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# Genetic and phenotypic diversity of *Flavobacterium psychrophilum* isolates from Czech salmonid fish farms

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## Abstract

**Background** The salmonid pathogen *Flavobacterium psychrophilum* poses a significant economic threat to global aquaculture, yet our understanding of its genetic and phenotypic diversity remains incomplete across much of its geographic range. In this study, we characterise the genetic and phenotypic diversity of 70 isolates collected from rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta* m. *fario*) from fish farms in the Czech Republic between 2012 and 2019 to compare their genomic content with all draft or complete genomes present in the NCBI database ($n = 187$).

**Results** The Czech isolates underwent comprehensive evaluation, including multiplex PCR-based serotyping, genetic analysis, antimicrobial resistance testing, and assessment of selected virulence factors. Multiplex PCR serotyping revealed 43 isolates as Type 1, 23 as Type 2, with sporadic cases of Types 3 and 4. Multi-locus sequence typing unveiled 12 sequence types (ST), including seven newly described ones. Notably, 24 isolates were identified as ST329, a novel sequence type, while 22 were classified as the globally-distributed ST2. Phylogenetic analysis demonstrated clonal distribution of ST329 in the Czech Republic, with these isolates lacking a phage sequence in their genomes. Antimicrobial susceptibility testing revealed a high proportion of isolates classified as non-wild type with reduced susceptibility to oxolinic acid, oxytetracycline, flumequine, and enrofloxacin, while most isolates were classified as wild type for florfenicol, sulfamethoxazole-trimethoprim, and erythromycin. However, 31 isolates classified as wild type for florfenicol exhibited minimum inhibitory concentrations at the susceptibility breakpoint.

**Conclusion** The prevalence of the Czech *F. psychrophilum* serotypes has evolved over time, likely influenced by the introduction of new isolates through international trade. Thus, it is crucial to monitor *F. psychrophilum* clones within and across countries using advanced methods such as MLST, serotyping, and genome sequencing. Given the open nature of the pan-genome, further sequencing of strains promises exciting discoveries in *F. psychrophilum* genomics.

**Keywords** Aquaculture, *Flavobacterium* psychrophilum, Farmed rainbow-trout, Antimicrobial susceptibility, Serotypes, Genetic diversity

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## Background

Global trends in aquaculture over the past 20 years have shown substantial and consistent growth in freshwater aquaculture production, alongside advancements in fish nutrition and the introduction of feed alternatives [1]. Similar patterns are observed in the Czech Republic, where aquacultural production predominantly consists of extensive and semi-intensive pond fish farming. While around 80% of total Czech aquacultural production is represented by common carp (*Cyprinus carpio*) cultivation in polyculture stocking, intensive farming is predominantly focused on non-native salmonids, such as rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*), with an annual production exceeding 700 t [2]. According to the latest annual report from the Ministry of Agriculture of the Czech Republic, production of salmonids in 2022 reached 635 t, accounting for 3.3% of total Czech fish production [3]. Although total fish production has remained relatively stable around 20,000 t per year, there has been a significant increasing trend in salmonid aquacultural production over the monitoring period 2010-2021, peaking in 2018 at 1106 t [4–14].

However, growth in intensive fish farming is naturally accompanied with increased prevalence of microbial diseases, with the majority of infections caused by bacterial pathogens, such as flavobacteriosis, furunculosis and Enteric Redmouth Disease (ERM). The most frequently isolated flavobacterial pathogen in Czech fish farms is *Flavobacterium psychrophilum*, known to cause Bacterial Cold Water Disease (BCWD) and Rainbow Trout Fry Syndrome (RTFS) [15]. Mortality rates among aquacultural salmonids can reach up to 50% for BCWD and 70% for RTFS, resulting in substantial economic losses. Due to current preference to use antimicrobial treatment of *F. psychrophilum* infections, recent studies have shown that strains of *F. psychrophilum* have developed resistance to various antibiotics, including oxytetracycline, enrofloxacin, oxolinic acid, erythromycin, flumequine, and ampicillin [16, 17].

As fish production continues to rise, the aquacultural industry is facing growing challenges to meet sustainability criteria, standards and environmental expectations. A crucial aspect of addressing this issue lies in enhancing aquacultural management practices, particularly through increased surveillance for pathogens and parasites that represent a significant thread to production systems. In other countries such as Nordic countries, Switzerland, France, USA, Chile and Japan epidemiological analyses including MLST profiles of *F. psychrophilum* isolates were determined [18–22]. Therefore purpose of this study is to highlight the lack of such data in the Czech Republic and the importance of determining the epidemiology of *F. psychrophilum* in Central Europe. We present a contemporary analysis exploring the nuanced population dynamics of *F. psychrophilum* in the Czech Republic, with a dedicated focus on its prevalence within the high-intensity aquaculture of salmonids. Additionally, we provide an up-to-date examination of the genomic population of *F. psychrophilum* in the Czech Republic, with a particular emphasis on the intense farming of salmonids. Furthermore, we compare these findings with genomes from other regions worldwide to gain insights into global patterns of *F. psychrophilum* diversity and distribution.

## Methods

### Fish sample processing and isolation of *F. psychrophilum*

Sampling of dead fish on farms was carried out by fish disease specialist veterinarians as part of routine health care. Live diseased fish were transported to the laboratories of the Institute of Infectious Diseases and Microbiology at the University of Veterinary Sciences Brno in isothermal boxes or in oxygenated water within two to four hours of collection by trained breeders or veterinarians specialised in fish diseases. Live diseased fish were then euthanized by an overdose of anesthetic in the water (clove oil 0.5 mL/l) and left in the euthanasia solution for 10 min after cessation of opercular movement, in compliance with Act No. 246/1996 Coll. and Act No. 418/2012 Coll. and EC1099/2009 regulations on animal welfare during euthanasia, as issued by the government of the Czech Republic and the European Union, prior to autopsy and sampling by trained professionals. Samples mostly came from rainbow trout ($n=66$), with just four samples obtained from brown trout (*Salmo trutta* m. *fario*; $n=4$). Tissues sampled included spleen ($n=29$ isolates), body cavity smears ($n=23$ isolates), liver ($n=5$ isolates), gills ($n=5$ isolates), kidneys ($n=4$ isolates), skin ($n=2$ isolates) and roes ($n=2$ isolates) (Table S1). All bacteria were isolated and routinely cultivated on Tryptone Yeast Extract Salts (TYES) agar containing 0.4% tryptone (Tryptone, Oxoid), 0.04% yeast extract (Yeast Extract, Oxoid), 0.02% $CaCl_2 \times 2H_2O$ (Sigma-Aldrich), 0.05% $MgSO_4 \times 7H_2O$ (Merck KGaA), 0.05% glucose (Erba Lachema), 1.3% bacteriological agar (Agar No. 1, Oxoid) and 5.0% bovine foetal serum (BFS) (Biosera, France). The isolates were then routinely cultivated at $17 \pm 0.5$ °C for 3–4 days. The presumptive *F. psychrophilum* colonies grown at each plate identified using PCR as described in Cepeda & Santos [23], and matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) using a MALDI Biotyper v3.0 system (Bruker Daltonics). All isolates were subcultivated at least twice from a single colony to verify purity prior to preservation in a cryoprotective medium comprising 74.1% distilled water, 25.0% glycerol (Sigma

Aldrich) and 0.9% of bacteriological peptone (Oxoid) at -80 °C until further processing.

