Psychrotolerant enterobacters inhabiting the gut of Antarctic fishes of the family *Nototheniidae* and description of *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov.

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Abstract

The gut microbiota of four Antarctic marine fish species – *Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansoni*, and *Trematomus newnesi* – were analysed, with a particular focus on the members of the *Enterobacter cloacae* complex. Nineteen isolated strains were characterised by rep-PCR, automated ribotyping, extended phenotyping, and MALDI-TOF MS analysis. Fingerprinting methods grouped the psychrotolerant isolates into two distinct groups, representing the dominant enteric bacteria in the gut contents of these fish. Following their preliminary identification as members of the *Enterobacter cloacae* complex, additional phylogenetic analyses were conducted using the 16S rRNA and *rpoB* genes, as well as fatty acid analysis and whole-genome sequencing of two representatives selected based on their fingerprints. All results indicated that the analysed group represents a new autochthonous *Enterobacter* taxon inhabiting the gut of fish of the family *Nototheniidae*. Based on average nucleotide identity (ANI) and in silico DNA–DNA hybridization (dDDH) values, the "*Enterobacter hoffmannii*", the effectively published but non-valid name, was the most closely related species to representatives of both groups of isolates from the gut of the above-specified notothenioid fish. The name *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. is proposed, with the type strain P5473^T (= CCM 8629^T = LMG 34031^T), for isolates of Group 1. At the same time, the name *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov. is suggested for isolates of Group 2, with the existing type strain DSM 14563^T = LMG 30171^T.

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Key words: *Enterobacter cloacae* complex, psychrotolerant, taxonomy, *Notothenia*, Antarctica

Introduction

All oceans on Earth are teeming with a great variety of microorganisms, and marine fish have a unique and close interaction with the microorganisms that coexist in these environments. The diversity of bacteria in the digestive tract of fish increases as fish develop. *Pseudomonadota* is the most abundant microbial phylum in the gastrointestinal tract of fish, as reported in several focused studies (Rombout et al. 2011, de Bruijn et al. 2018, Egerton et al. 2018). Research into the gut microbiota of fish has expanded significantly in recent decades, coinciding with the growth of the aquaculture industry (Egerton et al. 2018). Earlier studies suggested that the bacterial content in the fish gut is low; however, more recent studies using advanced molecular techniques have provided evidence of a relatively rich bacterial biota (Zarkasi et al. 2014).

There are more than 30,000 species of fish worldwide, inhabiting various environments, and some of these species are native to the Southern Ocean region. Their number is estimated to be about 400 species (Vacchi 2015). These Antarctic fishes are well-adapted to cold water (Bista et al. 2023), and the most species-rich family is the *Nototheniidae* (cod icefishes), which comprises the highly endemic fish fauna of the Southern Ocean (Ward et al. 2009). As the dominant fish taxa in Antarctica, they occupy ecological niches both on the sea floor and in the water column. A few years ago, two new bacterial species were discovered in black rock cod (*Notothenia coriiceps*): *Pedobacter nototheniae*, isolated from the spleen (Kämpfer et al. 2019a), and *Paracoccus nototheniae* from a kidney sample (Kämpfer et al. 2019b). The gut microbiota of notothenioid fish species has been studied in several investigations using both molecular methods (Ward et al. 2009) and culture-dependent methods (MacCormack and Fraile 1991). Our previous study analysed cultivable bacteria from the gut of 43 fresh, healthy fish (*Notothenia coriiceps*, *Trematomus bernacchii*, *Trematomus hansoni*, and *Trematomus newnesi*) and showed that the *Enterobacter cloacae* complex (ECC) group was the predominant bacterial gram-negative phenotype in the gut contents of these fish species (Sedláček et al. 2016a).

The genus *Enterobacter*, belonging to the family *Enterobacteriaceae*, comprises a large group with more than twenty named species and a similar number of genomospecies (Feng et al. 2021). Such diversity can complicate the precise identification of isolates (Janda and Abbott 2021). Previously, the ECC group included seven species with valid names: *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter mori* (Paauw et al. 2008, Davin-Regli et al. 2019, Parte et al. 2020), which are mainly associated with plants, soil, animals, and humans.

Recently, several taxonomic changes have been proposed to the ECC group, including the reclassification of *Enterobacter tabaci* as *E. mori*, *Enterobacter muelleri* as *E. asburiae*, and *Enterobacter nimipressuralis* as *Lelliottia nimipressuralis* (Parte et al. 2020). In addition, subspecies of *E. cloacae* and *E. hormaechei* were revised based on genomic analyses (ANI and dDDH calculations). *Enterobacter cloacae* subsp. *dissolvens* was recognised as a separate species, *Enterobacter dissolvens*, and subspecies *E. hormaechei* subsp. *hoffmannii*, elevated to the species rank, and the name "*Enterobacter hoffmannii*" was proposed (Sutton et al. 2018). Subsequently, the remaining subspecies of *E. hormaechei*, namely *E. hormaechei* subsp. *oharae*,

E. hormaechei subsp. *steigerwaltii*, and *E. hormaechei* subsp. *xiangfangensis* were shown to be synonyms of *E. xiangfangensis* (Sutton et al. 2018, Wu et al. 2020, Parte et al. 2020, Coutinho et al. 2024, Zouagui et al. 2025), but this change has not yet been validated. Members of the ECC are environmental organisms widely spread in nature, and opportunistic pathogens of plants (*E. cancerogenus*, *E. mori*) and humans (mainly *E. cloacae*, "*E. hoffmannii*", *E. hormaechei*, *E. xiangfangensis*) (Janda and Abbott 2021, Maguvu and Bezuidenhout 2021, Zong et al. 2021, Morhart et al. 2023). Among these, *E. xiangfangensis* and "*E. hoffmannii*" are most frequently implicated in human infections (Jiang et al. 2024).

Recently, several novel *Enterobacter* species, including *Enterobacter quasiroggenkampii* and *Enterobacter quasimori* (Wu et al. 2020), *Enterobacter wuhouensis* and *Enterobacter quasihormaechei* (Wang et al. 2020), *Enterobacter pseudoroggenkampii* (Wu et al. 2023), as well as *Enterobacter chinensis* and *Enterobacter rongchengensis* (He et al. 2024), have been isolated from human clinical samples. Routine identification of ECC members at the species level using commercial biochemical systems is problematic for the accurate identification of *Enterobacter* spp. (Ciufo et al. 2018, Jain et al. 2018, Davin-Regli et al. 2019, Wu et al. 2020, Morhart et al. 2023, Qiu et al. 2024). Therefore, a polyphasic approach is needed for the identification and/or reclassification of *Enterobacter* species (Janda and Abbott 2021).

Following a polyphasic approach to bacterial classification that incorporates physiology, fatty acid analysis, and genome features, we describe a novel psychrotolerant subspecies of "*Enterobacter hoffmannii"* in this study. We formally propose a description of *E. hoffmannii* sp. nov. as well as the name *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. and *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov. for two distinct groups of isolates recovered from the gut of Antarctic notothenioid fish.

