Introduction, expansion and coexistence of epidemic
Flavobacterium psychrophilum lineages in Chilean fish farms

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A B S T R A C T

Chile is one of the countries where the development of salmonid farming has been the most successful. The first importation of salmonids in Chile from the northern hemisphere dates back to the late 19th century and the country now ranks as the world second largest producer of farmed salmon. However, the fast increase of infections caused by the bacterium Flavobacterium psychrophilum is a growing concern for this local industry. This pathogen, also recognized as an important problem worldwide, has been first reported in Chile in 1993 and is currently affecting all three cultivated salmonid species: Atlantic salmon (Salmo salar), Coho salmon (Oncorhynchus kisutch) and rainbow trout (O. mykiss). Here we conducted a MLST (multi-locus sequence typing) analysis of the local genetic diversity of F. psychrophilum to better understand its origin and propagation in the country, and to suggest practices that could contribute to its control in the future. A total of 94 bacterial isolates, collected from the main production zones, were analyzed and compared to those of other origins already available. The data reveal the country-wide distribution of several genotypes closely related to those that are the most prevalent in European and North American fish farms, and overlapping host fish species of the different lineages. This population structure is probably the direct consequence of local fish farming practices that relied until recently on massive import of fish eggs (e.g., 78 million of eggs in 2012) and where mixed-species farms and fish transportation across the country are common.

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1. Introduction

Chile is currently the second largest producer of farmed salmon in the world (Ibieta et al., 2011) despite the absence of native population of salmonids in the Southern hemisphere. All salmon and trout species found in different water bodies (rivers, lakes, fjords and sea) of the country have been imported for aquaculture purpose.
It has been estimated that Chile has bought over the years about two billion of salmonid eggs from various countries and continents of the Northern hemisphere, especially USA, Norway, Scotland, Denmark and Finland (Ibieta et al., 2011), since the first import of eggs in 1885 (Bluth et al., 2003).

Besides the absence of native salmonid species and the over 80% importation of eggs from foreign sources through the 1990s, several other aspects also contribute to distinguish salmonid farming in Chile from the rest of the world. In particular, whereas in other salmonid farming countries facilities are generally dedicated to the production of a unique species, many Chilean fish farms grow different salmonid species (i.e., rainbow trout, Atlantic salmon and Coho salmon). Moreover, specialized production zones have emerged (i.e., for fry and juveniles; for smolts and adults; for broodstock and hatching) to optimize the use of the different water bodies. As a consequence, eggs and live fish need to be moved across the country to complete their full life cycle (Rosefield and Manley, 2010).

*Flavobacterium psychrophilum* (Bernardet et al., 1996), a Gram-negative, filamentous, psychrotrophic bacterium belonging to the phylum *Bacteroidetes*, is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) in freshwater salmonid fish worldwide (see review Nematollahi et al., 2003; Barnes and Brown, 2011). In Chile, this condition is observed in freshwater aquaculture facilities since 1993 and the incidence of *F. psychrophilum* has dramatically increased since then (Bustos et al., 1995; Avendaño-Herrera et al., 2009). The bacterium is now routinely isolated from rainbow trout and Atlantic salmon raised in open and closed flow systems as well as from lake fish cultures; a total of 1937 cases were reported by the diagnostic laboratories between 2005 and 2010 (Godoy and Avendaño-Herrera, 2012). According to the Chilean National Fishing Service and Aquaculture (SERNAPECSA), the bacterium is responsible for significant to high mortality rates (5–70%) in fingerlings, resulting in economic losses which rank second after those caused by *Piscirickettsia salmonis* (Valdebenito and Avendaño-Herrera, 2009). Importantly, *F. psychrophilum* infections in Chile are usually chronic because the water temperature in fish freshwater farms rarely exceed 14 °C and is therefore particularly appropriate for the development of the pathogen. To date, antimicrobial therapies represent the only recourse to control the condition in farmed fish: it has been estimated that 55 tons of florfenicol and 55 tons of oxytetracycline were applied at the Chilean farms to control outbreaks between 2006 and 2009 (Henríquez-Núñez et al., 2012).