#### Multiplex PCR-based serotyping

Isolates were revived from the glycerol cryostock and grown on TYES agar at 17 ± 0.5 °C for 72 hours, with all cultures sub-cultivated prior to analysis. DNA was extracted from cells using the boiling method [24]. Briefly, a full loop of bacterial culture was added to 500 µl of sterile distilled water, mixed well and the suspension heated in a thermoblock (Labnet International) at 100 °C for 10 min. The suspension was then centrifuged and the supernatant used immediately or stocked at -20 °C. Serotypes were determined using multiplex PCR, as described in Rochat et al., 2017 [25] with minor modifications. Primer sequences for determination of Type 4 (5'-TGAAGCAAAAGCAACAAACA-3' and 5'-CCCCAA ACTGCTTACCTAAT-3'), which were not included in the latter study, were kindly provided by J. F. Bernardet. The PCR was carried out at a total volume of 25 µl (12.5 µl PPP Master Mix (Top Bio s r.o.), 3.5 µl PCR Ultra $H_2O$ (Top Bio s r.o.), 10 pmol of each primer and 1 µl of DNA). In a multiplex PCR combining all five primer pairs, only the 188 bp product demonstrated clear visibility under UV visualization. The remaining amplicons were too faint for definitive analysis. Insufficient amplification for some targets was likely caused by an uneven consumption of reagents. To address this, subsequent multiplex PCRs focused solely on primer pairs specific for Types 1, 2, 3, and 4. Primers for Type 0 were then tested in a separate reaction. The PCR products were electrophoresed through 2.5% agarose gel stained with MIDORI Green (Nippon Genetics) and identified as Type 0 (188 bp only), Type 1 (188 bp, 549 bp), Type 2 (188 bp, 841 bp), Type 3 (188 bp, 361 bp) or Type 4 (188 bp, 992 bp) [25, 26], using the GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific) as the fragment size marker.

#### Antimicrobial susceptibility testing

Susceptibility testing was performed using the broth microdilution method, according to the protocol recommended for *F. psychrophilum* in CLSI guideline VET04-A2 [27]. In this study, we tested twofold serial dilutions of eight antimicrobial compounds, i.e. oxolinic acid (OA) 8-0.004 µg/mL, oxytetracycline (OTC) 16-0.008 µg/mL, flumequine (UB) 8-0.004 µg/mL, florfenicol (FFC) 32-0.015 µg/mL, sulphamethoxazole-trimethoprim (SXT) 746/40-0.3/0.015 µg/mL, enrofloxacin (ENR) 16-0.008 µg/mL, erythromycin (E) 128-0.06 µg/mL and gentamicin (CN) 4-0.008 µg/mL. Two reference strains (*Aeromonas salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 grown at 17 ± 0.5 °C) were included in each run as quality controls. Each strain was tested at least twice and the data measured analysed according to cut-off values proposed by CLSI guideline VET04 for OA, OTC, UB, FFC, ENR and E [28], and Van Vliet et al., 2017 [29], for SXT.

#### Sedimentation, gliding motility, biofilm formation and gelatinase activity

Protocols for testing virulence factors were based on the work of Pérez-Pascual et al., 2017 [30], with minor modifications. Each analysis was repeated in three independent experiments.

To test their ability to create sediment, each isolate was inoculated into 3 mL of cation-adjusted Mueller Hinton broth (CAMHB; 4g/L) and incubated for 72 h at 17 ± 0.5 °C. After incubation, the sample was homogenised in a vortex mixer, re-inoculated into 3 mL of TYES broth and incubated for a further 72 h at 17 ± 0.5 °C. Sample density was then adjusted to 3–4 McFarland turbidity standard and the tubes incubated for 72 h in a shaker incubator at 18 ± 0.5 °C and 200 rpm. Presence or absence of sediment was recorded visually against a dark background.

Biofilm development was evaluated by inoculating each isolate into 3 mL of CAMHB in a 10mL polystyrene tube for 72 h at 17 ± 0.5 °C. Biofilm formation was evaluated visually against a dark background.

To analyse gliding motility, isolates grown on TYES agar were inoculated into 5 mL of CAMHB and incubated for 72 h at 17 ± 0.5 °C. Density was adjusted to 0.5 McFarland turbidity standard and 8 µl administered onto 1/5 TYES agar containing 0.08% tryptone (Oxoid), 0.008% yeast extract (Oxoid), 0.004% $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich), 0.01% $MgSO_4{\times}7H_2O$ (Merck KGaA), 0.01% glucose (Erba Lachema) and 1.5% bacteriological agar (Agar No. 1, Oxoid). Gliding motility, visualised by subsequent spreading over the agar, was recorded after 72 h incubation at 17 ± 0.5 °C.

To test gelatinase activity, isolates grown on TYES agar were transferred into 3 mL of CAMHB and incubated for 72 h at 17 ± 0.5 °C. After incubation, the samples were mixed in a vortex mixer and the density adjusted to 0.5 McFarland turbidity standard, after which 8 µl of the suspension was spot inoculated onto 1/5 TYES agar supplemented with 0.75% of gelatine and the plates incubated for 72 h at 17 ± 0.5 °C. Gelatinase activity was evaluated as a zone of agar clarification around the *F. psychrophilum* colony.

#### DNA extraction and genome sequencing

Isolates grown on TYES agar were re-inoculated into 5 mL of TYES broth and the isolates cultivated in a shaker incubator (IKA-Werke GmbH & Co. KG) at 17 ± 0.5 °C and 130 rpm for 72 h. Biomass was harvested after 5 min centrifugation (5,000 g) and re-suspended in 5 mL

of sterile Phosphate Buffer Saline (PBS). The pellet was collected after 5 min centrifugation (5,000 g) and re-suspended in 180 µl of Lysis Buffer (T1; Macherey-Nagel). After adding 25 µl of Proteinase K (Macherey-Nagel), all samples were incubated for 3 h at 56 °C and 1000 rpm in a thermoshaker (Biosan). Further steps were performed according to the manufacturer's instructions provided with Nucleospin Tissue kit (Macherey-Nagel). Isolated DNA was sequenced at the University Technology Sydney Sequencing Facility [31] using an Illumina MiSeq sequencer and MiSeq V3 chemistry. Sequence read quality was initially assessed using FastQC v0.11.9 [32]. Illumina raw reads passing quality control were assembled into draft genome sequences using the A5 assembly pipeline vA5-miseq 20140604 [33]. Genome sequences, assemblies, multi-locus sequence typing (MLST) and raw data have been deposited in the pubMLST and NCBI databases (BioProject number PRJNA771239, individual accession numbers are listed in Supplementary Table S2).

#### Bioinformatics analysis

The draft genome sequences of the 70 examined Czech isolates were typed using in silico Multilocus Sequence Typing (MLST) through the PubMLST database [34] and curated *F. psychrophilum* MLST scheme based on seven loci (*atpA, dnaK, fumC, gyrB, murG, trpB*) [35]. Clonal complexes were identified using the PHYLOViZ 2.0 software, employing the goeBURST algorithm analysis [36]. The analysis incorporated all European isolates of *F. psychrophilum* with identified sequence types (STs) present in the pubMLST database, enriched by the examined Czech isolates [34] ($n = 851$, accessed 20.5.2024).