Materials and Methods

Fish sampling, isolation, and cultivation of bacterial strains

Cod icefishes, which belong to the suborder *Notothenioidei*, are mainly found in the Southern Ocean and off the coast of Antarctica. During the Czech Antarctic expedition in 2014, field research was conducted to investigate the shoreline fish community (Jurajda et al. 2016) and the metazoan parasites of notothenioid fish (Nezhybová and Mašová 2015). The fish were caught using Nordic multi-mesh benthic gillnets and fishing rods in the Prince Gustav Channel (depth approximately 5–45 m) in front of the Johann Gregor Mendel Station on James Ross Island. This study was based on the framework of the Czech Antarctic Research Programme[1]. From January to March 2014, a total of 43 specimens representing four fish species were analysed for parasites and intestinal bacteria: *Notothenia coriiceps* (10 individuals), *Trematomus bernacchii* (3), *Trematomus hansoni* (22), and *Trematomus newnesi* (8) (*Supplementary Figure* S1) (Sedláček et al. 2016a).

Nineteen bacterial strains from the ECC group were isolated at 20°C on Xyloselysine deoxycholate (XLD) agar (Oxoid) or Endo Agar (Oxoid) and stored at -70°C until analysed. Details of the sampling date, source of isolates, isolation medium, and preliminary phenotypic identification are provided in *Supplementary Table* S1. Selected strains representing the bacterial groups studied have been deposited in

the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic[2]) and in the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium[3]) under the following accession numbers: P5473^T (=CCM 8629^T = LMG 34031^T) and P5481 (=CCM 8630 = LMG 32783). The reference ECC type strains *Enterobacter asburiae* CCM 8619^T, *Enterobacter cancerogenus* CCM 2421^T, *Enterobacter cloacae* subsp. *cloacae* CCM 8574^T, *Enterobacter cloacae* subsp. *dissolvens* CCM 8575^T, *Enterobacter hormaechei* subsp. *hoffmannii* CCM 9140^T, *Enterobacter hormaechei* subsp. *hormaechei* CCM 8620^T, *Enterobacter hormaechei* subsp. *oharae* CCM 8660^T, *Enterobacter hormaechei* subsp. *steigerwaltii* CCM 8661^T, *Enterobacter hormaechei* subsp. *xiangfangensis* CCM 8608^T, *Enterobacter kobei* CCM 8576^T, *Enterobacter ludwigii* CCM 8602^T, and *Enterobacter mori* CCM 9053^T were obtained from the Czech Collection of Microorganisms and used for the following comparisons.

Phenotyping and basic isolates classification

Samples from fish were cultured on basic commercial culture media, including Yersinia Selective Agar (YSA), Tergitol 7 Agar (T7A), Nutrient Agar CM03, MacConkey Agar (MCA), Mueller-Hinton Agar (MHA), Brain Heart Infusion Agar (BHI), Reasoner´s 2A Agar (R2A) (all Oxoid), all incubated at 30°C for 48 hours. Obtained isolates were picked up, purified, and stored at -70°C for further experiments (*Supplementary Table* S1). The Gram-stained cells of the investigated strains were examined by light microscopy after cultivation on Tryptone Soya Agar (TSA) for 48 hours at 30°C and confirmed by the KOH lysis test method (Carlone et al. 1982). Basic phenotyping of the isolated strains was performed using conventional tests and commercial identification systems, ENTEROtest 24 (Erba Lachema, Czechia) and Biolog GEN III MicroPlate (Biolog, USA), as previously described (Sedláček et al. 2016b). Additional biotyping was performed using the API ZYM and API 50 CH kits (bioMérieux) according to the manufacturer's instructions. In addition to the commercial kit assays, we evaluated the strains' tolerance to different environmental stressors. Growth at different temperatures (1, 3, 5, 10, 15, 20, 25, 30, 37, 42, 45, and 48°C) and tolerance to various NaCl concentrations (0.5, 3, 6, 8, and 9% w/v) were determined by culturing in Tryptone Soya Broth (Oxoid) for up to 4 days (Sedláček et al. 2017). Basic phenotyping was performed using cells cultured on TSA at 30°C for 24 hours, employing conventional tube and plate tests relevant for Gram-negative fermenting rods, as previously described (Barrow and Feltham 1993, Atlas 2010, Kosina et al. 2013, Sedláček et al. 2017). Amylase and protease activities were tested using TSA plates supplemented with the appropriate substrate (Margesin et al. 2003).

Antibiogram tests were conducted to evaluate the susceptibility of the strains to selected antibiotics using the disc diffusion method on MHA plates for 2 days at 30°C. Sixteen antibiotic discs were used: ampicillin (10 µg), aztreonam (30 µg), cefalexin (30 µg), cefixime (5 µg), ceftazidime (10 µg), ciprofloxacin (5 µg), doripenem (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), imipenem (10 µg), cotrimoxazole (25 µg), nitroxoline (30 µg), ofloxacin (5 µg), piperacillin (30 µg), tigecycline (10 µg), and tobramycin (10 µg). The EUCAST standards for *Enterobacteriaceae* were followed when reading the inhibition zone diameters (EUCAST 2024[4]). All phenotypic characteristics were recorded in duplicate.

Automated ribotyping and repetitive sequence-based PCR fingerprinting

Genomic relatedness between the isolated strains was determined by automated ribotyping using the RiboPrinter identification system and by repetitive sequence-based PCR (rep-PCR) fingerprinting analysis with the ERIC primer. Numerical analysis and dendrogram construction were performed using BioNumerics software (Applied Maths N.V.) version 7.6 (Sedláček et al. 2016b).

16S rRNA and rpoB genes sequencing and phylogenetic analyses

Sequences of the 16S rRNA and *rpoB* genes from selected representatives were extracted from their corresponding whole-genome assemblies. The genomic techniques used followed the proposed minimum standards for prokaryote taxonomy (Chun et al. 2018). Initial identification of isolates at the genus level was based on pairwise sequence alignment of the 16S rRNA gene and calculation of similarity scores using the algorithm implemented in the EzBioCloud database (Yoon et al. 2017a). The evolutionary relationships of *Enterobacter* spp. were inferred using the Neighbor-Joining method (Saitou and Nei 1987). Evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura et al. 2004). All ambiguous positions were removed for each pair of sequences (pairwise deletion option). Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

Evolutionary analysis based on the RNA polymerase beta subunit (*rpoB*) gene was performed using the Maximum Likelihood method and the General Time Reversible model (Nei and Kumar 2000). The starting trees for the heuristic search were obtained by applying the Neighbor-Joining bottom-up method to a matrix of pairwise distances estimated by the Maximum Composite Likelihood (MCL) method. The evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

Genome sequencing and whole-genome phylogeny

Isolates P5473^T and P5481 were selected as representatives of *Enterobacter* sp. Group 1 and Group 2 and cultured in TSB medium at 30°C until mid-exponential phase. Cells were collected and enzymatically treated as previously described (Dvořák et al. 2024). Genomic DNA was extracted from the cell lysate using the Genomic DNA Clean & Concentrator-25 kit (Zymo Research, USA) according to the manufacturer's instructions. For Oxford Nanopore sequencing (Oxford Nanopore Technologies, UK), the library was prepared using the SQK-RAD004 Rapid Sequencing kit following the manufacturer's instructions. Sequencing was performed using the FLO-FLG001 flow cell (R9.4.1) in a MinION Mk1B device controlled by MinKNOW software v.22.10.10, which was also used for basecalling (super-accurate model with a minimum q-score threshold of 10) and adapter trimming. For Illumina sequencing, a 500 bp library was prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs). Samples were sequenced using a MID output cartridge in 150-bp paired-end mode on an Illumina NextSeq platform (Illumina, San Diego, CA, USA). Sequencing read quality was analysed using the FastQC v0.11.8 (Wingett and Andrews 2018). Low-quality bases and adapters were trimmed using the sliding window model in Trimmomatic Galaxy v0.38.1 (Bolger et al. 2014). Complete bacterial genome sequences were obtained by hybrid assembly using the Unicycler v0.5.0 (with a lowest k-mer size of 0.2, a

highest k-mer size of 0.95, and 10 k-mer steps for SPAdes assembly) (Wick et al. 2017). Assembled genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (Li et al. 2021). Mobile genetic elements were predicted using the MOBhunter[5].

