Reclassification of *Francisella noatunensis* subsp. *orientalis* Ottem et al. 2009 as *Francisella orientalis* sp. nov, description of *Francisella noatunensis* subsp. *chilensis* subsp. nov. and emended description of *Francisella noatunensis*

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The 10 whole genomes that were sequenced for this project have been deposited under the following DDBJ/ENA/GenBank accession numbers:

QPQI00000000

QPQJ00000000

QPQK00000000

QPQL00000000

QPQM00000000

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Abstract

Francisella noatunensis is a fastidious facultative intracellular bacterial pathogen that causes “piscine francisellosis”, a serious disease affecting both marine and fresh water farmed and wild fish worldwide. Currently two *F. noatunensis* subspecies are recognised i.e. *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis*. In the present study, the taxonomy of *F. noatunensis* was revisited using a polyphasic approach, including whole genome derived parameters such as digital DNA:DNA hybridization, whole genome average nucleotide identity (wg-ANIm), whole genome phylogenetic analysis, whole genome G+C content, metabolic fingerprinting and chemotaxonomic analyses. The results indicated that isolates belonging to *F. noatunensis* subsp. *orientalis* represent a phenotypically and genetically homogenous taxon, clearly distinguishable from *F. noatunensis* subspecies *noatunensis* that fulfils requirements for separate species status. We propose, therefore, elevation of *F. noatunensis* subsp. *orientalis* to the species rank as *Francisella orientalis* sp. nov. with the type strain remaining as Ehime-1^T (DSM 21254^T = LMG 24544^T). Furthermore, we identified sufficient phenotypic and genetic differences between *F. noatunensis* subsp. *noatunensis* recovered from diseased farmed Atlantic salmon in Chile and those isolated from wild and farmed Atlantic cod in Northern Europe to warrant proposal of the Chilean as a novel *F. noatunensis* subspecies i.e. *Francisella noatunensis* subsp. *chilensis* subsp. nov. with the strain PQ1106^T (CECT 9798^T = NCTC14375^T) as the type strain. Finally, we emend the description of *F. noatunensis* by including further metabolic information and the description of atypical strains.

Keywords

Francisella new species;

Francisella tilapia;

Francisella orientalis;

Francisella noatunensis subsp. *noatunensis;*

Francisella salmon Chile;

*Francisella noatunensi*s subsp. *chilensis*;

Introduction

In the last decade there has been a steady increase in the number of species and genera effectively and validly described, both cultured and uncultured, within the bacterial family *Francisellaceae* [1-13]. Although many of these studies have provided valuable insights into the population structure of the family as a whole, the lack of consistent clear criteria and cut-off values for delineation of closely related taxa has made fine scale taxonomy imprecise and led to numerous controversies including the initial description of *F. endociliophora* as "*Candidatus* F. noatunensis subsp. endociliophora", the reclassification of *F. novicida* as a subspecies of *F. tularensis* and the initial description of *F. noatunensis* as a subspecies of *F. philomiragia* [5, 14-21].

Francisella noatunensis is a facultative intracellular fastidious bacterial pathogen, which causes piscine francisellosis, an emerging disease reported worldwide affecting several farmed and captured fish species, in marine and fresh water ecosystems [22]. Currently *F. noatunensis* is divided into two subspecies i.e. *F. noatunensis* subsp. *noatunensis* (Fnn) and *F. noatunensis* subsp. *orientalsis* (Fno) [23].

Consistent with their fastidious nature, isolates of *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalsis* are biochemically unreactive to the standard tests that rely on bacterial growth and are commonly used in microbiology, complicating the comparative assessment of their physiological responses to different metabolites in taxonomic studies [23]. Recently, Ramirez-Paredes et al. [24] described a cost-effective and practical approach for characterisation of *F. noatunensis* subsp. *orientalis* based on a number of genomic, phenotypic and chemotaxonomic parameters and suggested a misplacement of this taxon within the species *F. noatunensis*.

In the present study, a polyphasic approach, including whole genome derived parameters such as digital DNA:DNA hybridization, whole genome average nucleotide identity (wg-ANIm), whole genome phylogenetic analysis, whole genome G+C content, metabolic fingerprinting and chemotaxonomic analyses was utilised on a large number of *Francisella* isolates representing all known validly described fish pathogenic lineages to revise the taxonomy of the species *Francisella noatunensis.*

Materials and methods

Bacterial strains and genome sequences

For this study 10 *F. noatunensis* strains i.e. Fno2-Fno9, Fno15 and Fnn6 (Table 1) were WG sequenced, assembled and annotated as previously described by Ramirez-Paredes [25] while the rest were retrieved from public databases. In total, 35 whole genome (WG) sequences comprising 13 *F. noatunensis* subsp. *orientalis*, 7 *F. noatunensis* subp. *noatunensis*, 6 *F. philomiragia*, all the validly described *Francisella tularensis* subspecies, *Francisella hispaniensis* and the type strain of the genus *Allofrancisella* were included (Table 1). The genome derived parameters here analysed i.e. digital DNA:DNA hybridization, whole genome average nucleotide identity (wg-ANIm), whole genome phylogenetic analysis and whole genome G+C content were in compliance with the standards recently for the taxonomy of prokaryotes outlined by Chun et al. [26]. In addition, for the phenotypic analyses 15 *F. noatunensis* subsp. *orientalis* and 15 *F. noatunensis* subp. *noatunensis* i.e. Fno1-Fno15 and Fnn1-Fn15 (Table 1) from different fish species and geographical origins, isolated over a period of 15 years and the type strain of the most closely related taxon i.e. *F. philomiragia* ATCC 25015^{T} isolated from diseased moribund muskrat were included.

Genomic characterisation

16S rRNA gene sequence similarity and phylogeny

Complete 16S rRNA gene sequences were retrieved from WG sequences by *in silico* PCR utilising universal primers EUBB 5'AGAGTTTGATCMTGGCTCAG'3 and EUBA 5'AAGGAGGTGATCCANCCRCA'3 described by Suzuki and Giovannoni [27] using the Sequence Manipulation Suite platform [28] with modified script to a limit input of 3,000,000 characters. The 16S rRNA sequences were aligned as DNA using MUSCLE [29] in MEGA version 6 [30]. Nucleotide composition and evolutionary divergence (pairwise distances) were then estimated using MEGA6. A Maximum Likelihood (ML) [31] phylogenetic tree was constructed using the Hasegawa-Kishino-Yano model (HKY) [32] with a discrete gamma distribution of rates across sites using X rate classes and deletion of gaps. The tree was bootstrapped with 1000 replications and nearest-neighbour-interchange branch swapping.

Multilocus sequence analysis (MLSA)

For the MLSA the following eight housekeeping genes were utilised: malate dehydrogenase (*mdh*), chromosomal replication initiator protein alpha subunit (*dnaA*), DNA mismatch repair protein (*mutS*), peptide chain release factor 2-beta subunit (*prfB*), bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase alpha subunit (*putA*), DNA-directed RNA polymerase alpha subunit (*rpoA*), DNA-directed RNA polymerase beta subunit (*rpoB*) and triose-phosphate isomerase alpha subunit (*tpiA*).

The suitability of these genes for phylogenetic analyses was previously confirmed by Ramirez-Paredes et al. [24]. The respective sequences were retrieved from available WG sequences using Prodigal version 2.6.2 [33]. Sequences for individual loci were concatenated and aligned as described by Ramirez Paredes et al. [24].