Furthermore, the core and pan-genome of *F. psychrophilum* were analysed. To conduct this analysis on a global scale, additional genome sequences of *F. psychrophilum* were included. The genome sequences, identified as reference typical genomes of *F. psychrophilum* as of April 1, 2024 ($n = 257$ including 70 examined isolates in this study), were downloaded from the Genome database deposited in NCBI (Supplementary Table S3). We selected only genomes that originated from mono-isolate sequencing studies and passed the NCBI quality check The draft genome sequences were annotated using Prokka v1.13 [37] and predicted coding sequences were further functionally characterised using eggNOG-mapper2 v.2.1.2. software [38]. Next, annotated GFF3 files provided by Prokka were analysed with Roary v3.13.0 to identify core and accessory genes [39]. Finally, the phage sequences were identified using PHASTEST v 3.0 [40].

To infer phylogenetic relationships, two methods were utilized: one based on the bacterial core gene set and the other on *F. psychrophilum*-specific core gene sets. The core gene sequences identified by Roary were aligned using MAFFT v7.310 [41]. An in-house script was used to extract only variable sites from the alignment. To ensure the quality of the sequence alignment, we performed a codon-aware alignment to improve accuracy and assessed the coverage of each site. Variable sites with low coverage or poor alignment quality were excluded to maintain the reliability of our phylogenetic analysis. A phylogenetic tree was then constructed under the GTR + Γ4 substitution model using the RAxML-NG v.1.2.2 tool [42]. Additionally, a phylogenetic tree was constructed using the up-to-date bacterial core gene (UBCG) v2 pipeline [43] which includes 81 bacterial core genes and a revised bioinformatic pipeline comprising MAFFT alignment and maximum likelihood RAxML analysis. The resulting phylogenetic trees were visualized using iTOL v.6.9 [44].

## Results

### Typing of 70 Czech *F. psychrophilum* isolates using: multiplex PCR

In this study, we employed a multiplex PCR-based serotyping approach to characterise 70 *F. psychrophilum* isolates collected from dead or diseased fish in the Czech Republic. The PCR scheme was utilized to screen all isolates in terms of their serotypes. The results revealed a diverse distribution among the serotypes, with 43 isolates classified as Type 1, 23 as Type 2, 1 as Type 3, and 3 as Type 4. Notably, no Type 0 isolate was detected (Fig. S1; Supplementary Table S4).

#### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the 70 isolates to oxolinic acid (OA) showed a significant bimodal distribution [45]; the first group of isolates (17.1% of all tested) was inhibited by concentrations of 8 µg/mL and higher, while the second group (82.9% of all tested) was inhibited by concentrations ranging from 0.03 to 1 µg/mL, with most isolates being inhibited by concentration of 0.5 µg/mL (Fig. 1, Supplementary Table S4). Based on cut-off values [28], 75.7% of tested isolates were categorized as non-wild type (NWT). MIC for oxytetracycline (OTC) exhibited a trimodal pattern [45], with most isolates having MIC values between 1–16 µg/mL. In total, 88.6% of tested isolates were classified as NWT. Notably, all isolates classified as wild type (WT) had an MIC at the calculated breakpoint for susceptibility. For flumequine (UB), MIC values for most isolates (88.6%) ranged between 0.5–4 µg/mL, with only sporadic occurrences outside this range. Based on cut-off values [28], 90.0% of isolates were categorised as NWT. Distribution of MICs for florfenicol (FFC) was unimodal [45], with a peak at 1–2 µg/mL, and 15.7% of the isolates were classified as NWT based on cut-off values [28]. Surprisingly, 44.3%

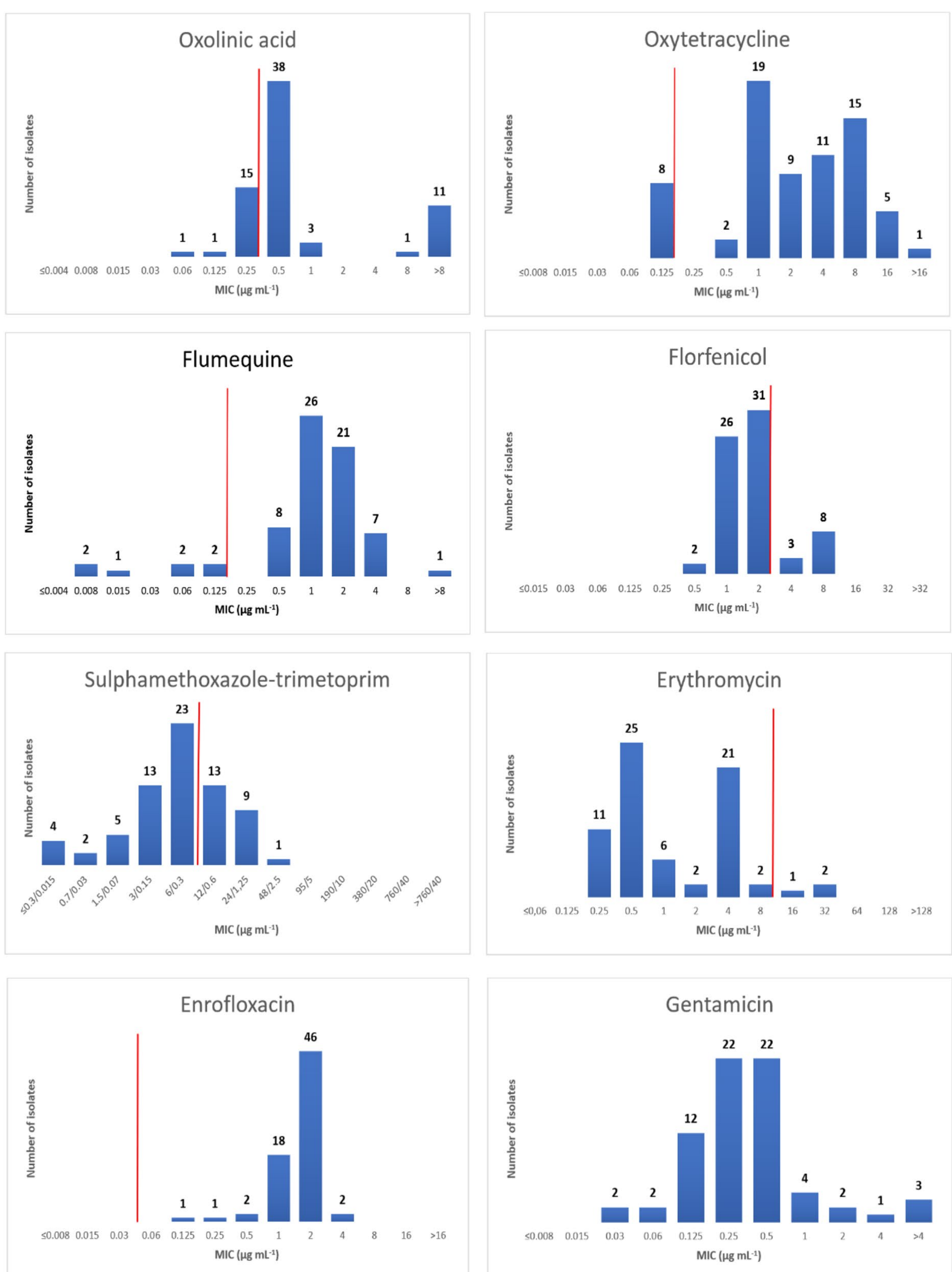


**Fig. 1** Distribution of the 70 Czech *F. psychrophilum* isolates tested for antimicrobial susceptibility using the broth microdilution method. Determination of WT/NWT isolates was conducted using cut-off values proposed by CLSI guideline VET04 and Van Vliet et al., 2017, with calculated cut-off values represented as red lines. No cut-off value was calculated for gentamicin