The average nucleotide identity (ANI) values between the whole-genome sequences of strains P5473^T, P5481, and those of the phylogenetically closest *Enterobacter* spp. from the ECC group were calculated using the OrthoANI tool (Yoon et al. 2017b). The digital DNA-DNA hybridization (dDDH) values were calculated using the Genome-to-Genome Distance Calculator (GGDC v2.1) (Meier-Kolthoff et al. 2022) following the recommended formula 2. Pairwise alignment of chromosomal sequences was performed using the LASTZ v1.04.15 plugin in Geneious Prime v2025.2.2[6] with the following parameters: Step Length 50, Seed Pattern 14 of 22, Allowed single transition in seed hit, Enabled chaining, Enabled gapped alignment, Search in both strands, HSP Threshold Score 3,000, Gapped Threshold Score 3,000.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry

MALDI-TOF MS (Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry) analysis using an ultrafleXtreme instrument (Bruker Daltonic) was employed to obtain the protein fingerprint spectra of the *Enterobacter* spp. under investigation. Samples were treated with the ethanol/formic acid extraction protocol (Freiwald and Sauer 2009), and MALDI-TOF MS analysis was performed as previously described (Nováková et al. 2020).

Fatty acid methyl ester characterization

The reference isolates, P5473^T and P5481, were grown on TSA (Difco), incubated at 30 ± 2°C for 24 hours to reach the late exponential growth stage, as determined by the four-quadrant streak method (Sasser 1990). The fatty acid methyl esters were extracted and analysed using an Agilent 7890B gas chromatograph (Agilent Technologies, USA) according to the standard protocol of the Sherlock MIDI Identification System (MIDI Sherlock version 6.2; MIDI database, RTSBA 6.21).

Results and Discussion

A group of Gram-stain-negative, fermenting rods isolated from the gut of notothenioid fish was initially classified within the ECC group based on phenotyping (Sedláček et al. 2016a). Subsequent analysis of the 16S rRNA and *rpoB* genes of representative isolates confirmed this classification in this study (*Supplementary Figures* 2a and 2b). The assignment of species and subspecies within the ECC group is generally problematic, and the taxonomy of the complex has undergone significant changes in recent years (Davin-Regli et al. 2019, Coutinho et al. 2024). Comprehensive phenotyping revealed that few biochemical tests distinguished Group 1 and Group 2 from each other and from the closest ECC species, and most biochemical and physiological characteristics were consistent across the group of isolates. The distinguishing characteristics between the fish isolates and members of the ECC species are summarised in Table 1.

[&]Tube/plate tests	Species										
	1	**2***	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
[§]Growth at 3°C	+	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	-	-	-	-	-	+	-	-	w	-	+
Growth in the presence of 9% NaCl	-	-	w	+	-	-	-	-	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	-	+	+	+	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	+	-
Pyrrolidonyl arylamidase	+	-	w	w	w	+	-	-	-	w	+
Hydrolysis of tyrosin	+	-	+	+	-	+	-	-	-	-	-
[&]**API 50 CH**, acid from:	+	+	-	w	-	-	+	-	-	-	-
$_{D}$-arabinose											
rhamnose	+	+	-	+	+	+	+	+	+	+	+
dulcitol	-	-	-	-	-	-	+	+	-	-	-
inositol	-	-	-	-	+	+	-	-	+	+	-
sorbitol	+	+	+	-	+	+	-	+	+	+	+
methyl-alpha D-mannopyranoside	-	+	+	-	+	+	+	+	+	+	+
hydrolysis of esculin	-	-	+	+	+	+	+	+	+	+	+
melibiose	+	+	-	-	+	+	w	+	+	+	+
raffinose	-	+	-	-	+	+	-	+	+	+	+
$_{D}$-fucose	+	w	-	-	-	-	+	-	-	-	+
$_{L}$-fucose	+	+	-	+	-	-	+	-	-	-	-

Table 1. Differentiation of *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. from type strains of the ECC representatives. *Note*: **1** – *E. hoffmannii* subsp. *nototheniae* (n = 12); **2** – *E. hoffmannii* subsp. *hoffmannii* CCM 9140^{T*}; **3** – *E. asburiae* CCM 8619^{T}; **4** – *E. cancerogenus* CCM 2421^{T}; **5** – *E. cloacae* subsp. *cloacae* CCM 8574^{T}; **6** – *E. cloacae* subsp. *dissolvens* CCM 8575^{T}; **7** – *E. hormaechei* CCM 8620^{T}; **8** – *E. kobei* CCM 8576^{T}; **9** – *E. ludwigii* CCM 8602^{T}; **10** – *E. mori* CCM 9053^{T}; **11** – *E. xiangfangensis* CCM 8608^{T}. *Abbreviations*: + – positive; - – negative; w – weakly positive. All data were obtained in this study. * – type strain features are identical with all results of *Enterobacter* group 2 (except for growth at 3°C and PYR test); & – read after 24-48 hours; § – read after 5 days.

PCR fingerprinting based on ERIC repetitive sequences (Fig. 1) and automated ribotyping using the RiboPrinter system (Fig. 2) separated the investigated fish isolates from all related reference ECC type strains. Both techniques divided the analysed isolates into two groups: Group 1, comprising 12 isolates, and Group 2, consisting of the remaining 7 isolates (Figs. 1 and 2). Based on the rep-PCR fingerprints obtained with the ERIC primer, Group 1 resembled the types of *Enterobacter mori* and *Enterobacter cancerogenus*, while the isolates of Group 2 clustered with the type strain of "*Enterobacter hoffmannii*". The dendrogram, based on cluster analysis of the *Eco*RI ribotype patterns obtained with the RiboPrinter system (Fig. 2) also clearly separated both groups of isolates from the reference types of ECC and showed

Enterobacter hormaechei as the most similar species. Interestingly, the type strain of "*E. hoffmannii*" CCM 9140^T showed a distinct ribotype pattern in relation to the analysed fish isolates, showing that ribotyping could preferably be used for intra-species classification rather than inter-species discrimination.