Housekeeping gene sequences were concatenated using an in-house Perl script (www.perl.org) (Supplementary File 1a) as previously described by Ramirez Paredes et al. [24]. The suitability of the data for Neighbour Joining (NJ) algorithm analysis was confirmed using the average pairwise Jukes-Cantor (JC) distance method [31] and a NJ phylogenetic tree based on concatenated sequences was constructed using MEGA6 [30]. Finally, pairwise percent similarity values for the concatenated sequences were estimated and pairwise distances inferred using MEGA6 [30].

Whole genome average nucleotide identity (wg-ANI$_m$), phylogenetic analysis and G+C content

In order to compare the genomes, the 35 WG sequences were aligned into a single multiple alignment. Prior to the alignment, the sequences of *Francisella hispaniensis* FhSp1^T, *Francisella tularensis* subsp. *mediasiatica* GIEM 543^T and *F. philomiragia* ATCC 25015^T, retrieved from sequence read archive, had to be reassembled (due to low assembly quality) using the parallel assembler for short read sequence data ABySS (Assembly By Short Sequences) version 1.3.4 [34]. To conduct the multiple alignment, the sequences were firstly aligned into different subsets of 12 or 13 genomes using "a sum-of-pairs breakpoint score" available in progressive Mauve [35] version 2.4.0 development snapshot (2015-02-13). Each subset included three assemble reference genomes or backbone genomes i.e. *F. philomiragia* ATCC25017 (accession number CP000937.1), *F. tularensis* subsp. *holarctica* FSC200 ^{T} (accession number CP003862.1) and *F. noatunensis* subsp. *orientalis* LADL-07-285A (accession number CP006875.1). To complete the alignment the resulting multiple alignments were merged into using the LADL-07-285A genome as reference with an in-house Perl script (Supplementary File 1b).

For the whole genome average nucleotide identity (wg-ANI), pairwise comparisons were performed with all the sequences within and between the taxa in JSpecies [36] using the MUMmer algorithm i.e. wg-ANIm [37]. For the phylogenetic analysis a Neighbour Joining (NJ) tree was constructed using the whole genome alignment and the average pairwise Jukes-Cantor (JC) distance model [31] using the software MEGA version 6 [30]. Finally the G+C content of the genome sequences was computed using the software MEGA version 6 [30].

In silico DNA:DNA hybridization (DDH)

Digital DDH values were estimated between all strains of the family *Francisellaceae* using the Formula-2 (identities / high-scoring segment pairs length) of the genome to genome distance calculator version 2.1 [38] available at http://ggdc.dsmz.de/ with the aligned WG sequences.

Phenotypic characterisation

Culture conditions and basic phenotyping

All isolates were cultured either on cystine heart agar supplemented 1:1 (v/v) with 2% bovine haemoglobin (BD, Oxford, UK) (CHAH) or in Modified Mueller-Hinton II cation adjusted broth supplemented with 2% IsoVitaleX (BD, Oxford, UK) and 0.1% D-(+)-glucose ACS reagent (Sigma-Aldrich, Dorset, UK) (MMHB) depending on the analysis.

The basic phenotypic characteristics included Gram stain, catalase, oxidase tests and motility. Colony morphology, optimal *in vitro* growth temperatures and the mean time to reach mid logarithmic phase of the type strains were investigated on CHAH as described by Ramirez-Paredes et al. [24]. Broth cultures were incubated at the optimal *in vitro* temperature for each taxon i.e. cold or warm water fish, mammals etc. and in all cases cells were harvested at mid log phase. The biomass subjected to extraction of quinones and polar lipids was grown at 20

°C in Bacto Heart Infusion Broth (BD, Oxford, UK) supplemented with 10g glucose l^{-1} and 1 g L-cystine l^{-1}.

Metabolic fingerprinting

The metabolic capabilities of 30 fish-pathogenic *Francisella* strains (15 *F. noatunensis* subsp. *orientalis* and 15 *F. noatunensis* subsp. *noatunensis*) and the *F. philomiragia* type strain ATCC 25015^T were investigated using the Biolog GN2 micro system (Biolog Inc., California, USA) according to the manufacturer's instructions with the modifications described by Ramirez-Paredes et al. [24]. The optimal density (OD_{600}) used to inoculate the Biolog GN2 microplates with *F. noatunensis* subsp. *orientalis* and *F. noatunensis* subsp. *noatunensis* was 0.85, whereas 0.65 was adequate for *F. philomiragia*. After inoculation, the microplates were incubated at the optimal *in vitro* temperature of each taxon. The tests were read visually at six and twelve hours, and digitalised with a computer scanner (Epson Perfection V370 Photo scanner; Epson, London, UK) for subsequent retrospective comparison.

Chemotaxonomic analyses

Cellular fatty acid methyl ester (FAME) composition

The cellular fatty acid methyl ester (FAME) composition of 20 *Francisella* strains including the type strains *F. noatunensis* subsp. *orientalis* Ehime-1^T, *F. noatunensis* subsp. *noatunensis* NCIMB 14265^T and *F. philomiragia* ATCC 25015^T was determined as previously described by Ramirez-Paredes et al. [24] with a minor modification. Briefly, the FAME extracts were purified on HPTLC (high performance thin layer chromatography) plates (10cm X 10cm X 0.15mm) pre-coated with silica gel 60 (without fluorescent indicator) *(*Merck KGaA, Darmstadt, Germany*)*.

Quinones, polar lipids and polyamines

The strains *F. noatunensis* subsp. *orientalis* Ehime-1^T (Fno1) from Japan, *F. noatunensis* subsp. *noatunensis* NVI 5330^T (Fnn1) from Norway, *F. noatunensis* subsp. *noatunensis* PQ1106 (Fnn5) from Chile and *F. noatunensis* subsp. *noatunensis* SVA74/04 (Fnn15) from Sweden were subjected to quinone, polar lipid and polyamine detection and quantification analyses. The quinones and polar lipids were extracted and analysed according to the protocol outlined by Tindall [39, 40] and Altenburger et al. [41]. For detection of total lipids 5% ethanolic molybdatophosphoric acid was used. Aminolipids, phospholipids and glycolipids were identified using 0.2 % ethanolic ninhydrin, Molybdenum blue reagent (Sigma-Aldrich, Dorset, UK) and α–naphthol-reagent, respectively. The polyamines were extracted following the protocols of Busse and Auling [42] and Busse et al. [43]. The HPLC equipment used for analysis of quinones and polyamines was described by Stolz et al. [44].

Results

Genomic comparisons

16S rRNA gene similarity and phylogenetic analysis

The 16S rRNA gene of the *F. noatunensis* subsp. *orientalis* and *F. noatunensis* subsp. *noatunensis* strains were 1520 nucleotides, 2 bp longer than *F. philomiragia* strains and 1 bp longer than *A. guangzhouensis*. All the *F. noatunensis* subsp. *orientalis* were 100% identical but not all the *F. noatunensis* subsp. *noatunensis* were identical as the Chilean strain had a lower value i.e. 99.8% when compared to the European isolates. The similarity between *F. noatunensis* subsp. *orientalis* and *F. noatunensis* subsp. *noatunensis* strains was 99.1-99.2%, between *F. noatunensis* subsp. *orientalis* and *F. philomiragia* was 99.1-99.2% and between *F. noatunensis* subsp. *noatunensis* and *F. philomiragia* 99.2-99.5. The estimates of evolutionary divergence and the percent similarity are shown in Supplementary File 2a.The phylogenetic tree generated for this gene is presented in Supplementary File 2b.

