the genetic diversity of this bacterium.

Seven housekeeping genes in a set of 66 isolates were investigated. They were recently collected from rainbow trout during clinical episodes in French farms from the two main geographical areas of production. A total of 5808 bp of sequence were analyzed for each isolate and showed relatively low levels of gene ($H = 0.4313$) and nucleotide ($\pi \times 100 = 0.31\%$) diversities. MLST identified 15 sequence types (STs), of which 14 have never been described. eBURST analysis separated the 15 STs in one clonal complex of 8 genetically related STs (with ST2 as founder) and 7 singletons. Genetic diversity was largely due to recombination, as demonstrated by a pairwise homoplasy index ($PHI = 5.35 \times 10^{-9}$) significantly different from zero ($p < 0.05$). The evolution of standardized association index (I_A^S) (all isolates: 0.6088, $p < 0.05$; single representative of STs: 0.4567, $p < 0.05$; and clusters of STs: 0.084, $p > 0.05$), showed an epidemic structure of the population.

These results emphasized the expansion of a limited number of dominant genetic variants in French clinical *F. psychrophilum* isolates from a single host species, with no geographic relationships.

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1. Introduction

Flavobacterium psychrophilum is a Gram negative bacterium, responsible for rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD), two clinical expressions associated with important economic losses in salmonid fish farming. All salmonid fish (especially coho salmon and rainbow trout) are susceptible to RTFS and BCWD, as well as some other non-salmonid fish, like cyprinids (Lehmann et al., 1991) and pale chub (*Zacco platypus*) (Iida and Mizokami, 1996). These infections are associated with skin ulcers, necrotic myositis, septicemia as well as exophthalmia (Nematollahi et al., 2003). Vaccines are currently still at the experimental stage (Plant et al., 2009) and treatments rely on antibiotics, with the risk of resistance emergence (Schmidt et al., 2000).

In France, RTFS has been reported as the second major bacteriosis, after furonculosis, affecting breeding fish, particularly rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Guichard, 2004). The impact of this disease on

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French fish production is considerable, as rainbow trout is the main fish species farmed in France. Information is required about the genetic diversity of *F. psychrophilum*, through the identification of epidemic clones or genetic variants with harmful behavior, in order to define appropriate management strategies to minimize the risks of pathogen introduction or transmission. This knowledge would be useful for future epidemiological monitoring. Several techniques like Random Amplified Polymorphic DNA (RAPD), ribotyping, Fragment Length Polymorphism of PCR products (PCR-RFLP), Pulsed Field Gel Electrophoresis (PFGE), and Multi Locus Sequence Typing (MLST) have been employed to attain this goal (Li et al., 2009).

In this study, we used the previously published MLST scheme (Nicolas et al., 2008) as MLST is a well-established method for characterizing bacterial populations on the basis of sequence polymorphisms on conserved housekeeping genes, and enables easy data sharing (Maiden, 2006). We attempted to assess the genetic diversity of *F. psychrophilum* isolates, recently collected from the main French areas of rainbow trout production (Aquitaine and Bretagne), and some from near-by counties in the Atlantic region of France (Pays de la Loire and Poitou Charente), from rainbow trouts with clinical signs.

2. Materials and methods

2.1. Bacterial isolates and phenotypic identification

Sixty six *F. psychrophilum* isolates were sampled between 2007 and 2010 from four French major rainbow trout producing areas, where cases of BCWD and RTFS are regularly reported. Their geographic origins are presented in Fig. 1. Isolates were collected from farmed trouts

Fig. 1. Geographic origins of French isolates. Regions are indicated by different colours: Bretagne in blue, Pays de la Loire in green, Poitou Charente in violet, and Aquitaine in red. STs recovered from each farm are indicated for the two mains salmonid production regions that are subdivided in departments: (a) Bretagne [Côte d'Armor (CA), Ile et Vilaine (IV), Morbihan (Mo) and Finistère (F)] and (b) Aquitaine [Dordogne (D), Lot et Garonne (LG), Landes (L), Gironde (G) and Pyrénées Atlantiques (PA)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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presenting clinical signs of flavobacteriosis, by veterinarians or technicians using different sampling protocols. Therefore, some isolates were collected from internal organs like spleen, kidney, liver, and encephalon, and others from external organs like skin, gills and fin (Table 1). Sometimes several samplings were performed at six or more months intervals at a given site (Fig. 1).

Each isolate was grown on FLP agar medium (Cepeda et al., 2004) at 17 °C for 48–72 h. A single developed colony was picked from the FLP agar plates and subcultured. The resulting colonies were selected after a preliminary biochemical and phenotypic characterization, based on Gram negative, catalase positive, and flexirubin pigment positive test results.

Table 1
MLST allele types and sequence types of 66 *F. psychrophilum* isolates. Geographic origins are indicated by region and watershed. Isolates were collected from internal (int), external (ext) or unknown organ (unk), and those included into the clonal complex (CC) are indicated by "in", and the others by "out". New ATs and STs are underlined.