of isolates had MICs at the calculated breakpoint, similarly to OTC susceptible strains, suggesting that resistance to OTC and FFC may be developing in fish farms. For sulfamethoxazole-trimethoprim (SXT), isolates exhibited a unimodal MIC distribution, ranging from 48/2.5 to ≤ 0.3/0.015 μg/mL, with 6/0.3 μg/mL being the most frequently observed value. Overall, 32.9% of isolates were classified as NWT [29]. MICs for erythromycin (E) ranged from 32 to 0.25 μg/mL and showed a bimodal distribution, with peaks at 4 and 0.5 μg/mL. Both peaks fell below the calculated cut-off values [28], and aside from three isolates, were all classified as WT. The MIC for enrofloxacin (ENR) showed a unimodal distribution, with most isolates (65.7%) exhibiting MICs at 2 μg/mL. With cut-off values applied [28], all isolates were classified as NWT. MICs for gentamicin (CN) showed a wide distribution, ranging from 4 to 0.03 μg/mL, with the most frequent (78.6%) values falling between 0.5 and 0.125 μg/mL. Three isolates had MIC values ≥ 4 μg/mL. Cut-off values for CN have not been calculated, so percentages of WT and NWT were not determined for this antibiotic. MICs for the two control strains (*A. salmonicida* ATCC 33658 and *E. coli* ATCC 25922) fell within the accepted CLSI ranges (CLSI 2014b) [46].

### Further experimental assessment: sedimentation, gliding motility, biofilm formation and gelatinase activity assays

The results of phenotypic testing of selected virulence factors are summarized in Supplementary Table S4. Of the 70 isolates tested for sedimentation ability, 58 produced a distinct sediment at the bottom of the test tube when cultured in liquid medium. Repeated testing on the remaining 12 isolates confirmed the negative reaction. In addition, even after repeated testing, the type strain ATCC 49418 also failed to produce a visible sediment. Among the 70 isolates tested for gliding motility on low nutrient agar, only 25 exhibited distinct spreading. Following prolonged incubation (six days), the spreading zone in these 25 isolates significantly increased, while the remaining 45 isolates classified as non-gliding showed no motility. All 70 isolates and the ATCC 49418 control strain formed visible biofilms against a dark background. Although the intensity of biofilm formation varied between the isolates, this variability likely resulted from differences in the initial inoculum concentrations, and thus these differences may not necessarily indicate disparities in biofilm creation capability. With only one exception, all isolates showed proteolytic activity when gelatine was used as a substrate in agar. After prolonged incubation (seven days), the zone of proteolysis further increased. The exception, isolate 96,236, showed no proteolytic zone, even after repeated analyses and prolonged incubation. This same isolate also exhibited negative reactions for sedimentation and gliding motility.

### Genomic feature of examined *F. psychrophilum* isolates

All 70 *F. psychrophilum* isolates of *F. psychrophilum* examined in this study were successfully sequenced using an Illumina MiSeq sequencer, and assembled. The number of contigs in each draft genome ranged between 113 and 548, with an average of 150 per genome. Draft L50 values showed a median of seven, with an average N50 value of 136,805 bp (Supplementary Table S2). The assembled genomes had an average size of 2.79 Mbp, with the smallest genome (isolate 9333 z) being 2.72 Mbp and the largest (isolate 9335 z) being 3.07 Mbp (Supplementary Table S2). The average GC% content was 32.42, with a mean of 32.36. For comparison, the reference genome of the type strain *F. psychrophilum* ATCC 48419$^{T}$ had a size of 2.63 Mbp and a GC% content 32.3. The number of coding genes did not vary significantly, ranging between 2399 and 3010 (average 2484; Supplementary Table S2).

### Core- and Pan-genome analyses: insights into genetic diversity

Next we compared the nucleotide sequences of 70 *F. psychrophilum* genomes characterised in this study and additional 187 *F. psychrophilum* genome sequences available in NCBI database and determined the core-genome and pan-genome of the investigated pathogen. As of April 1, 2024, these 257 genome sequences encompass all reliable *F. psychrophilum* genomes available in public databases. Prokka analysis identified a total of 628,548 protein-coding genes, which were subsequently clustered by Roary into 9790 gene groups, sharing more than 95% identity in gene content. Among these, 1082 core genes were present in 99% of the examined genomes ($n$ = 254-257), along with 545 soft core genes in 95-99% of genomes (245-253 genomes), 1183 shell genes spanning 15-95% of genomes ($n$ = 39-243), and 6980 cloud genes found in less than 15% of genomes ($n$ = 1-38) (Supplementary Fig. S2). The analysis also revealed that the number of core and soft core genes has stabilized, resulting in 1627 genes being common across most *F. psychrophilum* isolates. However, the pan-genome remains open, with the continual addition of new genomes leading to the expansion of accessory genes (Supplementary Fig. S3).

The datasets containing core (core and soft core) and accessory (shell and cloud) genes underwent alignment against the Cluster of Orthologous Group (COG) databases using eggNOG mapper. Analysis of COG functional categories revealed commonalities between core and accessory genes, particularly in categories of unknown proteins (either not classified or classified as COG category S). However, genes within category S, assumed to be

involved in cellular processes and signalling, were notably more abundant among core genes compared to accessory genes (21.52% vs. 11.98%, respectively), as depicted in Supplementary Fig. S4 and Supplementary Table S5. Furthermore, genes enriched in Translation, ribosomal structure, and biogenesis (COG category J) were more prevalent among core genes compared to accessory genes (8.17% vs. 1.60%, respectively). Conversely, uncharacterized genes (45.36% vs. 10.33%) and genes associated with Replication, recombination, and repair (COG category L, 11.11% vs. 4.30%) were more prevalent among accessory genes when compared to core genes. The prevalence of genes involved in Cell motility (category N) was relatively similar between core and accessory genes.

A pan-genome matrix was constructed to evaluate gene presence or absence across all isolates (Fig. 2). This matrix revealed distinctive patterns among isolates, notably with 70 genomes forming a distinct cluster marked by a significant absence of genes (visually highlighted in Fig. 2). Further analysis identified 82 deleted genes, mainly consisting of hypothetical proteins and phage-associated proteins (Supplementary Table S6). Comparison of isolates with and without the deletion using PHASTER indicated that the deleted region corresponds to an intact phage and adjacent genes (Supplementary Fig. S5). Of note, the type strain ATCC 48419$^{T}$ (GCF_002217405) belongs to the cluster which lacks the phage.