Multilocus sequence analysis (MLSA)

The alignment of concatenated housekeeping genes totalled 15,750 bp. The pairwise identity comparisons identified 99.9-100% similarity within *F. noatunensis* subsp. *orientalis*. Values of 94.4-94.5% were found between *F. noatunensis* subsp. *orientalis* and *F. philomiragia*, and values of 96.7-97.0% were seen between *F. philomiragia* and *F. noatunensis* subsp. *noatunensis* suggesting that *F. noatunensis* subsp. *noatunensis* is indeed more closely related to *F. philomiragia* than to *F. noatunensis* subsp. *orientalis*. An average value of 95.48% (lower than that between *F. noatunensis* subsp. *noatunensis* and *F. philomiragia*) was observed between *F. noatunensis* subsp. *orientalis* and *F. noatunensis* subsp. *noatunensis* suggesting that these represent two separate species. This analysis also revealed genetic differences between the European and Chilean *F. noatunensis* subsp. *noatunensis* that are similar (99.7%) to those seen between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *novicida*. Pairwise percent identities are presented in Supplementary File 3a.

Phylogenetic analyses of housekeeping genes provided a greater degree of resolution than 16S rRNA and were in agreement with the pairwise identity comparisons. This analysis revealed that *F. noatunensis* subsp. *orientalis*, *F. noatunensis* subsp. *noatunensis* and *F. philomiragia* represent a monophyletic group for which *F. noatunensis* subsp. *orientalis* represents the basal member. The Chilean strain remained within the *F. noatunensis* subsp. *noatunensis* taxon but in a separate branch from the European strains. Interestingly the Irish *F. noatunensis* subsp. *noatunensis* clustered closely with the Scandinavian *F. noatunensis* subsp. *noatunensis* but in a separate subclade within the European *F. noatunensis* subsp. *noatunensis* branches. The neighbour joining tree for the concatenated housekeeping genes is presented in Supplementary File 3b.

Whole genome, average nucleotide identity (wg-ANI$_m$), phylogeny and G+C content

Wg-ANI$_m$ indicated 99.9-100% similarity amongst *F. noatunensis* subsp. *orientalis* strains, lower value between *F. noatunensis* subsp. *orientalis* and *F. philomiragia* (93.1%) than between *F. philomiragia* and *F. noatunensis* subsp. *noatunensis* 95.2% confirming that *F. philomiragia* is more similar to *F. noatunensis* subsp. *noatunensis* than to *F. noatunensis* subsp. *orientalis*. While similarity values of 100% were identified among the Scandinavian *F. noatunensis* subsp. *noatunensis*, the Chilean had a lower similarity value i.e. 99.5% when compared to them. Some minor differences of 99.94-99.95% were seen between the Irish and Scandinavian. The average whole-genome nucleotide identities are summarised in Table 2.

The topology observed in the WG phylogeny was in agreement with wg-ANI values. For instance *F. noatunensis* subsp. *noatunensis*, *F. philomiragia* and *F. noatunensis* subsp. *orientalis* conformed a monophyletic branch with *F. noatunensis* subsp. *orientalis* departing from this lineage as a clearly independent species prior to separation of *F. noatunensis* subsp. *noatunensis* and *F. philomiragia*. The analysis also confirmed the Chilean strain to represent a novel *F. noatunensis* subsp. *noatunensis* subspecies while the Irish and Scandinavian *F. noatunensis* subsp. *noatunensis* isolates grouped into two different branches within the *F. noatunensis* subsp. *noatunensis* clade. The WG phylogenetic tree is presented in Figure 1.

The overall nucleotide composition (%) and G+C content (%) of each of the genomes here analysed are presented in Supplementary File4.

In silico DNA:DNA hybridization (DDH)

The DDH values between *F. noatunensis* subsp. *orientalis* and *F. noatunensis* subsp. *noatunensis* were between 59-60% (59% against the Chilean *F. noatunensis* subsp. *noatunensis*). *Francisella philomiragia* had higher values of association with *F. noatunensis* subsp. *noatunensis* i.e. 65-66% (65% against the Chilean *F. noatunensis* subsp. *noatunensis*) than with *F. noatunensis* subsp. *orientalis* (53-54%) confirming that *F. noatunensis* subsp. *noatunensis* is more closely related to *F. philomiragia* than to *F. noatunensis* subsp. *orientalis* and thus (as seen in the wg-ANI_m) the latter taxon constitutes a separate species. The values between the Chilean and the European *F. noatunensis* subsp. *noatunensis* were 98% while the values among the four validly published *F. tularensis* subspecies ranged between 88-98%. The values between the Chilean and the European *F. noatunensis* subsp. *noatunensis* were also 98%. All the other *Francisella* strains had values ranging from 24-49%, which confirms their status as independent species. The value that established a threshold between the two validly published genera within this family i.e. *Allofrancisella* and *Francisella* was 21%. The DDH values between all the strains are shown in Table 3.

Phenotypic comparisons

Culture conditions and basic phenotyping

A detailed description of the basic phenotypical features including optimal growth temperatures and colony morphology of fish pathogenic *Francisella* spp. from different geographic origin and the *F. philomiragia* type strain here analysed is presented in Supplementary File 5.

Metabolic fingerprint

A total of 24 differences were identified between *F. noatunensis* subsp. *noatunensis* and *F. philomiragia*, 17 between *F. noatunensis* subsp. *orientalis* and *F. philomiragia*¸ 10 between *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* including the inability of *F. noatunensis* subsp. *noatunensis* to metabolise glucose-1- phosphate (H11), glucose-6-phosphate (H12), and acetic acid (D1) and the inability of *F. noatunensis* subsp. *orientalis* to metabolise D-alanine (F5). Differences were also observed within *F. noatunensis* subsp. *noatunensis* with the largest number identified between the Norwegian and Chilean isolates (9 differences) and 3 between the Irish and the Scandinavian. A summary of the results obtained with the modified protocol of the Biolog GN2 microplates is presented in Supplementary File 6.

Chemotaxonomic analyses

Cellular fatty acid methyl ester (FAME) composition

The FAME 24:0 and the total amount of saturated and monounsaturated FAMEs were significantly different amongst the 3 taxa compared. The content of 24:1n-9 was significantly higher in *F. philomiragia* and the content of 22:1 was significantly lower in *F. noatunensis*

subsp. *orientalis*. No significant differences were seen between isolates belonging to the same taxon. The relative FAME composition per taxon is presented in Table 4 and the FAME content per isolate is shown in Supplementary File 7.