Isolate	Geographic origin	Watershed	Tissue	Year	Allelic profile							ST	CC
					trpB	*gyrB*	*dnak*	*fumC*	*murG*	*tuf*	*atpA*		
438	Aquitaine	Adour	int	2008	2	2	2	2	2	2	2	2	in
439	Aquitaine	Adour	int	2008	2	2	2	2	2	2	2	2	in
440	Aquitaine	Vignac	int	2008	2	2	2	2	2	2	2	2	in
441	Aquitaine	Garonne	int	2008	2	2	2	2	2	47	2	89	in
442	Aquitaine	Vignac	int	2008	1	1	1	1	1	2	1	90	out
444	Aquitaine	Adour	int	2008	2	2	2	2	2	2	2	2	in
445	Aquitaine	Adour	int	2008	2	2	2	2	2	2	2	2	in
446	Aquitaine	Adour	int	2008	2	2	2	2	2	41	2	91	in
448	Aquitaine	Adour	int	2008	2	2	2	2	2	47	2	89	in
449	Aquitaine	Adour	int	2008	2	2	2	2	2	47	2	89	in
737	Aquitaine	Magescq	int	2008	2	2	2	2	2	2	2	2	in
738	Aquitaine	Adour	ext	2010	1	1	1	1	1	2	1	90	out
739	Aquitaine	Adour	int	2009	2	2	2	2	2	47	2	89	in
740	Aquitaine	Adour	ext	2009	2	2	2	2	2	2	2	2	in
742	Aquitaine	Magescq	int	2008	2	2	2	2	2	2	2	2	in
743	Aquitaine	Lyere	ext	2010	2	2	2	2	2	2	2	2	in
744	Aquitaine	Magescq	int	2008	2	2	2	2	2	2	2	2	in
746	Aquitaine	Adour	int	2010	2	2	2	2	2	2	2	2	in
747	Aquitaine	Adour	int	2009	2	2	2	2	2	2	2	2	in
749	Aquitaine	Adour	int	2009	1	1	1	1	1	2	1	90	out
755	Aquitaine	Adour	int	2008	2	2	2	2	2	2	2	2	in
756	Aquitaine	Adour	int	2008	2	44	16	3	3	3	3	102	out
759	Aquitaine	Adour	ext	2010	2	2	2	2	2	2	2	2	in
760	Aquitaine	Adour	ext	2009	1	49	10	7	20	25	25	104	out
802	Aquitaine	Garonne	unk	2010	2	2	2	2	2	47	2	89	in
805	Aquitaine	Dordogne	int	2009	1	1	1	1	1	2	1	90	out
806	Aquitaine	Adour	int	2009	1	1	1	1	1	2	1	90	out
807	Aquitaine	Adour	ext	2010	2	2	2	2	2	2	2	2	in
808	Aquitaine	Adour	ext	2009	2	2	2	2	2	2	2	2	in
810	Aquitaine	Adour	int	2009	2	2	2	2	2	2	2	2	in
811	Aquitaine	Lyere	int	2010	2	2	2	2	2	2	2	2	in
812	Aquitaine	Dordogne	int	2008	2	2	2	2	2	2	2	2	in
814	Aquitaine	La Palue	int	2009	2	2	2	2	2	2	2	2	in
816	Aquitaine	Adour	int	2007	2	2	2	2	2	47	2	89	in
817	Aquitaine	Adour	int	2010	1	1	1	2	1	41	1	108	out
818	Aquitaine	Lyere	int	2009	2	2	2	2	2	2	2	2	in
820	Aquitaine	Adour	int	2007	2	2	2	2	2	2	2	2	in
822	Aquitaine	Lyere	int	2009	3	2	2	2	2	41	2	92	in
823	Aquitaine	Adour	int	2010	1	1	1	1	1	2	1	90	out
824	Aquitaine	Adour	int	2009	2	2	2	2	2	47	2	89	in
825	Aquitaine	Magescq	int	2009	2	2	2	2	2	47	2	89	in
829	Aquitaine	Adour	ext	2009	4	29	24	5	6	49	40	113	out
539	Bretagne	Le Guillec	int	2008	3	2	2	2	2	41	2	92	in
540	Bretagne	Hyere	int	2008	4	2	2	2	2	2	2	93	in
591	Bretagne	Rivière de Morlaix	unk	2009	4	2	2	2	2	41	2	95	in
607	Bretagne	Le Guillec	ext	2009	29	2	2	2	2	2	2	97	in
626	Bretagne	Penzé	int	2009	2	2	2	2	2	2	2	2	in
635	Bretagne	Aulne	int	2008	2	2	2	2	2	48	2	98	in
636	Bretagne	Douron	int	2008	2	2	2	2	2	47	2	89	in
637	Bretagne	Elorn	int	2009	2	2	2	2	2	2	2	2	in
687	Bretagne	Elorn	ext	2009	2	2	2	2	2	2	2	2	in
697	Bretagne	Quillimadec	int	2010	2	2	2	2	2	2	2	2	in
721	Bretagne	Le Gouët	int	2010	2	2	2	2	2	2	2	2	in

Table 1 (*Continued*)