The definition of the core-genome helped characterize the evolutionary relationships among *F. psychrophilum* genomes (Fig. 3). The resulting phylogenetic tree, constructed using the RAxML-NG method, revealed two major clusters: one consisting of 70 genomes missing the phage and the other comprising 177 genomes. Since phylogenetic relationships based on core-gene sequences can be influenced by varying mutation rates across different genes and positions, we subsequently analysed relatedness using the UBCG2 pipeline (Supplementary Fig. S6). This pipeline utilizes a bacterial core gene set, representing genes evolving under neutral selection. The tree also indicated that the 70 genomes with a deleted phage formed a separate cluster, while the remaining genomes clustered into three additional phylogenetic groups, labeled as Cluster I-IV.

### MLST analysis

Overall, MLST analysis identified seven novel sequence types of *F. psychrophilum* isolates from the Czech Republic. The most prevalent was ST329 ($n = 24$, Table S7), a

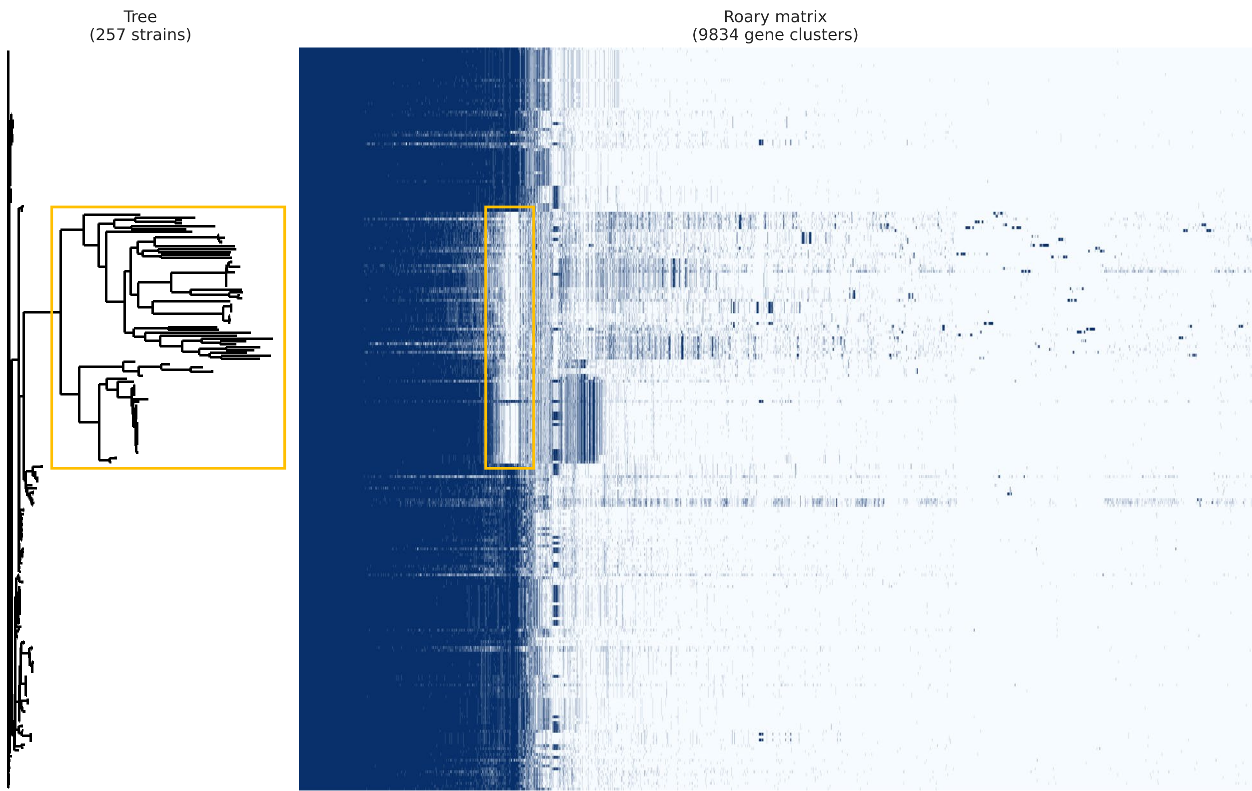


**Fig. 2** Pan-genome presence/absence matrix was constructed for the 628,548 protein-coding genes in the *F. psychrophilum* pan-genome using Roary. Alongside a maximum-likelihood tree generated from variable positions of core-gene alignment