Quinones, polar lipids and polyamines

The quinones analysis displayed a system which had Q-8 as the major ubiquinone (99-100 %) in the four compared strains. Additionally traces of Q-9 were detected in strains Fnn1 (*F. noatunensis* subsp. *noatunensis* NVI 5330^T) and Fnn15 (*F. noatunensis* subsp. *noatunensis* SVA74/04). The polar lipid profile for the 4 strains was almost identical differing only in the amounts of certain lipids and consisted of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, two unidentified phospholipids (PL2 and PL3) and an unidentified phosphoglycolipid (PGL). The polar lipid profile of the *F. noatunensis* subsp. *noatunensis* from Chile was highly similar to that of *Francisella noatunensis* subsp. *orientalis* Ehime-1^T regarding presence of major compounds but it differed by the presence of moderate amounts of the unidentified glycolipids GL and GL1, the lipids L1, L2 and L3 lacking a functional group and the aminophospholipid APL2 and lack of the phosphoglycolipid PGL. A comparison of the total polar lipid profiles of the strain Ehime-1^T and the *F. noatunensis* subsp. *noatunensis* from Chile is presented in Figure 2. The polyamine patterns of the four strains contained predominantly cadaverine and spermidine and minor amounts of 1, 3 diaminopropanem putrescine and/or spermine (Table 5).

Discussion

In prokaryotic taxonomy 2 concepts of species i.e. the “genomo” [45] and the “phylophenetic” [46] have been tacitly adopted by the International Committee on Systematics of Prokaryotes http://www.the-icsp.org/ [47]. The genetic differences to discriminate “genomo” species were

first outlined by Wayne et al. [45], later revised by Stackebrandt and Goebel [48] and most recently updated by Stackebrandt and Ebers [49]. The phylophenetic concept is based on the criteria used for the "genomo species" definition, but establishes that besides being genomically coherent, species should also be monophyletic (in a phylogenetic tree) and clearly diagnosable by a discriminative phenotypic properties [46].

The most updated guidelines for bacterial species delineation indicate that strains sharing 16SrRNA gene similarity of ≤ 98.65% and DNA-DNA (DDH) re-association values of ≤ 70% constitute separate species [50, 51]. Although these genetic thresholds have proven useful for wide variety of bacterial species, they have not been able to clearly discriminate closely related *Francisella* spp. [23, 24, 52-54, Supplementary Files 2a and 8].

Ramasamy et al. (2014) [55] firstly proposed the incorporation of genomic data into the description of novel bacterial species and more recently Chun et al. [26] and Ciufo et al. [56] proposed the use of whole genome identity analyses i.e. wg-ANI, GGD and *in silico* DDH as an alternative solution for the valid description of novel closely related species that share a high level of sequence similarity in 16S rRNA gene.

In recent years several novel *Francisella* spp. including *F. halioticida*, *F. oportunistica, F. salina. F. uliginis F. frigiditurris, F. persica*, *F. endociliophora*, *F. adeliensis* and *F. marina* have been reported in the scientific literature [2, 3, 6, 9-11]. Although *F. halioticida* and *F. marina* are associated with disease in farmed aquatic organisms these are not closely related to *Francisella noatunensis* (Supplementary File 8, 10). Moreover since no whole genome sequence is available for the first and the later has not been validly described these were not included in the current study.

In the present, the inclusion of whole genome sequences (WGS) of the most closely related validly published members of the family *Francisellaceae* allowed genetic comparisons at different levels of resolution and phylogenetic analyses.

As expected, with similarity values above 99.1%, 16S rRNA was not sufficient to discriminate between *F. noatunensis* subsp. *orientalis, F. noatunensis* subsp. *noatunensis* and *F. philomiragia*. Interestingly, all *F. noatunensis* subsp. *orientalis* were 100% similar despite their wide geographical origin while the Chilean *F. noatunensis* showed an evolutionary divergence of 3 nucleotides (99.8%) with respect to the European *F. noatunensis* subsp. *noatunensis* strains.

The MLSA provided a greater power of resolution than 16S rRNA, the pairwise identity comparisons of the concatenated housekeeping genes revealed that *F. noatunensis* subsp. *noatunensis* and *F. philomiragia* are more closely related to each other than *F. noatunensis* subsp. *orientalis* to *F. noatunensis* subsp. *noatunensis,* suggesting that the latter two are not part of the same taxon. The identity values suggested that these 3 taxa stand as individual species and not as subspecies of *F. philomiragia*. The homology seen amongst the *F. noatunensis* subsp. *orientalis* strains and amongst the European *F. noatunensis* subsp. *noatunensis* strains remained as 100% while the similarity between *F. noatunensis* subsp. *noatunensis* from Europe and Chile was 99.7% which was 0.1% lower than in the 16S rRNA analysis. The MLSA results were backed up by the phylogenetic analysis which demonstrated a monophyletic relationship between the fish pathogenic *F. noatunensis* subspecies and *F. philomiragia* with clear separation of the two fish pathogenic taxa in braches that would stand at the species level. Moreover a close up view of the subtree clustering of the *F. noatunensis* subsp. *noatunensis* isolates confirmed the divergence of the Chilean *F. noatunensis* and also

depicted some degree of dissimilarity between the *F. noatunensis* from Ireland and the Scandinavian strains.

Whole genome similarity analyses i.e. wg-ANI, GGD and *in silico* DDH are considered as ultimate level of resolution. These comparisons confirmed that *F. noatunensis* subsp. *noatunensis* and *F. philomiragia* are more closely related to each other than *F. noatunensis* subsp. *noatunensis* to *F. noatunensis* subsp. *orientalis* validating the proposal that the latter are indeed two separate species. These analyses also depicted that the separation between the Chilean and European *F. noatunensis* subsp. *noatunensis* has the same degree of divergence (98%) to that observed amongst the *F. tularensis* subspecies i.e. *F. tularensis* subsp. *tularensis* vs *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* vs *F. tularensis* subsp. *mediasiatica*, confirming that in the context of the current valid taxonomy they constitute two different *F. noatunensis* subspecies.

WG phylogeny was consistent with the previous topologies and demonstrated a monophyletic relationship between the two fish pathogenic *F. noatunensis* subspecies and *F. philomiragia* where *F. noatunensis* subsp. *orientalis* appeared as the basal member of this monophyletic clade with *F. philomiragia* lying in an intermediate position between the two clearly separated fish pathogenic taxa. The evolutionary divergence seen between the Chilean and European *F. noatunensis* subsp. *noatunensis* isolates in this tree, was in agreement with the proposal that the first represent a subspecies of *F. noatunensis*. Interestingly this analysis also confirmed the divergence seen in the MLSA tree between the Irish and the Scandinavian isolates. Although the Irish was placed on separate sub-branch, the level of genetic divergence was not as big as that seen between the Chilean and the European or between the 4 *F. tularensis* ssp. and thus was not considered sufficient to separate it as novel “genomo” subspecies.