Isolate	Geographic origin	Watershed	Tissue	Year	Allelic profile							ST	CC
					trpB	*gyrB*	*dnak*	*fumC*	*murG*	*tuf*	*atpA*		
724	Bretagne	Ster Goz	int	2010	2	2	2	2	2	2	2	2	in
729	Bretagne	Penzé	int	2010	1	1	1	1	1	2	1	90	out
730	Bretagne	Le Corroac'h	int	2010	1	1	1	1	1	2	1	90	out
733	Bretagne	Unknown	int	2010	3	2	2	2	2	41	2	92	in
763	Bretagne	Unknown	int	2010	3	2	2	2	2	41	2	92	in
771	Bretagne	Odet	int	2010	2	2	2	2	2	2	2	2	in
780	Bretagne	Rivière de Morlaix	int	2010	2	2	2	2	2	2	2	2	in
787	Bretagne	Le Corroac'h	int	2010	1	1	1	1	1	2	1	90	out
796	Bretagne	Douffine	int	2010	21	3	22	3	3	2	2	105	out
690	Pays de la Loire	Ruisseau des Loges	ext	2010	4	42	21	5	2	39	40	99	out
718	Pays de la Loire	Unknown	int	2010	2	2	2	2	2	2	2	2	in
741	Poitou Charente	Charente	int	2009	1	1	1	1	1	2	1	90	out
745	Poitou Charente	Charente	int	2010	3	2	2	2	2	41	2	92	in

Several bacterial strains were used as control: *F. psychrophilum* reference strains (JIP 02/86, NCIMB 1947^{T} and LNPAA PO1/88), *Flavobacterium columnare* (NCIMB 2248^{T}), *Flavobacterium johnsoniae* (CIP 100931), *Flavobacterium aquatile* (CIP 103744), *Flavobacterium succinicans* (CIP 104744), *Aeromonas salmonicida* (ATCC 14174), *Aeromonas hydrophila* (CIP 7614) and *Escherichia coli* (ATCC 25922). All *Flavobacteria* strains were grown on FLP agar medium at 22 °C during 72 h, except *F. psychrophilum* cultured at 17 °C and *F. johnsoniae* cultured during 48 h. *Aeromonas* and *E. coli* strains were cultured on trypticase soy agar (TSA) medium at 22 °C and 37 °C, respectively.

2.2. DNA extraction and molecular identification of F. psychrophilum

A duplex PCR was developed for the rapid identification of *F. psychrophilum* isolates. This PCR was carried out to amplify *gyrA* and *gyrB* genes, using previous designed primer pairs, namely GYRA-FP1F (5′-GAAACCGGTGCACAGAAGG-3′) and GYRA-FP1R (5′-CCTGTGGCTCCGTTTATTAA-3′) (Izumi and Aranishi, 2004), and PSY-G1F (5′-TGCAGGAAATCTTACACTCG-3′) and PSY-G1R (5′-GTTGCAATTACAATGTTGT-3′) (Izumi and Wakabayashi, 2000). The sizes of the expected PCR products were 396 and 1017 bp for *gyrA* and *gyrB* genes amplification, respectively. Three *F. psychrophilum* reference strains (JIP 02/86, NCIMB 1947^{T} and LNPAA PO1/88) were used as positive controls in the PCR assays, *E. coli* was used as negative control, and six other bacterial fish pathogen were used to test the PCR specificity: *F. columnare*, *F. johnsoniae*, *F. aquatile*, *F. succinicans*, *A. salmonicida* and *A. hydrophila*.

Genomic DNA of reference strains and French isolates were extracted and purified using a Wizard genomic DNA purification kit (Promega, France). A 25 μL reaction volume contained 5 μL of DNA, buffer 1X (Eurobio, France), 25 pmol of each *gyrA* primer, 37.5 pmol of each *gyrB* primer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$ and 0.5 units of *Taq* polymerase (Eurobio, France). The following thermocycling steps were used: 94 °C for 5 min, 35 cycles consisting of a denaturation step at 94 °C for 1 min, hybridization at 56 °C for 1 min and elongation at 72 °C for 1.5 min, and a final elongation phase at 72 °C for 5 min. The products were visualized on a UV transilluminator after separation on a 1.5% ethidium bromide agarose gel (30 min at 100 V).

2.3. Multilocus sequence typing

The seven housekeeping genes (*trpB*, *gyrB*, *dnaK*, *fumC*, *murG*, *tuf*, and *atpA*) used for the MLST scheme are described in Table 2. They were present in a single copy at each locus and distributed along the chromosome of the reference isolate, JIP 02/86, which is a virulent type isolate of *F. psychrophilum* (Duchaud et al., 2007). These loci are considered as typical core genome genes with relatively little selective polymorphism and have already been used in MLST studies of some bacteria and particularly of *F. psychrophilum* (Nicolas et al., 2008). The *F. psychrophilum* reference strain, JIP 02/86 was used as amplification positive control.

Briefly, the amplification mix contained 10 μL of genomic DNA (at ≈2 ng μL^{-1}), GoTaq Green Master Mix 1X, 1.87 mM of $MgCl_2$, 0.15 mM of each dNTP, 0.25 μM of each primer (Table 2) and one unit of *Taq* DNA polymerase (Promega, France). PCR amplifications were performed in the following conditions: 94 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 55 °C (−0.4 °C/cycle) for 30 s and 72 °C (+2 s/cycle) for 1 min; followed by 12 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C (+3 s/cycle) for 2 min, and a final extension at 72 °C for 10 min. Five microliters of the PCR products were resolved on a 1% ethidium bromide agarose gel to check amplification.

The PCR products were sequenced (LGC Genomics, Berlin) using M13 forward and reverse primers: M13_f, CAGGAAACAGCTATGACC and M13_r, TGTAAAACGACGGCCAGT, included in each specific gene primer (Table 2). The quality of the resulting chromatograms was checked visually and sequences of each locus of each isolate were assembled with Phred and Phrap programs (Ewing and Green, 1998; Ewing et al., 1998). The sequences were trimmed and aligned using respectively Consed (version

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Table 2
Housekeeping genes analyzed in this MLST study, primers and amplicon sizes.