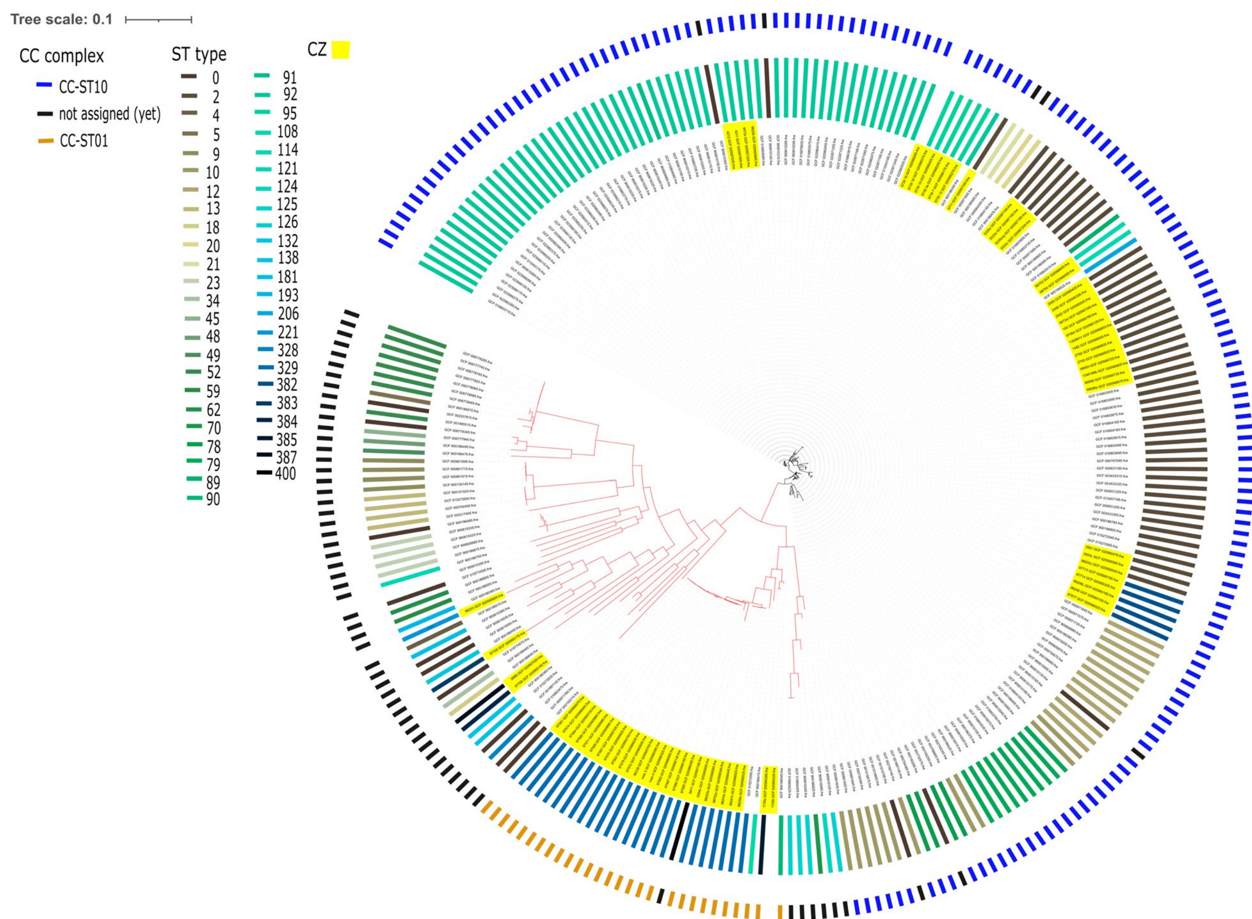


**Fig. 3** RAxML phylogenetic tree reconstructed from the Roary-generated core gene multi-FASTA alignment. Newly sequenced Czech *F. psychrophilum* isolates are highlighted in yellow, while the subgroup of 70 isolates containing a deletion resulting in the loss of a phage (Fig. 5) are depicted in red. Branch lengths are proportional to the number of nucleotide substitutions per position as indicated by the scale bar

recently identified sequence type belonging to clonal complex CC-ST01, which is now spreading in the Czech Republic. According to phylogenetic analysis (Fig. 3), a closely related strain, 9471, has been assigned as ST400, diverging from ST329 by the *atpA* allele. Other closely related isolates (1730s and 1729s) were identified as a novel sequence type, ST385, also belonging to clonal complex CC-ST01 (Fig. 4). Additionally, four isolates lacking a phage sequence in their genomes were assigned new sequence types: ST221 (isolate 96233), ST383 (97705), ST384 (97708), and ST387 (8888).

The remaining isolates ($n=39$) belonged to clonal complex CC-ST10 (Fig. 4, Table S7), predominantly consisting of ST2 isolates ($n=22$), followed by ST95 ($n=6$), the newly identified ST382 ($n=5$), ST92 ($n=4$), and ST114 ($n=2$). While ST95, ST382, ST92, and ST114 are clustered within phylogenetic trees consistently with ST typing (Fig. 3, Fig. S6), the isolates belonging to the widespread ST2 formed three distinct clusters in the phylogeny. Additionally, isolate 9711 as ST2, constituted an entirely separate lineage. A goeBURST algorithmic analysis [36], based on all European *F. psychrophilum* isolates present in the pubMLST database [34] ($n=851$ as of 20.5.2024), grouped the Czech flavobacterial isolates into three clonal complexes: CC-ST10 (ST2, ST92, ST95, ST114, ST382), CC-ST01 (ST329, ST385, ST400), and the newly formed CC-ST221 (ST221), along with three singleton STs (ST383, ST384, ST387) (Fig. 4). A summary of the MLST and goeBURST analysis for the Czech isolates can be found in Table 1. A more detailed description of the individual identified alleles for each isolate is provided in Supplementary Table S7.

## Discussion

*Flavobacterium psychrophilum* is a widespread bacterial pathogen of salmonids, causing significant economic losses in aquaculture worldwide [47]. Consequently, there is a real need for phenotypic and genetic characterisation of isolates for both epidemiological and ecological analyses to develope effective protective measures

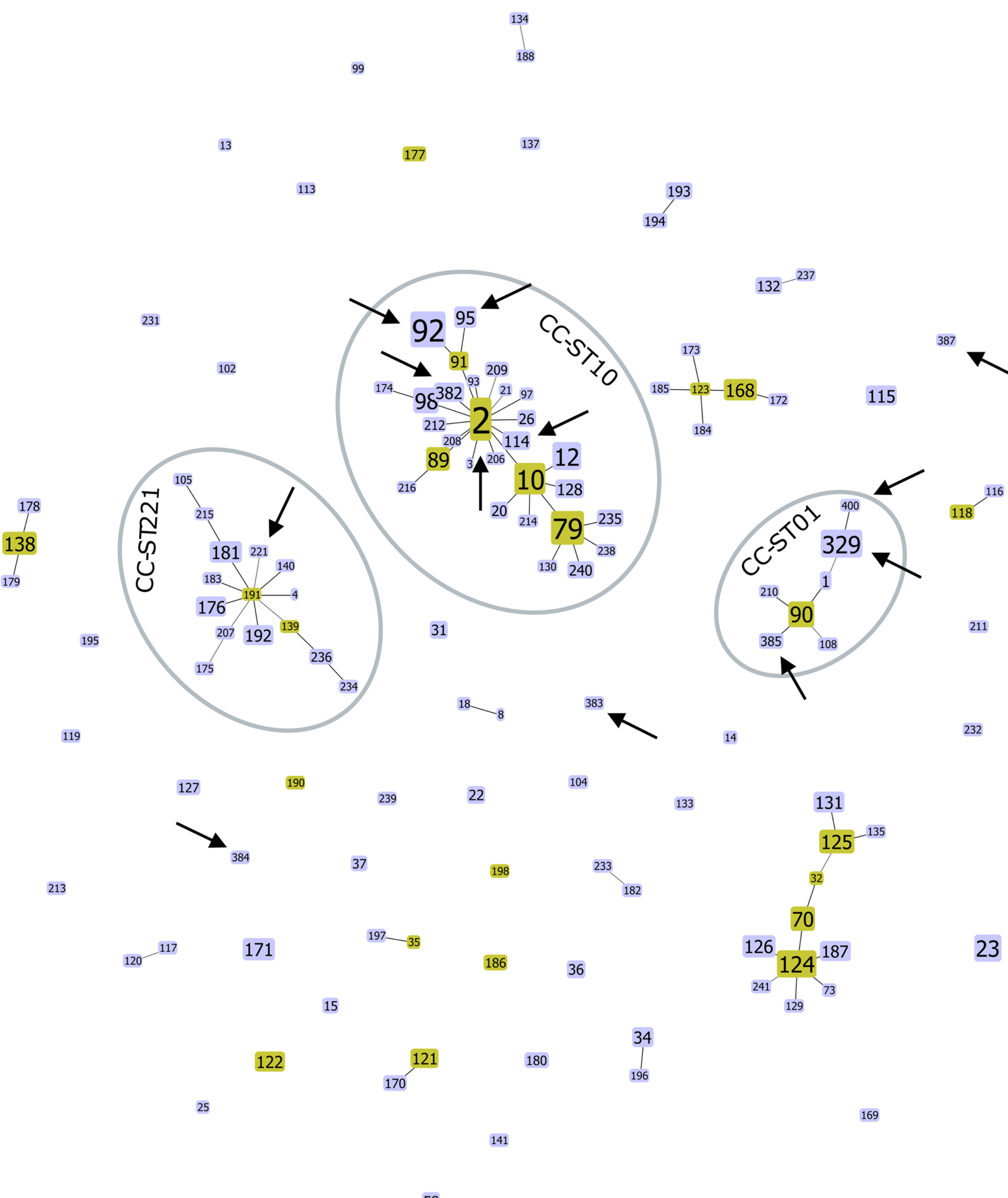


**Fig. 4** eBURST diagram of the 70 Czech *F. psychrophilum* isolates genotyped in this study combined with previously typed European isolates ($n = 851$). Arrows are pointing out STs identified within this study. Only clonal complexes (CC-STx) comprising the Czech isolates of *F. psychrophilum* are highlighted