Despite the impact of *F. psychrophilum* infections in Chile, relatively little is known about its genetic diversity in the country. This knowledge would be useful for understanding epidemiological factors associated with the bacterium (i.e., host species, geographical distribution, virulence), in order to define appropriate management strategies to minimize the risks of pathogen introduction or transmission. Valdebenito and Avendaño-Herrera (2009) using RAPD, 16S rRNA alleles and repetitive extragenic palindromic PCR (REP-PCR) observed a relative genetic homogeneity using 20 Chilean *F. psychrophilum* strains isolated from farmed Atlantic salmon and rainbow trout. Other molecular techniques have been used to study the genetic variability of this species but only a few Chilean isolates have been included (Chakroun et al., 1998; Soule et al., 2005; Ramsrud et al., 2007; Nicolás et al., 2008). Among these approaches, multi-locus sequence typing (MLST) is currently regarded as the gold standard for molecular typing of many bacterial species, including *F. psychrophilum* (Nicolás et al., 2008; Siekoula-Nguedia et al., 2012; Fujiwara-Nagata et al., 2013; Strepparava et al., 2013). Recently, Apablaza et al. (2013) reported MLST sequence data for 25 Chilean *F. psychrophilum* isolates obtained from 2006 to 2010, showing some genotypical links with isolates from Europe and North America. However, the MLST scheme used in this latter study did not fit the standards of the other *F. psychrophilum* MLST studies, hampering comparisons with previously published and future data.

Here, we subjected a large collection of *F. psychrophilum* isolates retrieved from farms located in the main Chilean areas of salmonid production to the MLST scheme of the previous studies (Nicolás et al., 2008; Siekoula-Nguedia et al., 2012; Fujiwara-Nagata et al., 2013; Strepparava et al., 2013). This allowed to analyze genetic diversity and the comparison of the genotypes to those found in other countries.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Only 3 *F. psychrophilum* isolates from Chilean origin were included in Nicolás et al. (2008). In this study, we genotyped a total of 91 additional isolates. This collection consists of isolates that were retrieved by the authors either from fish (rainbow trout, Atlantic salmon and Coho salmon) directly sampled in the field or from fish sent to diagnostic laboratories; most fish presented typical clinical signs of BCWD or RTFS. The bacterium was obtained from internal organs (kidney or spleen), or less frequently, from external organs (gill, fin and skin). The identity of each isolate was confirmed as *F. psychrophilum* by standard phenotyping procedures (Bernardet et al., 2002): colony morphology and pigmentation, cell morphology, gliding motility, Gram-staining, cytochrome oxidase and catalase activities, oxidation/fermentation reactions, presence of cell wall-associated flexirubin type pigments and absorption of Congo red.

The *F. psychrophilum* type strain ATCC 49418 was included as a positive control in all analyses. The bacteria were grown on TYES agar plates (tryptone yeast extract salts medium, consisting of: 0.4% tryptone, 0.05% yeast extract, 0.02% anhydrous calcium chloride, 0.05% magnesium sulphate heptahydrate and 1.2% agar, pH 7.2) and incubated aerobically at 15 °C for 3–5 days. Stock cultures were maintained frozen at −80 °C in Cryobible tubes (AES Laboratory) or in TYES with 15% glycerol.

### 2.2. DNA extraction and confirmation of the bacterium species

Chromosomal DNA was extracted using InstaGene Matrix (Bio-Rad) following the manufacturer’s recommendations.
The quality of DNA was visually checked by agarose gel electrophoresis and DNA concentration was adjusted to 10–20 ng μL\(^{-1}\) with a ScanDrop analyzer (Analytikjena); 1 μL of DNA solution was used in each PCR.