Locus	Gene product	Primers	Sequence (5′–3′)	Size of PCR amplicon (bp)	Length of the target sequence (bp)
trpB	Tryptophan synthase beta subunit	trpB_f trpB_r	M13-f_AAGATTATGTAGGCCGCCC M13-r_TGATAGATTGATGACTACAATATC	976	789
gyrB	DNA gyrase B subunit	gyrB_f gyrB_r	M13-f_GTTGTAATGACTAAAATTGGTG M13-r_CAATATCGGCATCACACAT	1260	1077
dnaK	Chaperone protein	dnaK_f dnaK_r	M13-f_AAGGTGGAGAAATTAAAGTAGG M13-r_CCACCCATAGTTTCGATACC	1068	882
tuf	Elongation factor Tu	tuf_f tuf_r	M13-f_GAAGAAAAAGAAAGAGGTATTAC M13-r_CACCTTCACGGATAGCGAA	981	795
fumC	Fumarate hydratase class II	fumC_f_new fumC_r_new	M13-r_CCAGCAAACAAATACTGGGG M13-f_GGTTTACTTTTCCTGGCATGAT	936	750
murG	Undecaprenyldiphospho-muramoylpentapeptide beta-N acetylglucosaminyltransferase	murG_f murG_r	M13-f_TGGCGGTACAGGAGGACATAT M13-r_GCATTCTTGGTTTGATGGTCTTC	869	681
atpA	ATP synthase alpha subunit	atpA_f atpA_r	M13-f_CTTGAAGAAGATAATGTGGG M13-r_TGTTCCAGCTACTTTTTTCAT	1019	834

22.0) (Gordon et al., 1998) and BioEdit (version 7.0.5.3) (Hall, 1999) softwares. The resulting sequences were chosen to be shorter than in the initial MLST scheme (Nicolas et al., 2008) to automate the trimming process and ensure the maintenance of good sequence quality.

The nucleotide sequences of each housekeeping gene used for MLST were assigned allele numbers according to the MLST web site (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Flavopsy.html). The numbered allele types (AT) at each locus of each isolate were combined in order to define a sequence type (ST).

2.4. Population genetic analysis

Each MLST locus was analyzed for descriptive characteristics, such as the number of polymorphic sites and the nucleotide diversity per site (π), performed by DnaSP version 5.10.01 (Librado and Rozas, 2009). The gene diversity (H) determined using LIAN 3.5 (http://pubmlst.org/analysis/), was calculated as: $H = [n/(n-1)](1 - p_i^2)]$, in which n is the number of samples and p_i, the relative frequency of the ith allele. The number of synonymous and non-synonymous mutations were counted using MEGA5 software (Tamura et al., 2011). The average non-synonymous/synonymous mutations ratio (dN/dS) and the linkage disequilibrium between alleles at the seven gene loci which is measured by standardized index of association (I_A^S) were calculated with START2 software (Jolley et al., 2001). The index of association provides an indication of the recombination events that have generated genetic variability, as pairwise homoplasy index (PHI) (Bruen et al., 2006) calculated by SplitsTree 4 version 4.12.3 (Huson and Bryant, 2006). Evidence for recombination between STs was also investigated using split decomposition tree, constructed with 1000 bootstrap replicates based on parsimony splits implemented in SpleesTree4 version 4.12.3.

3. Phylogenetic analysis

A neighbor-joining (NJ) tree of concatenated housekeeping genes was constructed based on the NJ method (Saitou and Nei, 1987) using MEGA5 software (Tamura et al., 2011), with support for branches provided by bootstrapping ($n = 1000$). The e-BURST v3 software was used to divide STs into groups of closely related ones (clonal complexes) (http://pubmlst.org/analysis/). These clonal complexes were statistically assessed by bootstrap method ($n = 1000$) and the analysis was performed with default settings (a minimum of 6 alleles shared) and relaxed settings (a minimum of 5 alleles shared) defining the group parameters.

3.1. Statistical analysis

Fisher's exact test is an appropriate statistical test for determining the presence of a non-random association between two categorical variables and was used to check whether the genetic variants of *F. psychrophilum* exhibited a geographical pattern. This statistical test was implemented with SAS software (SAS statistical software – version 9.2; SAS Institute Inc., Cary, NC, USA).

3.2. Nucleotide sequence accession numbers

Only nucleotide sequences corresponding to new allele types (19 sequences, Table 1) have been deposited in GenBank (accession numbers JQ595418, JQ595419, JQ595424, JQ595426, JQ595429, JQ595431, JQ595433 to JQ595435, JQ595437, JQ595439, JQ595442 to JQ595449).

4. Results

4.1. Phenotypic characterization and molecular identification of *F. psychrophilum*

The 66 selected isolates (Table 1) came from 45 farms (24 in Aquitaine, 17 in Bretagne, 2 in Pays de la Loire and 2

Table 3
Characteristics, polymorphism and recombination of MLST loci.