**Table 1** Identified sequence types and their association with clonal complexes of 70 Czech *Flavobacterium psychrophilum* isolates

| Identified ST | CC | Number of isolates | Fish farm | Tissue |
|---|---|---|---|---|
| ST2 | CC-ST10 | 22 | 1 | liver |
| | | | 2 | spleen |
| | | | 5 | body cavity |
| | | | 6 | kidney |
| | | | 9 | gills |
| | | | 10 | skin |
| | | | 11 | |
| ST92 | CC-ST10 | 4 | 2 | spleen |
| | | | 7 | body cavity |
| | | | 10 | |
| ST95 | CC-ST10 | 6 | 2 | spleen |
| ST114 | CC-ST10 | 2 | 4 | spleen |
| ST221[a] | CC-ST221[b] | 1 | 2 | spleen |
| ST329 | CC-ST01 | 24 | 1 | spleen |
| | | | 2 | kidney |
| | | | 3 | roes |
| | | | 4 | body cavity |
| ST382[a] | CC-ST10 | 5 | 1 | liver |
| | | | 2 | kidneys |
| | | | | body cavity |
| ST383[a] | S | 1 | 1 | spleen |
| ST384[a] | S | 1 | 1 | spleen |
| ST385[a] | CC-ST01 | 2 | 1 | gills, spleen |
| ST387[a] | S | 1 | 8 | gills |
| ST400[a] | CC-ST01 | 1 | 10 | spleen |

*ST* sequence type, *CC* clonal complex, *S* singleton

[a] novel ST identified in this study

[b] novel clonal complex identified in this study

against flavobacteriosis. To the best of our knowledge, there are presently no data available from *F. psychrophilum* isolates obtained in Central Europe. The data for isolates from the Czech Republic's aquacultures presented here can therefore be considered the first of its kind.

The most prevalent type observed in the 70 isolates obtained from Czech fish farms between 2012 and 2019 was Type 1 (61.4%), which corresponds to serotype Fd. Type 2, corresponding to serotype Th, was identified in 32.9% of isolates, while Types 3 and 4, both corresponding to serotype FpT, were only identified sporadically (5.8%). Serotype distribution differs greatly in different parts of the world and can also change over time [17, 18, 26, 48–51]. Based on our results, serotype distribution in the Czech Republic appears to be very similar to that found in northern Europe [18, 51]. Similar to elsewhere, our data showed changes in serotype prevalence over time, with Type 1 (serotype Fd) dominating in isolates collected between 2012 to June 2016 ($n=41$ isolates, Type $1=38$, Type $2=1$), but Type 2 isolates (serotype Th) dominating from July 2016 to 2019 ($n=29$ isolates, Type $1=5$, Type $2=22$). This trend was primarily influenced by the situation at two fish farms (in this paper referred to as Farm 1 and Farm 2), where only Type 1, and sporadically Type 4, were identified during the first period, and mainly Type 2 during the second, a change that may have been caused by the introduction of new strains into the farms with the new fish stocks.

In the present study, high percentage of isolates were categorized as non-wild type (NWT) forOA, similarly as in Miranda et al., 2016 [52], who tested isolates from Chile, where OA is frequently used for aquacultural antimicrobial therapy by some farmers [53], possibly leading to the presence of the resistant group of isolates. As OA is not registered for use in aquaculture in the Czech Republic, the reason for such a high occurrence of resistance in Czech farms is unclear, though it is possible that NWT isolates were imported into the country through cross-border stocking, since OA was widely used in other European countries until resistance developed [54]. OTC, on the other hand, is a very common antimicrobial agent in aquaculture, and has been commonly used to treat flavobacteriosis in several countries [54]. This has led to increased *F. psychrophilum* resistance in isolates from around the world [16, 53, 55–59]. However, its use in the Czech Republic is currently limited to the Cyprinidae family and, therefore, it should not be used for treatment of flavobacteriosis. The high percentage of NWT phenotypes recorded in Czech fish farms suggests either off-label use of OTC in Salmonidae, or the introduction of NWT strains through international trade in fish stocks. Most of the Czech isolates showed NWT phenotype for UB, a similar result to those found in isolates from Canada [58], Chile [52], Great Britain [55] and China [17], suggesting that *F. psychrophilum* resistance to UB is a worldwide issue. UB is a widely used antibiotic in aquaculture as most important fish bacterial pathogens display high susceptibility [60]. In the Czech Republic, UB is currently licensed in aquaculture for use only after identification of the pathogenic agent and determination of its susceptibility. Though only 15.7% of isolates were classified as NWT for FFC, 44.3% of isolates had MIC at the calculated breakpoint, suggesting a potential for rapid emergence of FFC resistance in Czech fish farms. The data obtained for FFC susceptibility in this study are disturbing as FFC, despite its recent introduction for veterinary use, has become the preferred therapy for flavobacteriosis in numerous countries [58]. While earlier studies reported low MIC levels for FFC [52, 59, 61], recent research, including our own, demonstrates a concerning trend of increasing MICs approaching the

breakpoint [17, 55]. Some studies even report the emergence of a significant FFC resistance in bacterial isolates [53, 58, 62]. Most of the isolates tested here were categoried as wild type (WT) when SXT tested. While SXT is not currently licensed for use in aquaculture in the Czech Republic, it is commonly used for treatment of fish bacterial infections in some other countries, such as Canada, where MIC levels are significantly higher [29, 58]. Interestingly, when comparing the most abundant STs in this study, ST329 ($n=24$) and ST2 ($n=22$), we found that reduced susceptibility to SXT in the ST2 group was 68.2% (15 of 22), while it was only 4.2% (one of 24) in the ST329 group. However, this may be representative of the disproportion between number of locations from which samples were taken, with ST329 isolated from only four locations and with 75.0% of isolates from just one location (Farm 2, 18 of 24 isolates), while ST2 was isolated from seven locations, with a more uniform distribution of isolates between localities (1–6 of 22 isolates). All isolates included in this study had NWT phenotype for ENR, which is a widely used antimicrobial drug as it is effective against most fish bacterial pathogens [63]. Most of the isolates tested were classified as WT for E. For CN, cut-off values have yet to be established, therefore isolates could not be divided into WT or NWT groups. SXT, ENR, E or CN is not currently registered in this country for use in uquaculture.