In addition to the phenotypic tests described above, a genetic confirmation of the identification of each isolate as *F. psychrophilum* was obtained prior to multi-locus sequence typing using two different PCR tests (Urdaci et al., 1998; Izumi et al., 2003). The amplification cycles used for denaturation, primer annealing and primer extension were carried out according to each published PCR protocol. Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each batch of PCR reaction. The presence of a single product with a size compatible with that obtained with the type strain ATCC 49418\(^T\); [1088 base pairs (bp)] for primers FP1–FP2 of Urdaci et al. (1998) and 1017 bp for primers PSY-G1F–PSY-G1R of Izumi et al. (2003)] was considered as a positive result. In practice, aliquots of 10 μL of PCR product were separated on a 1.5% (w/v) agarose gel in TAE 1 × (0.04 M Tris, 0.0001 M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 μg mL\(^{-1}\) of ethidium bromide (Bio-Rad) and photographed under UV light. The GeneRuler\(^\text{TM}\) 100 bp DNA Ladder Plus (100 – 3000 bp, Fermentas) was used as a molecular mass marker.

2.3. Multilocus sequence typing and sequence analysis

Seven housekeeping genes were selected for the MLST analysis on the basis of previously published MLST data for *F. psychrophilum* (Nicolás et al., 2008), and optimized according to Siekoula-Nguedia et al. (2012). The PCR products were visualized on a UV transilluminator after separation on agarose gel. The purified PCR products were sequenced on both strands. The quality of the resulting chromatograms was checked visually and sequences of each locus of each isolate were assembled using Phred/Phrap/Consed (Ewing et al., 1998). The data are available on the new *F. psychrophilum* MLST database (http://pubmlst.org/psypychrophilum/), based on BigbPdb (Jolley and Maiden, 2010). In addition, nucleotide sequences corresponding to new alleles have been deposited in GenBank (accession numbers: KJ370397 - KJ371033).

Following MLST standards, the different alleles found at given loci received unique arbitrary numbers, referred to as allele types (ATs). The combination of ATs at the seven loci defined the sequence types (STs), also identified by unique numbers. Genetic relationships among the STs were analyzed using the eBURST v3.0 program (http://eburst.mlst.net) (Feil et al., 2004). An operational definition of clonal complex (CC) was adopted that consists of STs connected by single locus variations (SLVs) but double locus variations (DLVs) are also discussed.

To display the similarity between STs at evolutionary distance beyond the SLV level we have also relied on a single-linkage hierarchical clustering tree based on the number of AT-level differences between ST pairs. For this purpose we used the “hclust” function in *R* to build the tree and the “ape” *R* library for plotting. SplitsTree 4 version 4.12.3 (Huson and Bryant, 2006) with default settings (i.e., methods UncorrectedP, NeighborNet and EqualAngle) was used to build a network of the relationships between the 15 STs based on the concatenated sequences of the 7 loci.

The gene diversity (H) corresponding to the probability that two randomly chosen isolates harbor the same AT at a given locus was determined using LIAN 3.5 (Haubold and Hudson, 2000; http://pubmlst.org/analysis/).

3. Results

3.1. Sampling of *F. psychrophilum* diversity in Chile and phenotypic characterization

All isolates were biochemically homogeneous regardless of the source of isolation (salmon or trout) and were identical to the type strain ATCC 49418\(^T\). The phenotypic tests showed that all bacterial isolates were Gram-negative, long, slender rods with gliding motility that were catalase positive and weakly cytochrome oxidase positive. All isolates contained cell wall-associated flexirubin-type pigments, but did not absorb Congo red. The FP1-FP2 and PSY-G1F–PSY-G1R primer pairs produced a unique and clear PCR product of the expected length and thereby provided another indication that all isolates studied belong to the *F. psychrophilum* species.

The collection of 91 isolates genotyped in this study is presented in details in Supplementary Table 1. With respect to the host fish, 49 isolates were obtained from