Locus	Size (bp)	No. (%) of variable sites		No. of types[a]		H[b]	π (bp^{-1})[c]	S[d]	N[e]	dN/dS[f]	PHI[g]
		Total	New[h]	Total	New						
trpB	789	5 (0.63%)	0	6	2	0.5408	0.00184	7	0	0.0000	0.022*
gyrB	1077	16 (1.48%)	0	7	4	0.4033	0.00332	23	0	0.0000	0.008*
dnaK	882	9 (1%)	1	7	4	0.4033	0.00176	10	1	0.0020	0.041*
tuf	795	15 (1.9%)	0	8	6	0.3837	0.00512	18	0	0.0000	0.806
fumC	750	4 (0.53%)	0	5	0	0.3795	0.00066	5	0	0.2738	1
murG	681	9 (1.32%)	0	5	1	0.5291	0.00343	7	5	0.0000	0.102
atpA	834	23 (2.7%)	1	5	2	0.3795	0.00633	28	1	0.0171	0.001*
Concatenate	5808	81 (1.4%)	2	15	14	0.4313	0.00317	135	9	0.0200	5.35×10^{-9}

[a] Number of allele types identified for each locus and number of sequence types identified for concatenate.
[b] Gene diversity.
[c] Nucleotide diversity per site.
[d] Number of synonymous substitutions.
[e] Number of non-synonymous substitutions.
[f] Ratio of non-synonymous to synonymous mutations.
[g] Pairwise homoplasy index.
[h] New by comparison with the results of Nicolas et al. (2008).
* Statistically significative trace of recombination ($p < 0.05$).

in Poitou Charente) (Fig. 1). They were negative for the Gram test and positive for the catalase and flexirubin tests. The *gyrA*/*gyrB* duplex PCR yielded products with the expected sizes: 396 and 1017 bp respectively (data not shown), confirming the identification of all isolates as *F. psychrophilum*. Amplicons were not observed for negative controls.

4.2. Analysis of sequence diversity in housekeeping genes

All seven housekeeping MLST genes of the 66 isolates were successfully amplified and sequenced, and the genetic characteristics of each locus are described in Table 3. Within the 5808 bp of the concatenated sequence of seven loci in the 66 *F. psychrophilum* French isolates, 81 polymorphic sites (1.39% of the positions) were observed. No insertions or deletions were detected within the loci analyzed. The number of single-nucleotide polymorphisms (SNPs) sites between loci varied from 4 (at locus *fumC*) to 23 (at locus *atpA*). Compared to the previous study data (Nicolas et al., 2008), new SNPs sites were identified in 2 loci (*dnaK* and *atpA*), each of them presenting one new SNP site. All SNPs sites were biallelic, except two that were triallelic (one in *tuf* and one in *gyrB*). The average number of allele types per locus was 6.1 (ranging from 5 at loci *atpA*, *fumC* and *murG*, to 8 at locus *tuf*) and new allele types were detected for all loci, except *fumC*.

The average gene diversity (H) over the seven loci was 0.4313, with H values for individual loci ranging from 0.3795 for *atpA* and *fumC*, to 0.5408 for *trpB*. Pairwise nucleotide diversity (π) ranged from 0.00066 for *fumC* to 0.00633 for *atpA*, with an average value of 0.00317. Synonymous substitutions were more common than non-synonymous substitutions, and the ratio of non-synonymous to synonymous mutations (dN/dS) was less than 1 for all loci, with dN/dS equal to 0 for *murG*, *gyrB*, *trpB*, and *tuf*, while ranging from 0.0020 to 0.2738 for the three other genes. A dN/dS ratio less than 1 indicates that the diversity present in all these genes is likely driven by purifying selection.

4.3. Analysis of recombination events

When the occurrence of recombination in each locus was investigated by PHI test, the results showed statistically significant recombination for four loci (*atpA*, *dnaK*, *gyrB* and *trpB*) (Table 3). Application of the PHI test to the concatenated sequences revealed statistically significant evidence of recombination ($p < 0.05$). To test further for recombination traces, SplitsTree program was used on

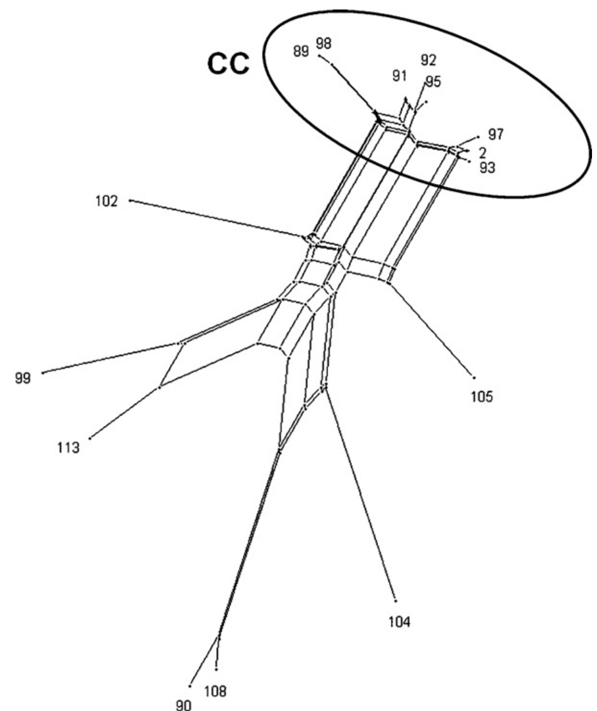

Fig. 2. Split decomposition analysis. The tree was constructed under neighbor net graph option, with the concatenated sequences of each ST. STs of the clonal complex are circled.

concatenated sequence of all STs. This analysis showed a bushy network structure indicative of recombination events (Fig. 2). Evidence of recombination was also assessed from the standardized index of association (I_A^S). This statistical clonality test attempts to measure the extent of linkage disequilibrium within a population by quantifying the amount of recombination occurring between a set of sequences and detecting association between alleles at different loci and gives indication on population structure (clonal, panmictic, or epidemic). Here, the entire dataset of *F. psychrophilum* isolates yielded an I_A^S of 0.6088, significantly different from 0 ($p < 0.05$). A lower value differing significantly from 0 ($p < 0.05$) was obtained when the dataset of all single representatives of each of the 15 STs retrieved by MLST (Table 3) was analyzed ($I_A^S = 0.4567$), indicating a linkage disequilibrium. This linkage disequilibrium disappears when STs are divided in clusters of genetically related STs (Supplementary data S1), with I_A^S value that drops sharply down to 0.084, significantly close to 0 ($p > 0.05$).