In the phenotypic analyses, the basic features here described for *F. noatunensis* subsp. *noatunensis* are in agreement with previous studies [19, 23] however the optimal growth temperature and the colony morphology of the "*orientalis*" strains differs to that reported by Ottem et al. [23] in the description of *F. noatunensis* subsp. *orientalis*. The use of the modified protocol for the Biolog GN2 microplates demonstrated to be a powerful and inexpensive tool to differentiate enzymatic activity between the monophyletic and genomically coherent clusters identified here on the genomic analyses. Each of the taxa or "clusters" here analysed had a unique metabolic profile. The *F. noatunensis* subsp. *orientalis* strains were highly homogeneous and distinct from the *F. noatunensis* subsp. *noatunensis* and *F. philomiragia*, and these two were clearly different from each other. Likewise the profile among the strains from Scandinavia was highly similar with only one difference seen between the Swedish and Norwegian strains however the strains from Chile and Ireland had a distinct profile where the Chilean had 9 differences and the Irish 3 with respect to the Norwegian isolates. In the chemotaxonomic analyses the *F. noatunensis* subsp. *orientalis* FAME results indicated that fatty acid 24:0, the total saturated and the total monounsaturated were significantly different among the *F. noatunensis* subsp. *noatunensis*, *F. philomiragia* and *F. noatunensis* subsp. *orientalis* groups, confirming that these can be considered as taxon-specific chemotaxonomical markers and a reference for future research on *Francisella* spp. taxonomy. This was not the case for the other chemical markers that were not able to distinguish between species/subspecies here analysed. Nevertheless valuable descriptive information was obtained from them such as the presence of a quinone system with the major ubiquinone Q-8 in all of the strains, the lack of several minor lipids reported to be present in *F. tularensis* subsp. *tularensis*, *F. philomiragia* and *Allofrancisella guangzhouensis* [5, 57] and polyamine patterns containing major amounts of cadaverine and spermidine in all the strains. The absence of the

unidentified aminophospholipid APL4 suggests that the four strains are less related to *F. tularensis* subsp. *tularensis* and *F. guangzhouensis* than to *F. philomiragia* which also lacks it.

Conclusion

Based on the results here obtained it is proposed to elevate *Francisella noatunensis* subsp. *orientalis* to the rank of species as *F. orientalis* retaining the type strain Ehime-1^T (=DSM 21254^T = LMG 24544^T). According to the rule 50b of International Code of Nomenclature of Bacteria [58] "*the name of the subspecies must be used as the specific epithet of the name of the species*" and thus the name as *F. orientalis* sp. nov. is proposed. According to rule 50b this elevation does not create a new combination [59]. In addition we also propose to create a new subspecies i.e. *F. noatunensis* subsp. *chilensis* subsp. nov., with the type strain PQ1106^T (CECT 9798^T = NCTC14375^T). Furthermore we emend the description of *Francisella noatunensis* by adding relevant phenotypic information including the description of atypical *F. noatunensis* subsp. *noatunensis* strains. The nomenclature and taxonomic classification here proposed for the fish pathogenic *Francisella* strains is presented in Supplementary File 9. The phenotypic differences that allowed differentiation between species and subspecies are summarised in Table 6.

Description of *Francisella orientalis* sp. nov.

Francisella orientalis (o.ri.en.ta‘lis. L. fem. adj. *orientalis* from the east, referring to where the type strain was isolated).

The description of this species is as that presented for *F. noatunensis* subsp. *orientalis* Ottem et al. [23] with the following corrections and additions:

The optimal *in vitro* growth temperature is 28-29 °C and the colonies are greenish-greyish on CHAH. Virulent strains are pathogenic to cichlids and other warm water fish species when the water temperature is below 25 °C. It is capable of metabolising dextrin, N-acetyl-Dglucosamine, D-fructose, α-D-glucose, D-mannose, methyl pyruvate, acetic acid, α-keto butyric acid, L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, L-proline, L-serine, L-threonine, inosine, uridine, glycerol, DL-α-glycerol phosphate, glucose-1-phosphate and glucose-6-phosphate. It is weakly reactive to lipase, α-chymotripsin and α-galactosidase enzymes. The polyamine pattern of the type strain contains predominantly cadaverine and spermidine. The quinone system contains predominantly ubiquinone Q-8. The polar lipid profile is composed of the major lipids phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, two unidentified phospholipids (PL2 and PL3) and an unidentified phosphoglycolipid (PGL). The type strain is Ehime-1^{T} (DSM 21254^{T} = LMG 24544^{T}) isolated from the farmed marine fish three-line grunt or isaki (*Parapristipoma trilineatum*) in 2001 in Ehime-prefecture, Japan. The whole genome G+C content of the type strain is 32.2%.

Description of *Francisella noatunensis* subsp. *chilensis* subsp. nov.

Francisella noatunensis subsp. *chilensis* (chi.len′sis. N.L. fem. adj. *chilensis* referring to the country Chile where the type strain was isolated).

473 This subspecies has only been isolated in South America i.e. Chile from diseased farmed or
474 captured fish Atlantic salmon and common galaxias or jollytail (*Galaxias maculatus*) in fresh
475 or brackish water in Chile [60-64].

476 In contrast to the strains of *F. noatunensis* subsp. *noatunensis* the colonies of this subspecies
477 are greenish-greyish and do not grow well at concentrations of NaCl greater that 2%. *F.*
478 *noatunensis* subsp. *chilensis* is unable to metabolise N-acetyl-D-glucosamine, mono-methyl-
479 succinate, L-alaninamide, D-alanine, L-alanylglycine, but it is positive for glycerol, L-
480 threonine, DL-lactic acid and acetic acid. The type strain is PQ1106^T (CECT 9798^T =
481 NCTC14375^T) isolated from Atlantic salmon farmed in the Lake Llanquihue Chile in 2006
482 [57] presents moderate amounts of the unidentified glycolipids GL and GL1, the lipids L1, L2
483 and L3 and lacks a functional group and the aminophospholipid APL2 and the
484 phosphoglycolipid PGL.

485 **Emended description of the *F. noatunensis* Ottem et al. 2009 [23]**

486 *Francisella noatunensis* (no.at.un.en′ sis. N.L. n. *noatun* (enclosure of ships) was the coastal
487 abode of the Norse god of fisheries and seamanship; L. fem. suffix *-ensis* suffix meaning
488 'belonging to'; N.L. fem. adj. *noatunensis* belonging to the coast/sea).

489 In addition to the description provided by Mikalsen et al. [19] and Ottem et al. [23] all the
490 strains of this species (including both subspecies and atypical strains) are capable of
491 metabolising D-fructose, α-D-glucose, D-mannose, methyl pyruvate, α-keto butyric acid, L-
492 alanine, L-asparagine, L-glutamic acid, L-proline, L-serine, inosine. In contrast to *F. orientalis*
493 the strains of both subspecies of this taxon are negative for dextrin, uridine, DL-α-glycerol
494 phosphate, glucose-1-phosphate, and glucose-6-phosphate. In the polyamine pattern
495 spermidine and cadaverine are predominant. The quinone system contains predominantly

ubiquinone Q-8. The polar lipid profile shows the major lipids phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, two unidentified phospholipids (PL2 and PL3) and an unidentified phosphoglycolipid (PGL). The whole genome G+C content of the type strain: 2005/50/F292-6C^{T} (NCIMB 14265^{T} = LMG 23800^{T}) isolated from Atlantic cod in Norway in Hordaland county Norway in 2005 is 32.5%. The strains of *Francisella noatunensis* subsp. *noatunensis* have only been isolated from diseased fish farmed or captured in northern Europe. The colonies of this subspecies are whitish and can grow in up to 6% of NaCl. Virulent strains are pathogenic to fish when water temperature is above 4 °C and are able to metabolise N-acetyl-D-glucosamine, mono-methyl-succinate, L-alaninamide, D-alanine, L-alanylglycine. Atypical strains have been recovered from diseased cod in Ireland, in contrast to the Scandinavian *F. noatunensis* subsp. *noatunensis* the atypical "Irish" strain (Fnn13) had the ability to metabolise acetic acid and glycerol but incapable to use D-alanine. A strain isolated from Sweden (Fnn15) is also incapable to metabolise D-alanine.