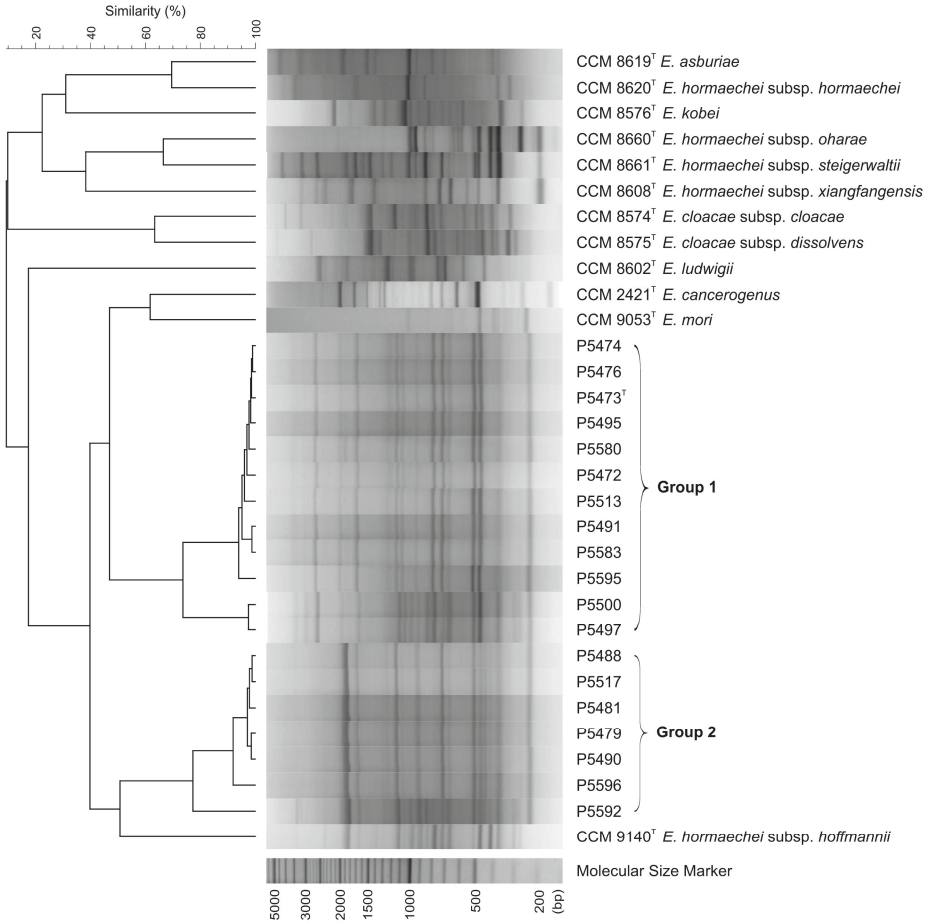

Fig. 1. Dendrogram based on cluster analysis of rep-PCR fingerprints obtained with ERIC primer from isolated *Enterobacter* sp. strains and the closest related reference ECC type strains. The dendrogram was calculated with Pearson's correlation coefficients with the UPGMA clustering method (*r,* expressed as percentage similarity values).

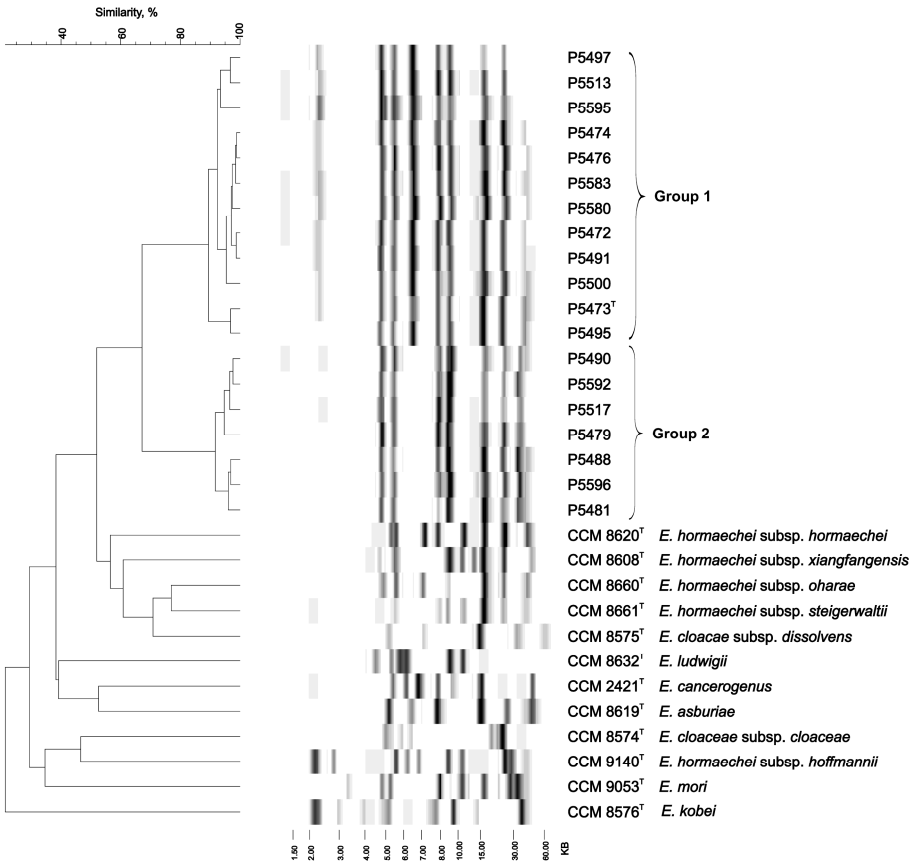

Fig. 2. Dendrogram based on cluster analysis of *Eco*RI ribotype patterns obtained using the RiboPrinter system from isolated *Enterobacter* sp. strains and the closest related reference ECC type strains. The dendrogram was calculated with Pearson´s correlation coefficients with the UPGMA clustering method (*r,* expressed as percentage similarity values).

Cluster analysis of the MALDI-TOF mass spectra grouped all 19 analysed ECC isolates from fish with the type strain "*E. hoffmannii*" CCM 9140^T (*Supplementary Figure* S3), indicating that the fish isolates are members of the species "*E. hoffmannii*", consistent with the genotyping results. The isolates from the guts of fish formed a single cluster, without clear separation between the two groups, as observed using rep-PCR and ribotyping techniques. MALDI-TOF MS appears more suitable for distinguishing between species than for differentiating within species, as in several cases, representatives of Group 1 and Group 2 were intermixed in the dendrogram(*Supplementary Figure* S3).

Preliminary identification of isolates based on TYGS classified representatives of Group 1 and Group 2 as members of the *Enterobacter hormaechei s.l.* cluster, and the analysed representatives were very close to the "*Enterobacter hoffmannii*" type strain (Fig. 3). The same cluster includes the recently described species "*Enterobacter intestinihominis*" (non-validated name) from the human gut, which is bona fide identical to the *E. hormaechei*

subsp. *hoffmannii* type strain based on the presented ANI value (Hitch et al. 2025). This indicates that all enterobacters in this sub-cluster are members of the species "*Enterobacter hoffmannii*".