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**Table 1**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Length (bp)</th>
<th>Polymorphic sites</th>
<th>Alleles</th>
<th>Gene Diversity (H)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Isolates (94)</td>
</tr>
<tr>
<td>atpA</td>
<td>834</td>
<td>24 (2.87%)</td>
<td>9</td>
<td>0.613</td>
</tr>
<tr>
<td>dnaK</td>
<td>873</td>
<td>9 (1.03%)</td>
<td>7</td>
<td>0.370</td>
</tr>
<tr>
<td>fumC</td>
<td>790</td>
<td>5 (0.67%)</td>
<td>6</td>
<td>0.383</td>
</tr>
<tr>
<td>gyrB</td>
<td>1077</td>
<td>18 (1.67%)</td>
<td>8</td>
<td>0.669</td>
</tr>
<tr>
<td>murC</td>
<td>681</td>
<td>10 (1.46%)</td>
<td>9</td>
<td>0.564</td>
</tr>
<tr>
<td>trpB</td>
<td>789</td>
<td>6 (0.76%)</td>
<td>6</td>
<td>0.332</td>
</tr>
<tr>
<td>tuf</td>
<td>795</td>
<td>12 (1.50%)</td>
<td>8</td>
<td>0.461</td>
</tr>
<tr>
<td>All</td>
<td>5799</td>
<td>84 (1.44%)</td>
<td>15 STs</td>
<td>0.485 ± 0.050</td>
</tr>
</tbody>
</table>

a Chilean specific ATs and STs with respect to the 436 isolates of the MLST database.

b Pairwise gene diversity H was computed either on the 94 Chilean isolates or on the 15 distinct STs found in Chile.

c Length, polymorphic sites and alleles (STs) were computed on the concatenate of the seven loci, H was averaged over the seven loci (± standard deviation).

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rainbow trout, 37 from Atlantic salmon and 2 from Coho salmon; no information on the host was available for 3 isolates. The geographical origin encompassed at least five Chilean states, from north to south: 1 strain came from Maule, 15 from Biobío, 6 from Araucanía, 58 from Los Lagos and 1 from Aysén del General Carlos Ibáñez del Campo (the precise geographical origin of the other isolates is not available) (Fig. 1). Most isolates were collected within a period of 5 years: 1 was obtained in 2005, 38 in 2006, 9 in 2007, 3 in 2008, 3 in 2009 and 12 in 2011 (25 isolates lacked precise associated information concerning their year of isolation). Of note, the number of *F. psychrophilum* isolates per year included in this study does not reflect the prevalence of the disease.

In our analyses we also included three more ancient Chilean *F. psychrophilum* isolates whose genotypes were already available (Nicolás et al., 2008): FC 1285/96 from rainbow trout in 1996, and MHC 1710K and MHC 1759SC obtained from Coho salmon in 2001.

3.2. Sequence polymorphism among the Chilean isolates

The seven housekeeping MLST genes were successfully amplified and sequenced, and the genetic characteristics of each locus are described in Table 1. Within the 5799 bp of the concatenated sequence of the seven loci in the 94 *F. psychrophilum* Chilean isolates, 84 polymorphic sites (1.44% of the positions) were observed. The number of single-nucleotide polymorphism sites between loci varied from 5 (at locus *fumC*) to 24 (at locus *atpA*). The average gene diversity *H* over the seven loci was $0.485 \pm 0.050$, with the lowest degree of diversity observed for *trpB* (0.332) and the higher value for *gyrB* (0.669). The analysis by the SplitTree program showed a reticulated network structure indicative of recombination events (Fig. 2).

A total of 15 distinct STs were found in the Chilean isolates. These STs were found at widely different frequencies (Fig. 3). The most frequent ST was ST21 that accounted for as much as 40% of the Chilean isolates, 4 other STs (ST2, ST12, ST38 and ST68) occurred individually in 7% to 14% of the isolates. Each of the 10 other STs were found in only 1 or 2 isolates, thus accounting for less than 2% of the total.

The strains with the five most frequent STs in our Chilean dataset (ST2, ST12, ST21, ST38 and ST68) were collected in at least two fish species (rainbow trout and Atlantic salmon), one strain each of ST2 and ST38 being also isolated from Coho salmon. Despite this clear indication of the possibility for each of the ST to infect multiple fish species, frequency of the STs differed statistically significantly between rainbow trout and Atlantic salmon (Fisher exact test *p*-value $<10^{-4}$). The most striking difference was for ST21 that accounted for as much of 70% of the isolates collected from Atlantic salmon,

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Fig. 1. Geographical origin of the 81 isolates with precise information available. The corresponding STs are also reported. The sampling encompasses five states, from north to south: Maule, Biobío, Araucanía, Los Lagos and Aysén del General Carlos Ibáñez del Campo.