Conflicts of interest

Declarations of interest: none. Authors have no competing interests to declare.

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FIGURES CAPTIONS

Figure 1. Whole genome phylogenetic tree depicting the evolutionary relationship of the family *Francisellaceae*.The evolutionary history was inferred using the Neighbor-Joining method and there were a total of 841,918 positions in the final dataset.

Figure 1. Whole genome phylogenetic tree depicting the evolutionary relationship of the family *Francisellaceae* (continued). Close up of the subtree clustering fish pathogenic strains and *F. philomiragia*.

Figure 1. Whole genome phylogenetic tree depicting the evolutionary relationship of the family *Francisellaceae* (continued). Comparison of the subtree clustering the *F. noatunensis* subsp. *noatunensis* (pathogenic to cold water fish) strains.

Figure 2. Total polar lipid profile of strain Ehime-1^{T} (a) and *F. noatunensis* subsp. *noatunensis* from Chile (b) after two-dimensional TLC and detection with 5 % ethanolic molybdatophosphoric acid. DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL2–3, unidentified phospholipids; PGL, unidentified phosphoglycolipid.

TABLES:

Table 1. Strains of *Francisellaceae* spp. analysed in this study.

Current species and subspecies	Designation	Genome Accession#	PRESENT ID	NVI	FOI	DSM	CCUG	BCCM/LMG	OTHER	Country	Location	Year	Host	Host	Nomenclature after classification here proposed
F. noatunensis subsp. *orientalis*	Ehime-1^{T}	PRJNA73447	Fo1	5887	FSC 771^{T}	21254^{T}	-	LMG 24544^{T}	-	Japan	Ehime prefecture	2005	Three-line grunt (Isaki)	*Parapristipoma trilineatum*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	F. Victoria	QPQI00000000	Fo2	-	FDC 191	-	-	-	-	Unknown	Unknown	Unknown	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	RUSVM-LA1	QPQJ00000000	Fo3	-	FDC 192	-	-	-	-	Unknown	Latin America	2012	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	F. CAL2	QPQK00000000	Fo4	-	FDC 193	-	-	-	-	USA	California	Unknown	Hybrid striped bass	*Morone chrysops x M. saxatilis*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	F. CAL1	QPQL00000000	Fo5	-	FDC 194	-	-	-	-	USA	California	Unknown	Hybrid striped bass	*Morone chrysops x M. saxatilis*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	LADL-07-285A	QPQM00000000	Fo6	-	FDC 195	-	-	-	-	Costa Rica	Alajuela	2007	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	LADL-11-060	QPQN00000000	Fo7	-	FDC 196	-	-	-	-	USA	Texas	Unknown	Tilapia	*Oreochromis* spp.	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	LADL-01-100	QPQO00000000	Fo8	-	FDC 197	-	-	-	-	USA	Midwest	2010	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	LADL 10-075 #5	QPQP00000000	Fo9	-	FDC 198	-	-	-	-	USA	Midwest	2010	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	Franc-COS1	-	Fo10	9535	-	-	-	-	-	Mexico	Queretaro	2013	Blue tilapia	*Oreochromis aureus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	Austria	-	Fo11	9449	-	-	-	-	-	Austria	Vienna	2013	Malawi cichlid	*Aulonocara maleri*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	STIR-GUS-F2f7	PRJNA297804	Fo12	-	FDC 410	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	STIR-MATT-F1f6	-	Fo13	-	-	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	PQ1104	PRJNA73389	Fo14	5409	FSC 770	-	-	-	-	Costa Rica	Unknown	2006	Tilapia	*Oreochromis* spp.	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	Cefas	QPQQ00000000	Fo15	8373	FDC 190	-	-	-	-	UK	England	Unknown	Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	STIR-AVU-F2f9	-	Fo16	-	-	-	-	-	-	Unknown	Europe	2012	Red Nile tilapia	*Oreochromis niloticus*	*Francisella orientalis*
F. noatunensis subsp. *orientalis*	Toba04	PRJNA82619	Fo17	-	-	-	-	-	-	Indonesia	Lake Toba	2004	Mozambique tilapia	*Oreochromis mossambicus*	*Francisella orientalis*
F. noatunensis subsp. *noatunensis*	NVI 5330^{T}	PRJNA73397	Fn1	5330	FSC 769^{T}	12596 ^{T}	-	LMG 23800^{T}	NCIMB 14265^{T}	Norway	Hordaland County	2005	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 5340	-	Fn2	5340	-	-	-	-	-	Norway	Sogn og Fjordane	2005	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 5396	-	Fn3	5396	-	-	-	-	-	Norway	Rogaland County	2006	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 5394	-	Fn4	5594	-	-	-	-	-	Norway	Møre og Romsdal	2006	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	PQ1106	PRJNA73449	Fn5	5888	FSC 772	-	-	-	-	Chile	Lake Llanquihue	2006	Atlantic salmon	*Salmo salar*	*Francisella noatunensis* subsp. *chilensis*
F. noatunensis subsp. *noatunensis*	NVI 6086	VIIR00000000	Fn6	6086	FDC 189	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6214	-	Fn7	6214	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6422	-	Fn8	6422	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6471	-	Fn9	6471	-	-	-	-	-	Norway	Møre og Romsdal	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6572	-	Fn10	6572	-	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6684	-	Fn11	6684	-	-	-	-	-	Norway	Nordland County	2009	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 7127	-	Fn12	7127	-	-	-	-	-	Norway	Rogaland County	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	F/134/09A	PRJNA73465	Fn13	7061	FDC 178	-	-	-	-	Ireland	Waterford	2009	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 8087	-	Fn14	8087	-	-	-	-	-	Norway	Møre og Romsdal	2011	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	SVA 74/04	PRJNA73463	Fn15	5518	FSC 846	-	-	-	-	Sweden	Southern Skagerrak	2004	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	NVI 6577	-	Fn16	6577	-	-	-	-	-	Norway	Sogn og Fjordane	2008	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	GM2212 (by NVI)	PRJNA73457	Fn17	5865	FSC 774	18777	-	-	-	Norway	Rogaland County	2004	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. noatunensis subsp. *noatunensis*	GM2212 (by FOI)	PRJNA73459	Fn18	-	FSC 775	18777	-	-	-	Norway	Rogaland County	2004	Atlantic cod	*Gadus morhua*	*Francisella noatunensis* subsp. *noatunensis*
F. philomiragia	O#319L^{T}	PRJNA32411	Fp1	5411	-	7535^{T}	19700^{T}	LMG7903^{T}	ATCC 25015^{T}	USA	Utah	1959	Muskrat	*Ondrata zibethica*	*F. philomiragia*
F. philomiragia	O#319-029	PRJNA73371	Fp2	-	FSC 037	-	-	-	ATCC 25016	USA	Utah	1960	Water	-	*F. philomiragia*
F. philomiragia	O#319-036	PRJNA27853	Fp3	5598	-	-	19701	-	ATCC 25017	USA	Utah	1960	Water	-	*F. philomiragia*
F. philomiragia	O#319-067	PRJNA73373	Fp4	-	FSC 039	-	-	-	ATCC 25018	USA	Utah	1960	Water	-	*F. philomiragia*
F. philomiragia	CCUG12603	PRJNA73377	Fp5	5596	FSC 145	-	12603	-	-	Sweden	Gothenburg	1982	Human	*Homo sapiens*	*F. philomiragia*
F. philomiragia	Swiss	PRJNA73381	Fp6	5597	FSC 154	-	13404	-	CDC E6588	Switzerland	Zurich	1979	Human	*Homo sapiens*	*F. philomiragia*
F. tularensis subsp. *holarctica*	GIEM 503^{T}	PRJNA16087	Ft1	-	FSC 200^{T}	-	-	-	GIEM 503^{T}	Sweden	Ljusdal	1998	Human	*Homo sapiens*	*F. tularensis* subsp. *holarctica*
F. tularensis subsp. *mediasiatica*	GIEM 543^{T}	PRJNA19571	Ft2	-	FSC 147^{T}	-	-	-	GIEM 543^{T}	Kazakhstan	Alma-Alta region	1965	Mid-day gerbil	*Meriones meridianus*	*F. tularensis* subsp. *mediasiatica*
F. tularensis subsp. *tularensis*	SCHU S4$^{\sharp}$	PRJNA239340	Ft3	-	FSC 237	-		-	-	USA	Ohio	1938	Human	*Homo sapiens*	*F. tularensis* subsp. *tularensis*
F. tularensis subsp. *novicida*	U112^{T}	PRJNA16088	Ft4	-	FSC 040^{T}	-	33449^{T}	-	ATCC 15482^{T}	USA	Utah	1951	Water	-	*F. tularensis* subsp. *novicida*
F. hispaniensis	FhSp1^{T}	PRJNA73391	Fh	-	FSC 454^{T}	22475^{T}	5802^{T}	-	FnSp1^{T}; F62^{T}	Spain	Unknown	2003	Human	*Homo sapiens*	*F. hispaniensis*
A. guangzhouensis	08HL01032^{T}	PRJNA271279	Fg	-	-	-	60119^{T}	-	NCTC 13503^{T}	China	Guangzhou city	2008	Water	-	*A. guangzhouensis*