The diversity within species and the definitions of intra-species units in the era of genomics have undermined the relevance of the prokaryotic rank subspecies, and the use of this taxonomic category has been questioned by some authors (Van Rossum et al. 2020, Venter et al. 2022, Rodriguez et al. 2024). However, several recent studies use the delineation of subspecies of different *Enterobacterales* taxa, for example, based on PCR fingerprints together with phenotyping (Brady et al. 2014), or with ANI values around 98% (Behrendt et al. 2021, Garcia et al. 2021, Pearce et al. 2021), which supports our results and the proposed subspecies status of the "*Enterobacter hoffmannii*" strains in our study.

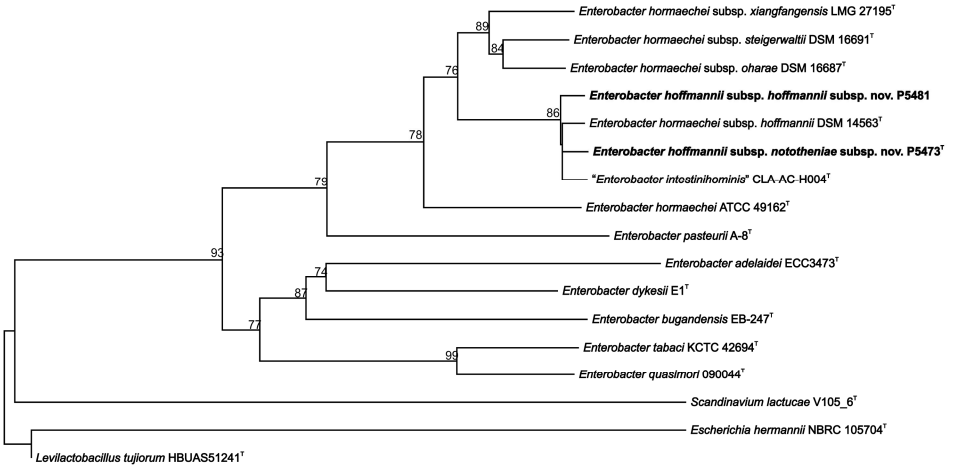

Fig. 3. A phylogenetic tree based on the comparison of the whole genome of the strains P5473^T and P5481 with reference type strains from the *Enterobacter cloacae* complex (ECC) and generated using the TYGS server.

The whole genome of Group 1 representative, strain P5473^T, consists of a 4.83 Mbp chromosome (GenBank: CM128657), a 61.2 kbp plasmid (JBRETZ010000002.1), and a 2.5 kbp plasmid (JBRETZ010000003.1). It encodes 4,705 genes, including 4,504 protein-coding genes (CDS), 92 pseudogenes, and 109 genes encoding RNAs, comprising 25 rRNAs (5S, 16S, and 23S rRNA), 78 tRNAs, and 6 ncRNAs. The genome of Group 2 reference strain P5481 (= CCM 8630) consists of a 4.74 Mbp chromosome (CM128643) and a large 315.6 kbp plasmid (JBRETY010000002.1). It encodes 4,875 genes, including 4,689 protein-coding genes (CDS), 85 pseudogenes, and 101 genes encoding RNAs (25 rRNAs, 69 tRNAs, and 7 ncRNAs).

The pairwise alignment of the complete chromosomal sequences of the strain P5473^T with reference *Enterobacter* spp. strains revealed a relative conservation of most of the P5473^T chromosome sequence compared to other strains (Fig. 4), which is in agreement with the ANI values (Table 2). The differences are apparent in size-limited regions, some of which are represented by distinctive chromosomal islands and prophages. The genome of strain DSM 16687^T has a large chromosomal inversion (Fig. 4).

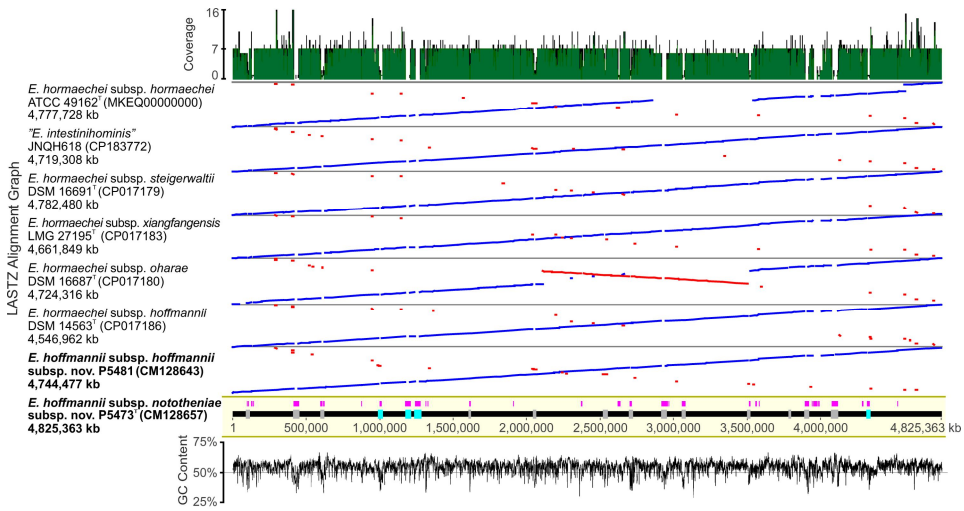

Fig. 4. Pairwise genome alignment of chromosomal sequences of *E. hoffmannii* subsp. *nototheniae* subsp. nov. P5473^T and other *Enterobacter* spp. representatives. A dotplot graph for LASTZ pairwise alignment is shown for each taxon. Reverse alignment is colored in red. The coverage graph shows the number of non-end-gap characters at each position. The scale bar has an indicator at the mean coverage level. Regions distinctive to strain P5473^T, with no coverage in reference genomes, are highlighted below the graph in magenta; chromosomal islands are depicted in gray, and prophages are shown in azure. The NCBI Genome reference chromosomal sequence of strain "*E. intestinihominis*" JNQH618 was used instead of the type strain sequence, which is highly fragmented. The alignment to the ATCC 49162^T strain sequence was affected by the incompleteness of its genome assembly, available only as a set of four contigs.

Obtained results, based on ANI and dDDH analysis data of selected isolates and *Enterobacter hormaechei s.l.* members (Table 2), show that enterobacters isolated from fish guts, previously identified as the ECC complex (Sedláček et al. 2016a), are members of the effectively published "*Enterobacter hoffmannii*". This taxon was first proposed by Sutton et al. (2018) under the valid name *E. hormaechei* subsp. *hoffmannii* with the type strain DSM 14563^T; however, no phenotypic or chemotaxonomic features have been described for this subspecies, as it has been defined solely by genomic features (Coutinho et al. 2024). Representatives of *E. hormaechei* subsp. *hoffmannii* have been isolated from clinical samples and sewage (Sutton et al. 2018, Maguvu and Bezuidenhout 2021), and it is currently likely the second most common enterobacter in human clinical material after *E. xiangfangensis* (Wu et al. 2020, Jiang et al. 2024). Wu et al. (2020) showed that all five validly named subspecies of *E. hormaechei* were incorrect, and concluded that *E. hormaechei* subsp. *hoffmannii* is a species rather than a subspecies and should be renamed "*Enterobacter hoffmannii*" with type strain DSM 14563^T = LMG 30171^T = CCM 9140^T. However, this species name has not yet been validated. In our study, we followed this updated taxonomy, as it fits all genotypic and phenotypic findings. In our view, it is necessary to integrate genomic data with phenotypic classification results when distinguishing subspecies of *Enterobacter* spp.