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Fig. 2. Split decomposition analysis. The tree was constructed under neighbour net graph option of the SplitTree program, with concatenated sequence of the 15 STs found in Chile. Scale bar: nucleotide changes per bp.

Fig. 3. AT profiles and STs of the 94 F. psychrophilum Chilean isolates and their observed incidence in the different host fish species: rainbow trout, Atlantic salmon and Coho salmon. The tree used to display the genetic relationships between isolates was obtained by hierarchical clustering using single-link distance of AT-profiles (order: trpB, gyrB, dnaK, fumC, murG, tuf, and atpA).

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but for only 22% of the isolates collected from rainbow trout. In general, the STs were much more evenly distributed in rainbow trout than in Atlantic salmon where the aforementioned five most frequent STs displayed incidences ranging between 10% and 24% of the isolates. In contrast, the second most frequent ST in Atlantic salmon (ST38) accounted for 11% of the corresponding isolates while the other STs individually accounted for less than 6%. Our data carry much less information on the distribution of the STs in Coho salmon since only four samples from this species were available. However, there are some indication that this distribution differs both from the distribution found in Atlantic salmon ($p$-value = 0.02) and in rainbow trout ($p$-value = 0.05). In particular ST9 was found twice, but only in Coho salmon.

The five dominant STs are apparently not specific of a particular region of origin. They were all found in the Los Lagos state in which the sampling intensity was the highest; four of these STs were also found in at least a second Chilean state: ST21 in four states, ST2 and ST12 in three states, and ST38 in two states.

### 3.3. Evolutionary relationships between Chilean isolates and with isolates found abroad

The extremely high rate of homologous recombination in the species *F. psychrophilum* (Nicolás et al., 2008; Vos and Didelot, 2008) makes it difficult to retrace the deep evolutionary relationships between isolates from the nucleotide sequences. Indeed, depicting relationships with classical phylogenetic trees is not appropriate in this context. As an attempt to better account for the significance of recombination in the history of the 15 STs, a network was reconstructed with SplitsTree (Huson and Bryant, 2006). In this representation (Fig. 2), most of the different STs appear at approximately equal distance from a reticulated central network that reflects the panmictic population from which emerge the epidemic lineages. Only the eight STs showing no more than three divergent ATs with ST2 (i.e., ST2, ST10, ST12, ST21, ST38, ST72, ST87 and ST88) display clear evidence of greater relatedness (Fig. 3).

Another approach, underlying for instance the eBURST analyses (Feil et al., 2004), focuses on AT-profiles rather than on nucleotide sequence. This simplification does not allow resolving the deep relationships but captures the information on more recent events. It has also the advantage of providing evolutionary distance estimates that are more robust with respect to recombination events. In practice, a unique homologous recombination event between strains with distinct STs will usually introduce a random number of differences at the nucleotide level (all localized at the same locus). This number is not relevant here and all mutation and recombination events should be put on an equal footing, which is achieved by comparing AT-profiles instead of nucleotide profiles. In order to summarize the relationships between the 15 Chilean STs, we have built a hierarchical clustering tree based on a distance that corresponds to the number of distinct ATs between pairs of STs (Fig. 3). We also relied on an eBURST population snapshot of the whole collection of MLST data available for *F. psychrophilum* to investigate the links between the genotypes found in Chile and those found abroad (Fig. 4). The eBURST approach lead to a

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**Fig. 4.** eBURST population snapshot of the whole collection of *F. psychrophilum* isolates with MLST data available (436 isolates). The eBURST diagram shows a selection of SLV-links between the STs that correspond to eBURST inferred lines of descent. DLV-links are not represented but the derived grouping of STs is indicated by gray shades. Dot sizes are proportional to the number of isolates within each ST. The STs found in the 94 Chilean isolates are highlighted.
decomposition of the population in terms of clonal complexes, each one consisting of closely related strains—most likely sharing a recent common ancestor—in which STs are connected by SLV-links.