NVI: Norwegian Veterinary Institute; FOI: Swedish Defence Research Agency; DSM: German Culture Collection of Microorganisms and Cell Cultures; CCUG Culture Collection, University of Göteborg, Sweden; Belgian Coordinated Collections of Microorganisms; NCIMB: National Collection of Industrial, Food and Marine Bacteria; ATCC: American Type Culture Collection; CDC: Centre for Disease Control and Prevention; GIEM: Gamalei Institute of Epidemiology and Microbiology; NCTC: National Collection of Type Cultures; (-) not applicable; $^{\sharp}$ Is the prototypic virulent strain not the type strain. FSC774 was supplied by NVI and FSC775 was requisitioned from DSM by FOI.

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Table 2. Whole genome average nucleotide identity ANIm (%) within the family *Francisellaceae*.

	Fno1	Fo2	Fo3	Fo4	Fo5	Fo6	Fo7	Fo8	Fo9	Fo12	Fo14	Fo15	Fo17	Fn1	Fn5	Fn6	Fn13	Fn15	Fn17	Fn18	Fp1	Fp2	Fp3	Fp4	Fp5	Fp6	Ft1	Ft2	Ft3	Ft4	Fh1	Ag1
Fo1	---																															
Fo2	100.0	---																														
Fo3	100.0	100.0	---																													
Fo4	100.0	100.0	100.0	---																												
Fo5	100.0	100.0	100.0	100.0	---																											
Fo6	100.0	100.0	100.0	100.0	100.0	---																										
Fo7	100.0	100.0	100.0	100.0	100.0	100.0	---																									
Fo8	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																								
Fo9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																							
Fo12	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																						
Fo14	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																					
Fo15	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																				
Fo17	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	---																			
Fn1	94.5	94.6	94.5	94.5	94.5	94.5	94.5	94.6	94.5	94.6	94.5	94.6	94.5	---																		
Fn5	94.4	94.4	94.4	94.4	94.4	94.4	94.4	94.5	94.4	94.5	94.4	94.5	94.4	99.5	---																	
Fn6	94.5	94.5	94.5	94.4	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.4	100.0	99.5	---																
Fn13	94.4	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.4	94.5	94.4	100.0	99.5	100.0	---															
Fn15	94.5	94.6	94.5	94.5	94.5	94.5	94.5	94.6	94.5	94.6	94.5	94.6	94.5	100.0	99.5	100.0	100.0	---														
Fn17	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.6	94.5	94.5	94.5	94.5	94.5	100.0	99.5	100.0	100.0	100.0	---													
Fn18	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	94.5	100.0	99.5	100.0	100.0	100.0	100.0	---												
Fp1	93.1	93.2	93.1	93.1	93.2	93.1	93.1	93.2	93.2	93.2	93.1	93.2	93.1	95.3	95.2	95.2	95.2	95.2	95.2	95.2	---											
Fp2	93.1	93.2	93.1	93.1	93.2	93.1	93.1	93.2	93.2	93.2	93.1	93.2	93.1	95.3	95.2	95.2	95.2	95.2	95.2	95.2	98.0	---										
Fp3	93.1	93.2	93.2	93.2	93.2	93.2	93.2	93.3	93.2	93.3	93.1	93.3	93.1	95.3	95.2	95.2	95.2	95.2	95.2	95.2	98.0	100.0	---									
Fp4	93.1	93.2	93.1	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.1	93.2	93.1	95.3	95.2	95.2	95.2	95.2	95.2	95.2	98.0	100.0	100.0	---								
Fp5	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.2	93.1	95.3	95.2	95.2	95.3	95.3	95.3	95.3	98.0	97.8	97.8	97.8	---							
Fp6	93.0	93.1	93.0	93.0	93.1	93.0	93.0	93.1	93.0	93.1	93.0	93.1	93.0	95.2	95.1	95.2	95.2	95.2	95.2	95.2	98.0	97.9	97.9	97.9	98.0	---						
Ft1	84.9	85.1	85.0	84.9	85.0	84.9	84.9	84.9	85.0	84.9	85.4	84.9	84.9	85.2	85.2	85.2	85.0	85.2	85.2	85.1	85.0	85.4	85.4	85.4	85.3	85.3	---					
Ft2	85.0	85.3	85.1	85.3	85.3	85.3	85.3	85.4	85.3	85.4	85.4	85.4	85.0	85.3	85.3	85.1	85.1	85.2	85.3	85.2	85.0	85.5	85.5	85.5	85.4	85.3	99.3	---				
Ft3	84.9	85.1	85.0	85.0	85.0	84.9	84.9	84.9	85.0	84.9	85.3	84.9	84.9	85.2	85.2	85.2	85.0	85.2	85.2	85.2	85.0	85.5	85.5	85.5	85.3	85.3	99.3	99.4	---			
Ft4	85.2	85.7	85.3	85.7	85.7	85.6	85.7	86.1	85.7	86.1	85.7	86.1	85.2	85.4	85.4	85.2	85.3	85.4	85.4	85.4	86.0	86.0	86.0	86.0	85.7	85.6	98.1	98.1	98.2	---		
Fh1	85.8	86.1	85.8	86.0	86.0	86.0	86.0	86.2	86.1	86.1	85.9	86.1	85.8	85.8	85.7	85.7	85.7	85.7	85.8	85.7	86.0	86.1	86.1	86.1	86.0	85.9	91.5	91.6	91.6	91.7	---	
Ag1	83.7	84.6	84.0	84.7	84.7	84.7	84.7	85.4	84.8	85.3	83.7	85.3	83.7	83.6	83.6	83.2	83.5	83.6	83.6	83.6	84.0	83.7	83.8	83.7	84.0	83.7	83.3	83.8	83.4	84.0	83.8	---

In grey *F. noatunensis* subsp. *orientalis* vs *F. noatunensis* subsp. *noatunensis* (average 94.5%); In purple *F. philomiragia* vs *F. noatunensis* subsp. *orientalis* (average 93.1%); In blue *F. philomiragia* vs *F. noatunensis* subsp. *noatunensis* (average 95.2%); In yellow % similarity within the 4 *F. tularensis* ssp.; In green % similarity of the Chilean isolate with respect to the other *F. noatunensis* subsp. *noatunensis.* All the strains are identified according to Table 1.