4.4. Phylogenetic relatedness of French F. psychrophilum isolates

This MLST study identified 15 STs among the 66 French *F. psychrophilum* isolates analysed, 14 of which have not been described before (Table 1 and Table 3). The STs occurred at unequal frequencies. The majority of STs (11/15) was found only once, each representing 1.51% of the total isolates, and the genetic variant most frequently encountered was ST2, representing 46.9% of the total isolates. The other most common genetic variants were ST90, ST89 and ST92 (7.5–15.5% of the total sample).

The predicted evolutionary descent of the 66 isolates was then investigated by clustering method, eBURST (Feil et al., 2004), using a default group definition in which STs differing at only one single locus (single locus variant or SLV) are grouped together. With these default parameters, STs that are linked share six common locus types and are grouped in a clonal complex at the center of which, a founder (common ancestor) is predicted. When this method was applied to our sample, the 15 STs retrieved could be divided into one clonal complex or CC (composed of 8 STs and representing 75.7% of the total isolates) and 7 singletons STs (Fig. 3). ST2 was identified as the potential founder and was connected with ST89, ST93, ST97 ST98 and ST91 which also shared alleles with ST92 and ST95. *F. psychrophilum* reference strains JIP 02/86 (isolated from rainbow trout) and NCIMB 1947^T (isolated from coho salmon) were added in analysis in order to highlight possible relationship with French isolates. e-BURST analysis including JIP 02/86 and NCIMB 1947^T respectively ST20 and ST13, show those STs as singleton.

The relatedness between STs can also be expressed by relaxed parameter that shows all pairs of STs sharing two ATs. ST20, the reference strain JIP 02/86 ST showed connections with ST2, ST93 and ST97. On the other hand, this relaxed double locus variants (DLV) criterion revealed that ST90 was linked to ST108.

The phylogenetic relationship between isolates was further analyzed by constructing a NJ unrooted tree, using

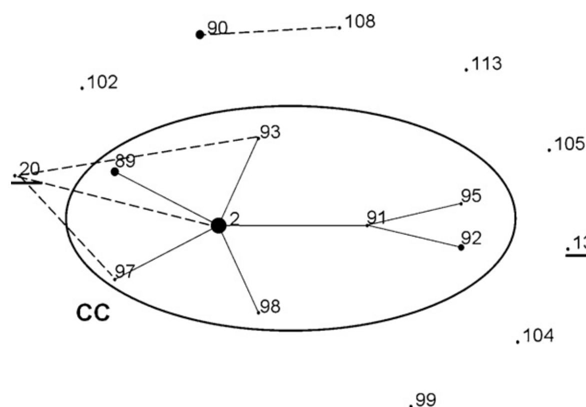

Fig. 3. eBURST diagram. Single locus variants (sequence types that differ by one allele type) are joined by straight lines, and circled in the same clonal complex (CC). Double locus variants (sequence types that differ by two allele types) are joined by dotted lines. Dot sizes are proportional to the number of isolates within each ST, and the bigger one is the founder of the CC, ST2. The underlined STs, ST13 and ST20, are the genotypes of reference strains NCIMB 1947^T and JIP 02/86 respectively.

the number of differences method. This tree was constructed from the concatenated nucleotide sequences of the seven loci in all 66 isolates and of the reference strains JIP 02/86 and NCIMB 1947^T (Fig. 4). The CC revealed by eBURST analysis was apparent as cluster on the phylogenetic tree, with good percentage values of the bootstrap support (Fig. 4). Examination of the NJ tree showed that very diverse STs could be found within a given French region. This was confirmed by applying an exact Fisher test to the *F. psychrophilum* sample. The presence and the absence of ST2 in the four French regions studied, as it is the most represented ST, were tested and the Fisher exact test did not reveal any significant geographical pattern in the distribution of the ST2 isolates ($p < 0.05$).

5. Discussion

MLST has emerged as the technique of choice for studying the population structure of many bacterial species (Maiden, 2006). This typing method became possible as a result of access to DNA sequences of bacteria. In the case of the recognized threat of *F. psychrophilum* to the salmonid farming industry, the recent publication of the complete genome sequence of this bacterium (Duchaud et al., 2007) had already led to the development of a MLST scheme (Nicolas et al., 2008). In the present study, we used the 7 loci (*trpB*, *gyrB*, *dnaK*, *fumC*, *murG*, *tuf*, and *atpA*) from this previously published MLST scheme to gain insight into the genetic diversity of clinical isolates of *F. psychrophilum* collected from rainbow trout farmed in France.