Species (GenBank No.)	Strain	Chromosome Assembly Size	P5473^T		P5481	
			Ortho-ANI (%)	dDDH (%)	Ortho-ANI (%)	dDDH (%)
***E. hoffmannii* subsp. *nototheniae* subsp. nov. (CM128657)**	**P5473^T**	4,825,363	100	100	99.1	92.2
***E. hoffmannii* subsp. *hoffmannii* subsp. nov. (CM128643)**	**P5481**	4,744,477	99.1	92.2	100	100
E. hormaechei subsp. *hoffmannii* (CP017186)	DSM 14563^T	4,546,962	99.1	93.1	99.1	93.1
E. hormaechei subsp. *oharae* (CP017180)	DSM 16687^T	4,724,316	95.8	66.2	95.9	66.6
E. hormaechei subsp. *steigerwaltii* (CP017179)	DSM 16691^T	4,782,480	95.9	66.1	95.9	66.2
E. hormaechei subsp. x*iangfangensis* (CP017183)	LMG 27195^T	4,661,849	95.9	66.0	95.5	66.0
E. hormaechei subsp. *hormaechei* (NZ_MKEQ01000001, NZ_MKEQ01000002, NZ_MKEQ01000003)	ATCC 49162^T	4,777,728	94.4	57.4	94.5	57.7
"E. intestinihominis" (CP183772)	JNQH618$^{\#}$	4,719,308	99.1	92.8	99.1	92.5

Table 2. Sequence similarity values (%) of OrthoANI and dDDH among *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. P5473^T, *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov. P5481, and reference type strains of *Enterobacter hormaechei sensu lato* group calculated from bacterial chromosome sequences. *Note*: #Reference genome (NCBI Genome) of "*Enterobacter intestinihominis*".

Conclusion

Enterobacters isolated from the gut of *Nototheniidae* fish were divided into two groups by rep-PCR and ribotyping (Figs. 1, 2). The isolates of Group 1 differed from the type strain "*E. hoffmannii*" CCM 9140^T in the results of the fingerprinting method (Figs. 1, 2), biotyping (Table 1), and isolation sources (Table S1). Combining genomic data with the distinct biochemical test results, it is reasonable to propose that Group 1 isolates be classified as novel subspecies of "*E. hoffmannii*". Therefore, a formal description of the *E. hoffmannii* sp. nov. is given and the name *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. is proposed for the Group 1 isolates from the intestinal contents of cod icefishes analysed in this study. The second group of isolates, designated Group 2, exhibited a phenotype pat-

tern identical to that of the type strain "*E. hoffmannii*" CCM 9140^T (= DSM 14563^T) and thus represented *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov. This subspecies was automatically generated according to Rule 40d of the International Code of Nomenclature of Prokaryotes (Oren et al. 2023), based on the available description of *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov.

Formal description of *Enterobacter hoffmannii* sp. nov.

Enterobacter hoffmannii (hoff.man'ni.i. N.L. gen. masc. n. *hoffmannii*, in honor of Harald Hoffmann, a German microbiologist who helped elucidate the phylogenetic structure of the *E. cloacae* complex).

The species description is in conformity with the published data (Lindh and Ursing 1991, Hoffmann and Roggenkamp 2003, Grimont and Grimont 2005, Chavda et al. 2016, Wu et al. 2020, Jiang et al. 2024), and corresponds with effective publication by Sutton et al. (2018), with the type strain DSM 14563^T characterization, and with the data of isolates obtained in this study.

Description of *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov.

Enterobacter hoffmannii subsp. *nototheniae* (no.to.the'ni.ae. N.L. gen. n. *nototheniae* of the striped notothenioid fish *Trematomus hansoni*, the isolation source).

The cells are Gram-stain-negative, non-spore-forming, short rods, occurring predominantly singly or in irregular clusters, and are motile. Colonies on TSA are smooth, shiny, circular with irregular margins, flat with a thick center, and have a diameter of about 2–3 mm after 24 hours of cultivation at 30°C. Aerobic growth occurs on Endo Agar, MacConkey Agar, Tergitol-7 Agar, Brain Heart Infusion Agar, Blood Agar, and Nutrient Agar at 30°C. On TSA, the growth is observed between 3°C and 42°C, but not at 48°C. The cells grow well in Tryptone Soya Broth (TSB) within a salinity range of 0% to 8% NaCl; however, 9% NaCl (w/v) inhibits the growth. Fermentation of glucose in the OF test medium is positive, with gas production from glucose. Catalase, ornithine decarboxylase, arginine dihydrolase, Simmons citrate, malonate, Voges-Proskauer (acetoin), and pyrrolidonyl arylamidase are positive. Oxidase, indole, H_2S, urease, phenylalanine, and lysine decarboxylase are negative. ONPG and tyrosine hydrolysis are positive, while gelatine, Tween 80, DNA, esculin, casein, lecithin, and starch hydrolysis are negative. Alkaline phosphatase, acid phosphatase, leucine arylamidase, and β-galactosidase are positive in the API ZYM commercial kit. Esterase (C4), esterase-lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are negative in API ZYM. Results for naphthol-AS-BI-phosphohydrolase and α-glucosidase are strain-dependent in the API ZYM test.

Acid is produced aerobically (API 50 CH kit) from glycerol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, sorbitol, N-acetylglucosamine, arbutin, cellobiose, maltose, melibiose, sucrose, trehalose, gentiobiose, D-fucose, L-fucose, and 2-ketogluconate(48 h/30°C). Weak acid production is observed from lactose, salicin, melibiose, and gluconate. Erythritol, L-xylose, adonitol, dulcitol, methyl D-xyloside, sorbose, inositol, methyl D-glucoside, methyl D-mannoside, amygdalin, inulin, melezitose, raffinose, starch, glycogen, xylitol, turanose, lyxose, tagatose, D-arabitol, L-arabitol, and 5-ke-

togluconate are negative in API 50 CH.

Positive for the utilisation (Biolog, GEN III MicroPlate) of dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, β-methyl-D-glucoside, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D-galactosamine (weak), α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-fucose, L-rhamnose, D-serine, D-sorbitol, D-mannitol, glycerol, D-glucose-6-PO4, D-fructose-6-PO4, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, D-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, D-saccharic acid, p-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, citric acid, L-malic acid, bromo-succinic acid, and acetic acid.

D-turanose, stachyose, D-raffinose, α-D-lactose, D-salicin, N-acetyl-neuraminic acid, D-arabitol, myo-inositol, D-aspartic acid, gelatine, L-arginine, L-pyroglutamic acid, quinic acid, α-keto glutaric acid, D-malic acid, γ-aminobutyric acid, α-hydroxybutyric acid, β-hydroxy-D,L-butyric acid, α-keto butyric acid, acetoacetic acid, propionic acid, and formic acid from the Biolog kit were negative as carbon sources. Strain-specific results were obtained for dextrin, D-melibiose, 3-methyl glucose, inosine, glycyl-L-proline, D-lactic acid methyl ester, and Tween 40.

