Pseudomonas anatoliensis sp. nov. and *Pseudomonas iridis* sp. nov. isolated from fish

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ABSTRACT

In a study carried out between 2013 and 2018 in fish farms in Turkey, several putative novel species were isolated. The 16S rRNA nucleotide sequences of fourteen strains of Gram-negative rods, which were isolated from asymptomatic and symptomatic rainbow trouts (*Oncorhynchus mykiss*), placed them under the genus *Pseudomonas*. The similarity values of the concatenated nucleotide sequences of the *rpoD*, *rpoB*, *gyrB* and 16S rRNA genes clustered these isolates into the *P. fluorescens* phylogenetic group of species and into the *Pseudomonas koreensis* subgroup, close to *Pseudomonas helmanticensis* and *Pseudomonas baetica*. An isolate of a totally different origin, strain CCUG 67011, clustered with these isolates. Phenotypic characterization, together with the chemotaxonomic data, whole-cell MALDI-TOF MS and fatty acids methyl esters analyses were performed. The DNA G + C content was 58.7 mol% for isolate P9^T and 58.8 mol% for isolate P42^T. The phylogenomic analysis and whole genome nucleotide sequences of four of these isolates confirmed that the isolates P9^T, P25 and P141, represent a novel species for which the name *Pseudomonas anatoliensis* sp. nov. is proposed, with P9^T as the type strain (=CCUG 74755^T = CECT 3172^T). The isolates P1, P2, P10, P27, P30, P24a, P42^T, P117, P139, P152 and CCUG 67011 represent another novel species, for which the name *Pseudomonas iridis* sp. nov. is proposed, with P42^T as the type strain (=CCUG 74870^T = CECT 3174^T).

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Introduction

In a surveillance study, which focused on *Aeromonas*, *Flavobacterium* and *Pseudomonas* species carried out in farm fisheries in Turkey, between 2013 and 2018, several putative novel species were isolated and characterized by a polyphasic approach [7]. Of these species, one was first described in 2020 as *Pseudomonas sivasensis* and comprised strains isolated from cultured fish species, but also from human specimens [6]. Another species described in the same study, *Pseudomonas haemolytica* was reported for the first time in this habitat [12]. Furthermore, other *Pseudomonas* species recently isolated from freshwater salmonids, have been reported as fish pathogens [22]. Farm fisheries may serve as a reservoir of potentially pathogenic *Pseudomonas* species and should be

considered as posing a potential risk for transmission to humans. Therefore, the study and implementation of surveillance programs, and the identification of the causal agents are essential to both avoiding the spread of bacteria to different environments and sustaining the application of the One Health paradigm.

Ninety strains isolated from samples taken in the aquaculture settings from water, fish, eggs, or milt in the spawning period, and also from alevins, were identified as *Pseudomonas* species. Sixty-eight percent of the 90 strains were assigned to known *Pseudomonas* species and 32% corresponded to putative novel *Pseudomonas* species. This study presents a polyphasic taxonomic characterization of a group of strains of two putative novel species not yet described before, but isolated in a surveillance program. The bacterial isolates were recovered from symptomatic and asymptomatic rainbow trouts farmed in Central Anatolia (Kayseri and Sivas provinces) and the Aegean region (Mugla province) between 2013 and 2018. A total of 14 *Pseudomonas* strains were considered as representatives of two putative novel species based on 16S rRNA sequence similarity and multi-locus sequence

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analysis (MLSA) of the *gyrB*, *rpoB* and *rpoD* genes. A strain of a totally different origin, strain CCUG 6701, isolated from a cystic fibrosis patient in Sweden [19] was phylogenetically close-related and thus included in the present study. The delineation of the novel species was based on the biochemical characterization of all isolates by substrate utilization tests (API 20 NE, BIOLOG GN III) chemotaxonomic characterization (MALDI-TOF MS protein and fatty acids profiles) and the whole genome nucleotide sequencing of selected strains.

Materials and methods

Bacterial isolation

Fourteen strains were isolated from rainbow trouts grown in fish farms located in the Central Anatolia region (10 strains from Kayseri province, 2 from Sivas province) and the Aegean region (2 strains from Mugla province), during the period from 2013 to 2018. Data, location, GPS coordinates, and the weight of the fish are shown in Table S1. The samples were aseptically collected from the skin of fish alevins with sterile swab sticks and plated on tryptic soy agar (Merck, 105458 TSA) and blood agar (BA; with 5% sheep blood). The plates were incubated at 28 °C. Sampling was carried out in accordance with the guidelines for the diagnosis of fish diseases and in compliance with international guidelines for animal welfare [23] as previously reported [6]. The strain CCUG 67011, was isolated from the sputum of a cystic fibrosis patient in Gothenburg (Sweden) [19].

Morphological, biochemical and physiological tests

The strains were routinely cultured in Luria broth medium (LB, Luria-Bertani, Difco) at 28 °C. Biochemical tests were performed using API 20 NE strips (bioMérieux, Marcy l'Etoile, France) and Biolog GEN III MicroPlates (Biolog, Hayward, CA) for the phenotypic characterization of the strains, in line with the manufacturer's recommendations. Different growth temperatures (4, 10, 25, 30, 37 and 42 °C) and pH levels (4–11) were tested in LB medium. Growth in the presence of NaCl (0–10% w/v) was tested in NB medium (Nutrient Broth, Difco). The fluorescence test was performed in King B medium as previously described [6,16]. Pyocyanin production was tested in King A medium (Himedia, M1543). The bacteria were also tested for haemolytic activity on blood agar (supplemented with 5% sheep blood) at 28 °C. Cell size, morphology and flagella insertion were determined by the transmission electron microscopic observation of cells in the exponential growth phase in LB medium. A Hitachi model H600 electron microscope was used at 75 kV. The samples were negatively stained with phosphotungstic acid (1%, pH 7.0) as previously described [14].