Analysis of the pattern of polymorphism in the entire dataset revealed a few polymorphic sites, characterized by low concatenate sequence nucleotide diversity per site ($\pi \times 100 = 0.31\%$) and weak average gene diversity ($H = 0.4313$). When the same seven MLST loci considered in the present study were analysed in light of the data of Nicolas and colleagues (2008), obtained for twenty four

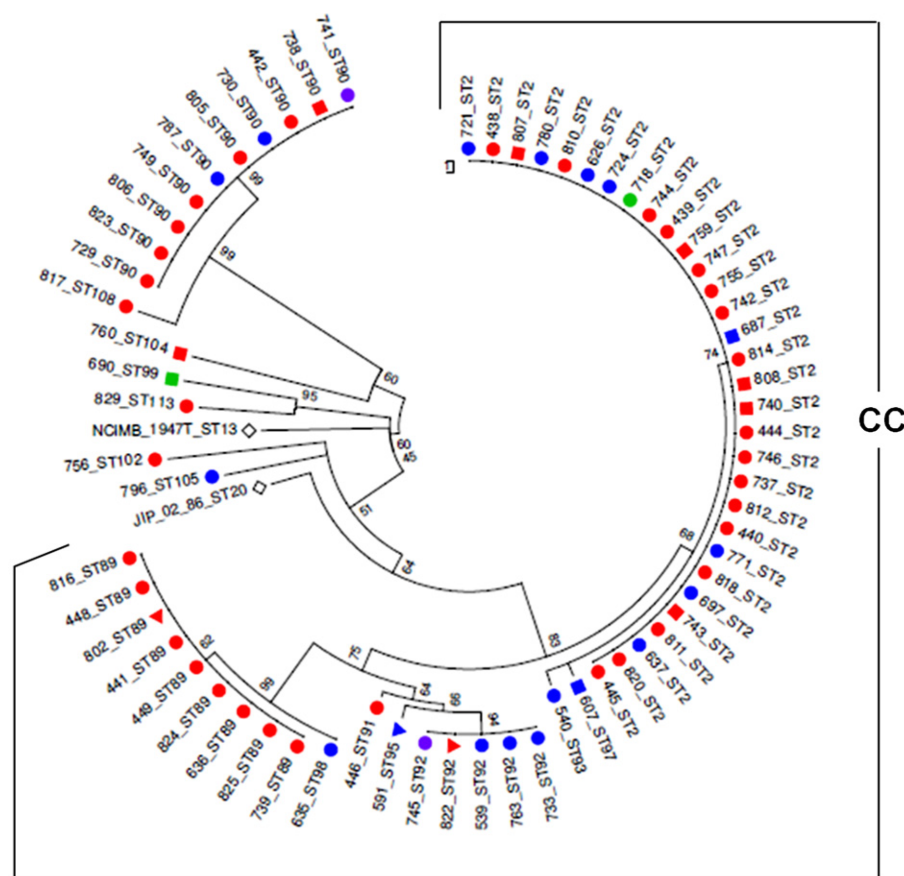

Fig. 4. Neighbor-joining unrooted tree of 66 French *F. psychrophilum* isolates, including *F. psychrophilum* reference strain JIP 02/86 and NCIMB 1947[T]. For each isolate, the French region, the tissue location and ST number are indicated. The evolutionary distances were computed using the number of differences method and are in the units of the number of base substitutions per site. French regions are indicated by different colors: Aquitaine in red, Bretagne in blue, Poitou Charentes in violet, and Pays de la Loire in green. Tissue locations are represented by different forms: internal by a circle, external by a square and unknown tissue by a triangle. Reference strains are indicated by diamonds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isolates from rainbow trout collected worldwide over a period of 14 years, the same order of gene diversity ($H = 0.5333$) was observed. Thus the French rainbow trout isolates exhibited almost as much diversity as the diversity observed in rainbow trout isolates from previously examined countries, thereby emphasizing the strong association between genetically closely related isolates and the rainbow trout host, as discussed below.

Clonal populations are identified by an I_A^S value that differs significantly from zero and population under panmixia has an I_A^S value close to zero. Limited and non-random sampling may lead to over-representation of particular STs, as in this study, and to the observation of a "clonal" population structure (Smith et al., 1993). This problem can be avoided by using single representatives of each ST as units for I_A^S measurement. On the other hand, Smith et al. (1993) demonstrated that when STs are divided into clusters of genetically related STs and each cluster is treated as a unit (Caugant et al., 1987), an epidemic structure of the population can be observed. In this case, the clonal structure observed for the entire dataset of the isolates and the all single representatives of each STs disappears. Here, the I_A^S value was significantly higher than 0 ($I_A^S = 0.6088$, $p < 0.05$) for all 66 *F. psychrophilum* samples which indicates that the alleles were in strong disequilibrium. The I_A^S value for the 15 STs ($I_A^S = 0.4567$, $p < 0.05$) was lower than 0 for the full dataset of 66 isolates, indicating that the number of recombination events which did not strongly affect the linkage between alleles was limited and attesting the possible clonal population structure of *F. psychrophilum* rainbow trout isolates in France. Equivalent I_A^S values were re-calculated from the data of Nicolas et al. (2008), and are globally similar to our data: 0.5984 for the entire sample (50 isolates) and 0.4936 using single representatives of the STs as units, both values being significantly higher than 0. However, when STs were clustered according to their genetic relatedness, the clonal structure of our sample disappeared ($I_A^S = 0.084$, $p > 0.05$), revealing an epidemic structure of the population. A similar example was observed with *Neisseria meningitidis* where the analyzed population appeared first clonal despite recombination events, to finally prove to be epidemic after I_A^S calculation following clustering (Smith et al., 1993). This demonstrates

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the epidemic structure of the *F. psychrophilum* population analyzed here.