Sensitive to aztreonam, cefixime, ceftazidime, ciprofloxacin, doripenem, gentamicin, chloramphenicol, imipenem, cotrimoxazole, nitroxoline, ofloxacin, piperacillin, tobramycin, and tigecycline. Resistant to ampicillin and cefalexin. Major cellular fatty acids of the type strain P5473^T are $C_{16:0}$ (28.75%), summed feature 8 ($C_{18:1}$ ω7c/$C_{18:1}$ ω6c) (31.46%), summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c) (13.52%), $C_{17:0}$ cyclo (11.84%), and summed feature 2 ($C_{14:1}$ 3OH/$C_{16:1}$ iso I) (8.75%).

The type strain of *E. hoffmannii* subsp. *nototheniae* subsp. nov. is P5473^T (= CCM 8629^T = LMG 34031^T). The whole chromosome of the type strain *E. hoffmannii* subsp. *nototheniae* subsp. nov. P5473^T (GenBank: CM128657) has a G+C content of 55.1%.

Description of *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov.

Enterobacter hoffmannii subsp. *hoffmannii* (hoff.man'ni.i. N.L. gen. masc. n. *hoffmannii*, referring to Hoffmann).

The creation of *Enterobacter hoffmannii* subsp. *hoffmannii* subsp. nov. is proposed to comply with Rule 40d of the International Code of Nomenclature of Prokaryotes (Oren et al. 12023), with the type strain DSM 14563^T = LMG 30171^T = CCM 9140^T.

The genome of the *E. hoffmannii* subsp. *hoffmannii* subsp. nov. type strain DSM 14563^T (GenBank: CP017186) has been published previously (Chavda et al. 2016) and has a G+C content of 55.4%.

Both *E. hoffmannii* subspecies exhibited almost identical phenotypes; however, they could be distinguished by fingerprinting techniques and their differing phenotypic characteristics. *E. hoffmannii* subsp. *nototheniae* subsp. nov. utilises dextrin but neither D-raffinose nor D-melibiose, whereas *E. hoffmannii* subsp. *hoffmannii* subsp. nov. does not utilise dextrin but utilises D-raffinose and D-melibiose. Tyrosine hydrolysis is positive for *E. hoffmannii* subsp. *nototheniae* subsp. nov. and mostly negative for *E. hoffmannii* subsp. *hoffmannii* subsp. nov. Using the API 50 CH kit, *E. hoffmannii* subsp. *hoffmannii* subsp. nov. produced acid from methyl-αD-glucopyranoside and D-raffinose, but all tested isolates of *E. hoffmannii* subsp. *nototheniae* subsp. nov. were clearly negative in these tests.

Data availability

All sequencing data were deposited in DDBJ/ENA/GenBank on September 30, 2025, under the project accession number PRJNA688026. The whole genome sequence of the type strain *Enterobacter hoffmannii* subsp. *nototheniae* subsp. nov. P5473^{T} has been deposited under accession number JBRETZ000000000. The whole genome sequence of reference strain *E. hoffmannii* subsp. *hoffmannii* subsp. nov. P5481 has been deposited under accession number JBRETY000000000.

Statements and Declarations

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Conflict of Interest Statement.

The authors declare no competing interests.

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Supplementary Materials

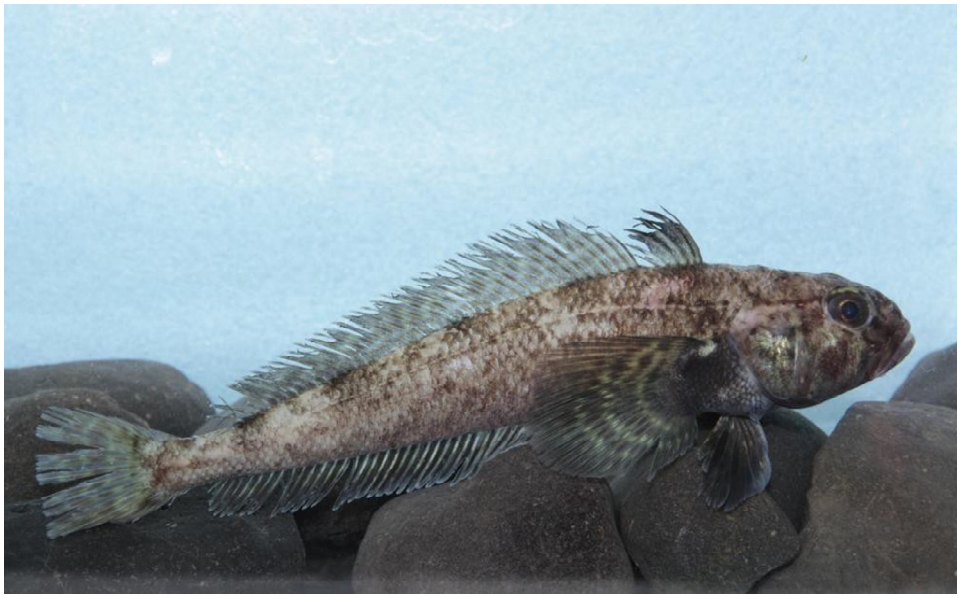

Figure S1. Sample of *Trematomus hansoni* fish.

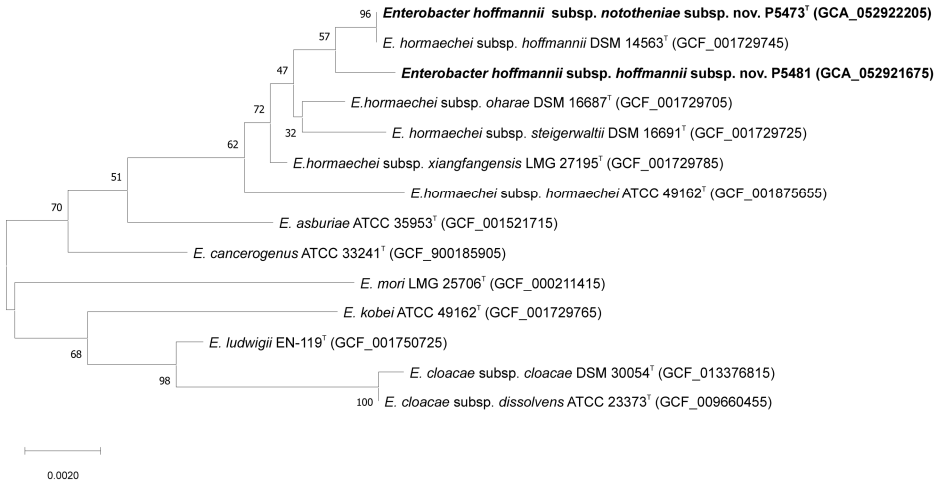

Figure S2a. Evolutionary relationships of *Enterobacter* spp. inferred using the Neighbor-Joining method[1] based on complete 16S rRNA sequences extracted from whole genome assemblies (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches[2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[3] and are in the units of the number of base substitutions per site. This analysis involved 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1506 positions in the final dataset. Evolutionary analyses were conducted in MEGA11[4].