Table 3. Digital DNA-DNA hybridisation (DGG) values (%) for all members of the family *Francisellaceae* analysed in this study.

---	Fno1	Fno2	Fno3	Fno4	Fno5	Fno6	Fno7	Fno8	Fno9	Fno12	Fno14	Fno15	Fno17	Fnn1	Fnn5	Fnn6	Fnn13	Fnn15	Fnn17	Fnn18	Fp1	Fp2	Fp3	Fp4	Fp5	Fp6	Ft1	Ft2	Ft3	Ft4	Fh	Ag1
Fno1	---																														
Fno2	100	---																													
Fno3	100	100	---																												
Fno4	100	100	100	---																											
Fno5	100	100	100	100	---																										
Fno6	100	100	100	100	100	---																									
Fno7	100	100	100	100	100	100	---																								
Fno8	100	100	100	100	100	100	100	---																							
Fno9	100	100	100	100	100	100	100	100	---																						
Fno12	100	100	100	100	100	100	100	100	100	---																					
Fno14	100	100	100	100	100	100	100	100	100	100	---																				
Fno15	100	100	100	100	100	100	100	100	100	100	100	---																			
Fno17	100	100	100	100	100	100	100	100	100	100	100	100	---																		
Fnn1	60	60	60	60	60	60	60	60	60	60	60	60	60	---																	
Fnn5	59	59	59	59	59	59	59	59	59	59	59	59	59	98	---																
Fnn6	60	60	60	60	60	60	60	60	60	60	60	60	60	100	98	---															
Fnn13	60	60	60	60	60	60	60	60	60	60	60	60	59	100	98	100	---														
Fnn15	60	60	60	60	60	60	60	60	60	60	60	60	60	100	98	100	100	---													
Fnn17	60	60	60	60	60	60	60	60	60	60	60	60	60	100	98	100	100	100	---												
Fnn18	60	60	60	60	60	60	60	60	60	60	60	60	60	100	98	100	100	100	100	---											
Fp1	53	53	53	53	53	53	53	53	53	53	53	53	53	66	65	66	66	66	66	66	---										
Fp2	53	53	53	53	53	53	53	53	53	53	53	53	53	66	65	66	66	66	66	66	85	---									
Fp3	53	53	53	53	53	53	53	53	53	53	53	53	53	66	65	66	66	66	66	66	85	100	---								
Fp4	53	53	53	53	53	53	53	53	53	53	53	53	53	66	65	66	66	66	66	66	85	100	100	---							
Fp5	54	54	54	54	54	54	54	54	54	54	54	54	54	66	65	66	66	66	66	66	85	85	85	85	---						
Fp6	53	53	53	53	53	53	53	53	53	53	53	53	53	66	65	66	66	66	66	66	84	86	86	86	86	---					
Ft1	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	---				
Ft2	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	97	---			
Ft3	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	98	98	---		
Ft4	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	88	88	88	---	
Fh	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	25	47	47	48	48	---
Ag1	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	19	19	19	19	19

In grey *F. noatunensis* subsp. *orientalis* vs *F. noatunensis* subsp. *noatunensis* (average 59%); In purple *F. philomiragia* vs *F. noatunensis* subsp. *orientalis* (average 53%); In blue *F. philomiragia* vs *F. noatunensis* subsp. *noatunensis* (average 66%); In yellow % similarity within the 4 *F. tularensis* ssp.; In green % similarity of the Chilean isolate with respect to the other *Fnn.* All the strains are identified according to Table 1.

Table 4. Relative fatty acid composition (%) of the taxa analysed

Fatty acid	*F. noatunensis* subsp. *orientalis*	*F. philomiragia*	*F. noatunensis* subsp. *noatunensis*	Average
14:0	8 ± 2	8 ± 2	10 ± 1	9
16:0	8^{b} ±1	11^{a} ±1	9^{b} ± 1	9
18:0	7 ± 1	4 ± 1	5	5
20:0	4	4 ± 1	5 ± 1	4
22:0	9^{ab} ± 1	6^{b} ± 2	10^{a} ± 3	9
24:0	11^{a} ± 1	2^{c} ± 1	6^{b} ± 2	6
Total saturated	51^{a} ± 2	36^{c} ± 3	44^{b} ± 4	44
18:1n-9	16 ± 1	18 ± 1	17 ± 2	17
20:1n-9	2^{b}	3^{a} ± 1	3^{a}	3
22:1	3^{b}	7^{a} ± 2	7^{a} ± 1	6
24:1n-9	23^{b} ± 2	29^{a} ± 1	23^{b} ± 3	25
26:1	2	4 ± 1	2 ± 1	3
Total monounsaturated	49^{c} ± 2	63^{a} ± 4	55^{b} ± 4	56
Total	100	100	100	100

Data are presented as mean + standard deviation (range per taxon). The values bearing different letters are significantly different ($P < 0.05$).

Table 5**. Polyamine compositions of the 4 *Francisella noatunensis* strains analysed**

Strain	DAP	PUT	CAD	SPD	SPM
Fno Ehime-1^{T}	0.2	2	22.6	11.5	0.2
Fnn NVI 5330^{T}	-	0.1	8.3	13.8	0.6
Fnn PQ1104	-	0.1	22.8	3	0.6
Fnn SVA74/04	0.1	1.9	21.7	9.7	0.1

DAP, 1,3-diaminopropane; PUT, putrescine; CAD, cadaverine; SPD, spermidine; SPM, spermine.

745

Table 6. Differential phenotypic characteristic of fish pathogenic *Francisella* spp. using the taxonomic classification here proposed

Characteristic	***F. orientalis***	***F. noatunensis***		
	F. orientalis	*F. noatunensis* subsp. *noatunensis*		*F. noatunensis* subsp. *chilensis*
Origin/ distribution	worldwide	Europe (Scandinavia)	Europe (Ireland)	Chile
Optimal temperature °C	28-29	22	22	22
Colony colour on CHAH	greenish-greyish	white	white-greyish	greyish-greenish
Assimilation of:				
Mono-methyl- succinate	-	+	+	-
Acetic acid	+	-	+	+
D,L-Lactic acid	-	-	-	+
D-Alanine	-	+	-	-
L-Alanylglycine	+	+	+	-
L-Threonine	+	-	-	+
Glycerol	+	-	+	+
Glucose-6-phosphate	+	-	-	-