Gliding motility is considered to be one of the most important virulence factors. In this study, using a diluted culture medium to represent a nutrient-poor environment, only 38.7% isolates showed colony spreading over the medium. It is worth noting that the method used is less sensitive then phase-contrast microscopy, meaning that slower-moving bacteria may appear non-motile using this culture method [64]. The ability to create sediment when cultured in liquid media is thought to be necessary for proper biofilm formation and is thus considered an important virulence factor [30]. Indeed, several previous studies have described a significant relationship between biofilm formation and sedimentation, with Pérez-Pascual et al., 2017 [30] and Barbier et al., 2020 [65] showing that strains with inactivated genes for the type IX protein secretion system T9SS (*gldD*, *gldG* and *gldN*) also lose their ability for gliding motility and sediment creation. In this study, among the 45 isolates showing no gliding motility, 34 were able to create visible sediment, while only 11 could not. This disproportion may have been caused by using the less sensitive method for testing gliding motility. Interestingly, one isolate positive for gliding motility did not create visible sediment, even after repeated testing. Consistent with previous studies [66, 67], no negative reaction for biofilm formation was detected in this study. Although the method used here does not allow a precise evaluation of the intensity of biofilm formation, there was an obvious difference between type strain ATCC 49418 and the wild strains. The bacterial suspensions of the wild strains cleared during cultivation and formed significant biofilms, while the test tube containing the type strain remained cloudy, with only a minor biofilm on the tube wall. This finding agrees with previous experimental results describing ATCC 49418 as “a strain with a low ability to create a biofilm” [67]. Gelatinase activity is common feature of *F. psychrophilum* [51, 57, 62, 66, 68–71]. Nevertheless, some papers have reported strains with a reduced ability, or complete inability, to cleave gelatine in agar substrate [72, 73]. Álvarez et al. (2006), for example, observed lack of gelatinase production in *tlpB* defective strains [74], which are also non-motile, while Pérez-Pascual et al. (2017) described gelatinase-negative strains after mutation of *gldD* and *gldG* genes, along with reduced sedimentation ability [30]. Among the isolates tested in this study, only 96236 failed to create a zone of proteolysis on agar supplemented with gelatine. This same isolate also failed to create a visible sediment and showed no gliding motility on diluted TYES agar.

MLST revealed 12 STs among the 70 Czech isolates tested, seven of which were newly described (ST221, ST382, ST383, ST384, ST385, ST387, ST400). Since these STs have not been identified in other countries, they appear to be geographically specific to the Czech Republic. Each of these STs was found in only one farm, so we assume that they are rather sporadic types. In only one outbreak was a newly described ST identified as the only pathogen (ST385 in farm 1). In the other cases, another sequence types or another pathogen was identified at the same time. In the case of sequence type 387 (isolate 8888), it was actually only a secondary finding on the gills, while *Aeromonas salmonicida* was found massively present in the internal organs. These data may suggest that the virulence of the newly described sequence types is low. Notably, recently identified ST329 [18] displayed a clonal outbreak, evident in the phylogenetic trees. Alongside ST385, ST400 and ST329 formed a cluster of genomes lacking a phage in the chromosome, similar to a typical *F. psychrophilum* type strain ATCC 49418. Other common strains spread nowadays posses a prophage in their genome [75]. The second most common type, ST2 (belonging to CC10) was found in 22 of 70 isolates (31.4%). It was isolated from six different tissue types, while ST329 from only three tissue types and roe samples. This may indicate that ST2 is slightly more virulent than ST329 (Table 1). ST2 appears to be more widely distributed in the Czech Republic than ST329 as it was found at seven different locations, compared

to four sampling sites for ST329. The ST2 has also been identified as the most common sequence type in several European countries [19–21]. Interestingly, noticeable changes in ST prevalence were observed over time in this study; the most isolates sampled between 2012 and 2015 ($n=37$) were identified as recently described ST329 (56.8%), while most isolates sampled between 2016 and 2019 ($n=33$) were identified as ST2 (51.5%), and only three isolates as ST329 (9.1%). This is a similar trend to that observed in serotype prevalence described above, and may be indicative of either increased international trading resulting in the introduction of new STs from external sources [76] or the development of new STs through allele recombination [19, 20]. Söderlund et al. (2018), when compared MLST results with antimicrobial patterns in Swedish isolates from 1988 to 2016, revealed an increasing occurrence of strain ST92 resistant to OTC and OA since 2011 [76]. In our collection, four isolates from three different farms were identified as ST92 between 2014 and 2019, all of which were classified as NWT for both OTC and OA. This could suggest that the resistant ST92 sequence type has extended its range outside of Sweden. Finally, Nicolas et al. (2008) identified a significant correlation between STs and host fish species [22]. However, the data in our study was highly skewed with 66 of the 70 isolates in our study being sampled from rainbow trout, 22 of which were ST2, and only four originating from brown trout, all of which were identified as ST2; consequently, no correlation could be assessed.

Our core-genome analysis reaffirmed that the distribution and genetic consistency of core virulence-related factors align with previous theories regarding *F. psychrophilum* isolates [66, 75]. Specifically, genes encoding adhesion, colonization, and tissue destruction in fish are expected to be widespread. Conversely, the pan-genome exhibits an open structure, suggesting that new strains will likely introduce novel genes associated with DNA repair mechanisms or previously unidentified features. Therefore, it is crucial to monitor the genomic evolution of *F. psychrophilum* strains worldwide.

Our analysis revealed the absence of known virulence genes in the Czech isolates of *F. psychrophilum*, as determined through the VirulenceFinder 2.0 database [77]. This finding prompted us to delve deeper into the accessory genome, where we encountered a significant number of hypothetical proteins. Although these proteins are not well characterized, they represent a reservoir of potential virulence factors and other functional elements that could play crucial roles in the biology and pathogenicity of *F. psychrophilum.*

## Conclusions

This study revealed changes in Czech *F. psychrophilum* ST/serotype prevalence over time, most likely attributed to the importation of new isolates via international trading. These findings underscore the necessity of tracing *F. psychrophilum* clones, both within and between countries, using tools such as MLST and serotyping. By identifying geographical areas with epidemiologically significant *F. psychrophilum* clones, proactive measures can be taken to prevent the spread of these clones to other uncontaminated aquacultural facilities.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03490-3.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.
Supplementary Material 9.
Supplementary Material 10.
Supplementary Material 11.
Supplementary Material 12.
Supplementary Material 13.

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**Authors' contributions**
Conceptualization, AC, SK, DC; Methodology, AC, SK, DC; Formal Analysis, VV, SK, DC; Investigation, VV, SK, DC, JS, JN; Resources, AC, MP; Data Curation, VV, SK, DC; Writing Original Draft Preparation, VV, SK, DC; Writing – Review & Editing, VV, SK, DC, JS, JN, MP, AC; Visualization, VV, SK, DC; Supervision, AC, DC; Project Administration, AC, VV; Funding Acquisition, AC, VV, MP. All authors read and approved the final manuscript.

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**Availability of data and materials**
Sequence data that support the findings of this study was deposited in the NCBI databases (BioProject number PRJNA771239).

## Declarations

**Ethics approval and consent to participate**
Animals were treated in accordance with Act No. 246/1996 Coll. and Act No. 418/2012 Coll. and EC1099/2009 rules (Certificate of the Ethics Commission

No. MSMT-17863/2022-3; Expert commission for ensuring the welfare of experimental animals, University of Veterinary Sciences Brno (https://www.vfu.cz/odborna-komise-pro-zajistovani-dobrych-zivotnich-podminek-pokusnych-zvirat).

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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