Those data emphasizes that the genetic diversity in *F. psychrophilum* was mainly driven by homologous recombination, as previously demonstrated (Nicolas et al., 2008; Vos and Didelot, 2008). Moreover, PHI values for the concatenate sequences, which differed significantly from zero, supported the recombinogenic nature of *F. psychrophilum*. The PHI test also indicated that *atpA*, *dnaK*, *gyrB* and *trpB* are the housekeeping genes which induced diversity through recombination. This is consistent with the observed conflicts on the trees at the different loci (Supplementary data S2), and strongly indicative of the role of homologous recombination in the generation of diversity in *F. psychrophilum*, also deducible by the bushy network structure done by split decomposition analysis.

The recently published MLST analysis of *Aeromonas* spp. (*A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii*, *A. jandaei*, *A. schubertii*, *A. enteropelogenes*, *A. encheleia*, *A. allosaccharophila*, *A. popoffii*, and *A. sharmana*) commonly isolated from diseased and healthy fish, showed a very different pattern (Martino et al., 2011). Although the genetic diversity was very high, the methods used to detect evidence of recombination indicated that the intra-specific recombination was very low. This was the case for *A. veronii*, although a relevant effect of recombination was reported in another study (Silver et al., 2011). Thus, the different sampling schemes and methodologies used to detect bacterial recombination also clearly influence the conclusions drawn. In the present case of *F. psychrophilum*, recombination was shown to be a major evolutionary force driving genetic diversity in the French ecological niche, as observed worldwide (Nicolas et al., 2008).

Conjugating plasmids, phages and transposons that require bacterial competence, are the three vectors involved in bacterial recombination. Plasmids (Alvarez et al., 2004; Duchaud et al., 2007), a number of *F. psychrophilum* bacteriophages (Kim et al., 2010) and transposons (Alvarez et al., 2004) have been identified on *F. psychrophilum* but their involvement in recombination of this bacterium remains unknown.

In the present study, 15 sequence types were identified, including 14 new STs derived from only 2 additional single nucleotide polymorphic (SNP) sites. This implies that most of the described allele diversity originated from new intragenic associations of previously described SNPs, thus supporting the important role of homologous recombination in the evolution of this bacterium. The eBURST analysis with SLV criterion separated the 15 STs detected into one clonal complex (CC) where ST2 was identified as the primary founder, and several STs that did not share any similarity with this CC. In a previous publication (Nicolas et al., 2008), three clonal complexes (CC1, CC2 and CC3) had been detected and CC1 was also shown to have ST2 (isolated only from rainbow trout) as its primary founder. CC1 and CC can therefore be considered as similar. No members of the previously described CC2 and CC3 were identified in our isolates. With DLV criterion, analysis including the reference strains JIP 02/86 and NCIMB 1947^T, showed that only the ST (ST20) of the reference strain isolated from rainbow trout (JIP 02/86) shared ATs with STs included in the CC.

The occurrence of identical STs in geographically distant areas (worldwide) as described previously (Nicolas et al., 2008), and highlighted in Fig. 1, by the NJ tree (Fig. 4) and the statistical test, suggests a likely role of brood fish, egg fish and fish trading in the dissemination of *F. psychrophilum*, at least for some specific STs. Moreover, ST2 seems to be strongly associated to rainbow trout as it has only been found in this fish species (Nicolas et al., 2008). However, many singletons STs have also been characterized in rainbow trout both worldwide (8 in the study of Nicolas et al., 2008) and in France (7 in the present study). One explanatory hypothesis for the presence of these singletons STs, might be that these STs are not linked to the trading of salmonid products but are related to endemic isolates. Those endemic isolates might occur rarely and/or could be less virulent than the others or less well adapted to the rainbow trout species. Indeed *F. psychrophilum* has been found in water (Madetoja and Wiklund, 2002) and non-fish host that could act as reservoir (Amita et al., 2000; Izumi et al., 2005). Natural fish population can also serve as reservoirs of *F. psychrophilum*, and it was shown that the associated bacteria could demonstrate a greater genetic variability than observed in reared fish population (Chen et al., 2008). Then horizontal transmission through circulating water could be assumed. A detailed study of the genetic diversity of isolates on a more limited geographic scale (a fish farm) might reveal local clonal complexes otherwise masked by the CC1 members over-distributed as a result of fish trade.

In conclusion, MLST analysis supports the hypothesis of an epidemic population structure of this bacterium due mainly to the expansion of genetically closely related isolates. Additional analyses are now required to complete our knowledge of the genetic variants present at an even smaller geographical scale (a farm for example), to examine if singleton STs represent local and environmentally associated types with a local clonal structure. Such analyses could be simplified by designing an allele-specific real-time PCR for the most informative SNPs on each locus (Sheludchenko et al., 2010). As shown in the present study, very few new polymorphic sites were found in the 66 isolates analyzed (only 2 on the 429 kbp sequenced in this study), and the finding of new SNPs will probably be rare in this context (rainbow trout as host fish studied in France). A bar-coding technique would avoid the expensive and labor-consuming step of systematic sequencing and could be applied to a large set of isolates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2012.07.022.

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