In our data, we identified the coexistence of two groups of strains that accounted for the five most frequent STs in the Chilean dataset: CC-ST2 and its close relatives found in CC-ST21 (connected to CC-ST2 by DLV-links), and CC-ST90 represented only by ST68 in Chile. Among these three clonal complexes, CC-ST2 and CC-ST90 are also largely represented outside Chile in particular in rainbow trout which was the fish species the most represented worldwide in the F. psychrophilum MLST database. Up to now CC-ST21 has not been found outside Chile, still it is closely related to CC-ST2 and none of the ATs of ST21 are specific from Chile.

The seven STs that belonged neither to the top five in terms of frequency nor to their three clonal complexes (CC-ST2, CC-ST90 and CC-ST21) were: ST218, ST71, ST23, ST9, ST87, ST69 and ST70. Among them, two were also already found elsewhere (ST9 in North America and Japan, ST23 in Europe), and none harbored less than two ATs also found outside Chile. Of note, ST9 is part of another clonal complex that was also found infecting Coho salmon in North America and Japan, and may be tightly associated to this fish species.

4. Discussion

The development of sustainable aquaculture requires a better epidemiological knowledge of the circulating pathogens. In Chile, the rapid development of the fish farming industry raised sanitary problems and pathogen control now becomes a major preoccupation. Using diverse typing methods, an important homogeneity among the Chilean F. psychrophilum isolates has been previously noticed (Valdebenito and Avendaño-Herrera, 2009). However these studies were performed on a rather limited number of isolates and the methods used did not allow comparisons at a worldwide scale. In contrast, MLST provides sequence data sets that are easy to compare between studies and are more relevant to analyze population structures and epidemiological processes. In this study, we applied the F. psychrophilum MLST scheme initially applied on a worldwide collection of 50 isolates (Nicolás et al., 2008) and subsequently used on several other collections originating from Europe (Siekoula-Nguedia et al., 2012; Strepparava et al., 2013) and Japan (Fujiwara-Nagata et al., 2013).

In Chile, 94 genotyped isolates collected from three fish species allowed to identify 15 distinct STs and the average gene diversity ($H$) amounted to 0.485 ± 0.050. These results correspond to a substantial gene diversity combined with a comparatively more limited number of STs. Indeed, this gene diversity is comparable to that found in 115 Swiss isolates collected from three fish species ($H$ = 0.493 ± 0.030; Strepparava et al., 2013) and higher than that found in 66 French isolates retrieved from a single fish species ($H$ = 0.431 ± 0.027; Siekoula-Nguedia et al., 2012). However, in these two datasets the ratio between the number of distinct STs and the number of isolates was higher (27 STs in the Swiss study, 15 STs in the French study). The Japanese study encompassing 120 isolates reported higher gene diversity ($H$ = 0.658 ± 0.026) and number of STs (35) but the number of sampled fish species was also considerably higher (15). These comparisons emphasize the coexistence in Chile of several F. psychrophilum lineages whose genetic divergence (they belong to separate clonal complexes) contribute to gene diversity ($H$) but whose limited number translates into a moderated number of distinct STs.

Historically, Chilean-spawned eggs were available as early as 1980, but Chilean hatcheries remained largely dependent on fertilized eggs from foreign broodstock through the 1990s (Ibieta et al., 2011). The MLST data leave no doubts that most of the F. psychrophilum lineages found in Chile have been introduced in the country by international trade of fish and/or eggs. In fact, a majority of the isolates found in Chile belong to clonal complexes also found abroad in similar fish species. In particular, this is true for CC-ST2, the dominant clonal complex found in rainbow trout in Europe, Japan and North America. A second clonal complex, CC-ST90, was reported to infect rainbow trout in France and Switzerland (Siekoula-Nguedia et al., 2012; Strepparava et al., 2013): our data show that it has also a representative in Chile (ST68). Another clonal complex, CC-ST9, apparently specific to coho salmon, received less attention due to the lower sampling of this fish species but was also found in North America and Japan (Nicolás et al., 2008; Fujiwara-Nagata et al., 2013). To date, the only clonal complex of our dataset that has not been found in the northern hemisphere so far is CC-ST21. However, ST21 is apparently particularly prevalent in Atlantic salmon for which the number of isolates available outside Chile is still very limited (three from Tasmania, one from Oregon, and none from its native geographical range). Furthermore, as noticed in the results section, this clonal complex is closely related to CC-ST2 dominant in rainbow trout (a DLV-link exists between CC-ST2 and CC-ST21) and none of its ATs appear to be specific from Chile. It is thus highly likely that CC-ST21 was also imported from abroad with rainbow trout or Atlantic salmon.