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[2] FELSENSTEIN, J. (1985): Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791.

[3] TAMURA, K., NEI, M. and KUMAR, S. (2004): Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (USA), 101: 11030-11035.

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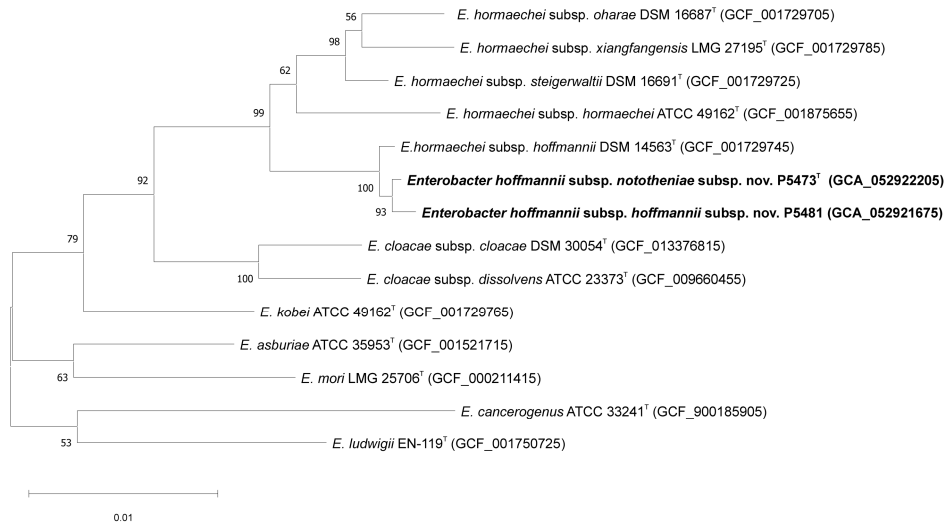

Figure S2b. Evolutionary analysis based on RNA polymerase beta subunit gene (*rpoB*) using the Maximum Likelihood method and General Time Reversible model[1]. The percentage of trees in which the associated taxa clustered together (500 bootstrap replications) is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 14 nucleotide sequences. There were a total of 4029 positions in the final dataset. *rpoB* gene was extracted from whole genome assemblies given in patentheses. Evolutionary analyses were conducted in MEGA11[2].

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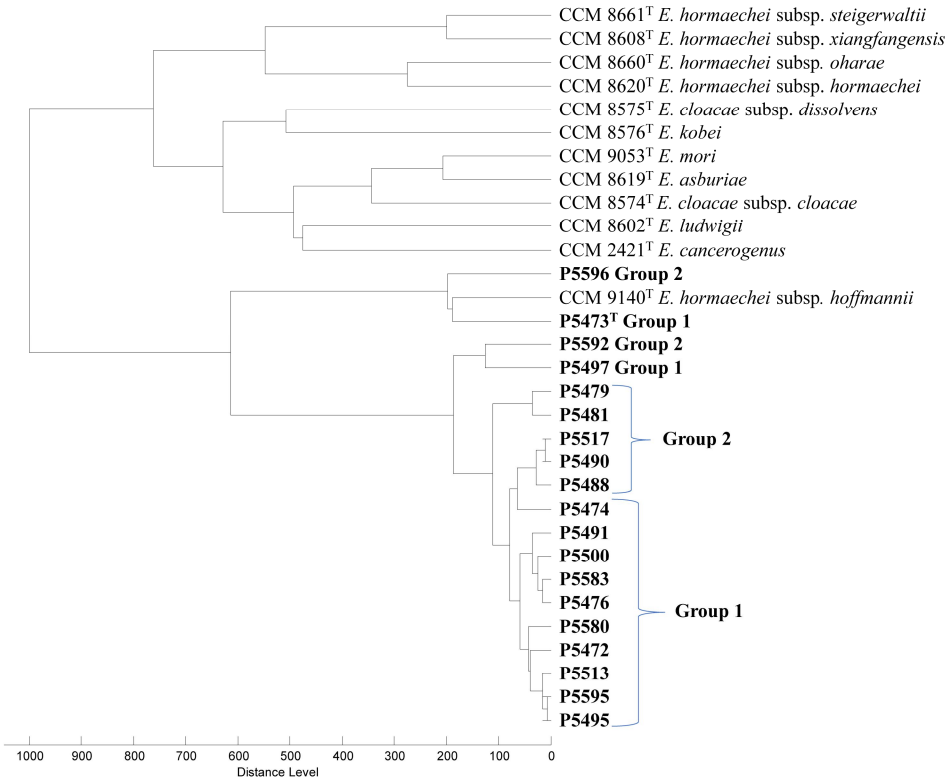

Figure S3. Cluster analysis of MALDI-TOF mass spectra of analysed isolates from fish intestine and the related reference types from *E. cloacae* complex. The dendrogram was generated with Biotyper 3 software using Pearson´s product-moment coefficient as a measure of similarity and the UPGMA as a grouping method.

Fish name (number of samples)	**Strain number**	**Sampling date**	**Isolated on[&]**	**Group[$]**	**Preliminary classification** (Biolog, GEN III MicroPlate)
Notothenia coriiceps (n = 10)	P5595	15.2.2014	XLDA	1	*Enterobacter hormaechei*
	P5491	17.2.2014	XLDA	1	*Enterobacter* sp.
	P5596	15.2.2014	XLDA	2	*Enterobacter* sp.
	P5490	16.2.2014	XLDA	2	*Enterobacter* sp.
	P5592	15.2.2014	XLDA	2	*Enterobacter cloacae*
Trematomus bernacchii (n = 3)	P5583	3.2.2014	Endo	1	*Enterobacter* sp.
Trematomus hansoni	P5472	15.2.2014	XLDA	1	*Enterobacter* sp.
	P5473[T]	16.2.2014	XLDA	1	*Enterobacter* sp.
	P5474	16.2.2014	XLDA	1	*Enterobacter hormaechei*
	P5476	16.2.2014	XLDA	1	*Enterobacter* sp.
	P5495	16.2.2014	XLDA	1	*Enterobacter cloacae*
	P5497	16.2.2014	XLDA	1	*Enterobacter cloacae*
	P5500	17.2.2014	XLDA	1	*Enterobacter hormaechei*
	P5513	23.2.2014	XLDA	1	*Enterobacter* sp.
	P5479	16.2.2014	XLDA	2	*Enterobacter cloacae*
	P5481	16.2.2014	XLDA	2	*Enterobacter cloacae*
	P5517	23.2.2014	XLDA	2	*Enterobacter cloacae*
Trematomus newnesi	P5580	13.2.2014	XLDA	1	*Enterobacter* sp.
	P5488	23.2.2014	Endo	2	*Enterobacter* sp.

Table S1: Sources of isolates from *Enterobacter cloacae* complex inhabiting fish intestine. *Note:* [&] – XLDA, Xylose Lysine Desoxycholate Agar (Oxoid); Endo, Endo agar (Oxoid); [$] – Group based on rep-PCR and RiboPrinter results.