The occurrence with very low individual frequency of STs not connected to the main clonal complexes was interpreted in Europe and Japan as a possible contribution of endemic F. psychrophilum population; in line with this hypothesis the Japanese study, that included electrofishing of a variety of wild fish regardless of their disease status, demonstrated that a considerable genetic diversity existed in the F. psychrophilum population of a single river. Until now, F. psychrophilum has also been isolated from a variety of sources including water, sediment and non salmonid fish species (see for instance Madetoja and Wiklund, 2002; Nematoilahi et al., 2003; Izumi et al., 2005; Fujiwara-Nagata et al., 2013) but the existence of F. psychrophilum populations native from the Southern hemisphere remains to be demonstrated. In principle, the presence of endemic F. psychrophilum population might contribute to the occurrence of sporadic STs disconnected from the main epidemic clonal complexes in Chile (ST23, ST69, ST70, ST71, ST87, and ST218) but our data does not provide sound arguments to support this hypothesis. Instead, ST23
was also reported once in Europe and all the Chilean STs harbor at least two ATs also found outside Chile. Currently, our working hypothesis is thus that this less prevalent STs have also been imported to Chile by international trade of fish or eggs. However, the presence of *F. psychrophilum* in puyre (*Galaxias maculatus*), a native Chilean non-salmonid fish species, has been reported (Irgang et al., 2011). Future MLST analysis of *F. psychrophilum* sampled from native non-salmonid fish species might reveal endemic isolates and would help to quantify more precisely the low or null-contribution of native *F. psychrophilum* populations to infections in Chilean salmonid farms.

Most of the STs found in our data overlap host fish species and geographical ranges. Strepparava et al. (2013) already reported an absence of strong association between clonal complexes and host fish in Swiss fish farms and the tightly interconnected Swiss fish farms were suspected to be responsible for this. One can hypothesize that the Chilean fish farming practices have not only favored the expansion of imported lineages but also contributed to create overlapping host ranges. Indeed, most fish are transported once or twice over their life time, often over long distances, in order to complete their development (Godoy and Avendaño-Herrera, 2012) in the different fresh water facilities that include lake-based, tank and cage systems, estuary cage systems, stream-based flow-through systems and recirculation tank systems disseminated across the country (Ibieta et al., 2011). For instance, in 2012, only 7 among 169 freshwater fish farms performed the full fish cycle. In this complex system both the hatcheries and the fish farms contribute to the mixing process: most hatcheries supply smolts for grow-out in farms owned by their parent company, but many hatcheries also contract to supply smolts to unrelated farms, either using eggs from their own broodstock lines or using eggs provided by the contracting farms (Olson and Criddle, 2008); in parallel, fish farms often mix stocks from different areas (Avendaño-Herrera et al., 2009).

5. Conclusion

The MLST data on Chilean *F. psychrophilum* diversity reveal the country-wide distribution of several genotypes closely related to those that are the most prevalent in European and North American fish farms, and overlapping host fish species of the different lineages. This population structure is probably the direct consequence of local fish farming practices that relied until recently on massive import of fish eggs (e.g., 78 million of eggs in 2012) and where mixed species farms and fish transportation across the country are common. Our observations should help designing epidemiological monitoring schemes and rational farming practices to limit the spreading of *F. psychrophilum*.

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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.jvetmic.2014.02.009.

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