Chemotaxonomic analysis

Whole-cell fatty acid methyl ester (FAME) analyses were performed at the Spanish Type Culture Collection, CECT, Valencia, Spain (<http://cect.org/identificaciones>) by gas chromatography. For this purpose, an Agilent 6850 unit equipped with the MIDI Microbial Identification System using the RTSBA6 method and the Microbial Identification Sherlock software package version 6.1 were used. The selected method was applied as previously described [6]. The main protein profiles of the 14 strains and their close-related species type strains were obtained by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of whole cells. Data were obtained by the Scientific-Technical Services of the University of Balearic Islands (Spain) and were analyzed as previously described [28].

DNA extraction, PCR amplification and DNA sequencing conditions

The DNA extraction method, and PCR amplification and DNA sequencing conditions applied in this study have been previously reported [6,20]. The genes that were amplified for phylogenetic analysis included 16S rRNA (1350 nt), *gyrB*, (RNA gyrase subunit B, 802 nt), *rpoB* (RNA polymerase beta subunit, 916 nt) and *rpoD* (RNA polymerase subunit D, 689 nt).

Phylogenetic analysis

An individual phylogenetic 16S rRNA tree was constructed with the sequences of the 14 isolates recovered in this study, together with the species type strains of the *P. koreensis* subgroup (SG), and *P. jesssenii* SG and with the representative type strains of the closest subgroups, namely, *P. mandelii*, *P. fluorescens*, *P. gessardii*, *P. chlororaphis* and *P. corrugata*. Distance matrices were generated by Jukes and Cantor method (JC) [13], and the tree was reconstructed with neighbor-joining (NJ) [27]. Trees were also constructed by Maximum likelihood (ML) [9] and Maximum Parsimony (MP) [21] methods as implemented in the MEGA package [29]. The multilocus sequence analysis (MLSA) tree was constructed by concatenating a total of 3757 nucleotides corresponding to partial sequences of four housekeeping genes (16S rRNA, *rpoD*, *gyrB*, *rpoB*), with the same algorithms (JC-NJ, ML, MP).

Multilocus sequence analysis (MLSA) based on 79 genes was performed on the autoMLST web server as described by Alanjary and collaborators [1]. Briefly, the uploaded genome sequences were analyzed together with their closest relatives detected by MASH ANI in the server database. The best single copy ubiquitous housekeeping genes were selected by the program and a maximum of 100 nucleotide sequences were concatenated and aligned. Phylogenetic distance calculations and tree building by maximum likelihood were performed on the autoMLST web server [1].

The core genome of *P. anatoliensis* and *P. iridis* strains was also calculated in the web server M1CROB1AL1Z3R [3]. Briefly, the putative open reading frames (ORFs) of each genome was extracted using Prodigal and a homology search in which each ORF was queried against the ORFs of the other genomes by using tBlastX. Orthologous groups were defined using default parameters: maximal e-value cutoff: 0.01; identity minimal percent cutoff: 80.0%; minimal percentage for core: 100.0%. Additionally, a maximum-likelihood phylogenetic tree was reconstructed based on the concatenated protein sequences of all core genes and using the genome of *P. baetica* type strain as outgroup. A UPGMA dendrogram was also constructed based on the ORFs shared by each pair of strains. The presence or absence in each genome of all orthologous genes found was coded as 1 and 0 and similarities were calculated using the Dice coefficient in the PAST program [11].

Genome sequencing and analysis

DNA was extracted from the strains P9^T, P42^T, P141 and CCUG67011 with a QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden-Germany). The amount and purity of DNA in each sample were measured at wavelengths of 260 nm and 260/280 nm using a spectrophotometer (Multiscan Go, Thermo, USA). The sequencing library was prepared using the Nextera XT DNA Library Preparation Kit, and sequencing was performed on the Illumina NovaSeq 6000 platform using the 2x250-bp paired-end (PE) sequencing mode with a 1000-cycle MiSeq reagent kit. The reads with low-quality scores (<20) or poly-Ns and adaptor contamination were trimmed using BBDuk implemented in Geneious Prime. High-quality reads of the genomes were assembled into contigs by de novo assembly using the SPAdes assembler

3.13.0. Genomes were annotated using the Rast tool kit available in the Pathosystems Resource Integration Center (PATRIC) database (PATRIC 3.6.3), as part of the online all-bacteria Bioinformatics Resource Center. In addition, the annotated protein-encoding sequences were identified using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAP) [31]. Analyses were performed for GC content and genome size. The draft genome sequences were submitted to GenBank.

The genome relatedness of the strains P9^T and CCUG 67011 to the whole-genome shotgun sequences of all species type strains in the *P. koreensis* phylogenetic subgroup available in public databases was determined based on the tetranucleotide frequency correlation coefficients (TETRA), average nucleotide identity (ANI) determined with the BLASTN algorithm (ANiB), and the results of analysis with the MUMMER ultrarapid alignment tool (ANIm). ANiB and ANIm were calculated using the JSpecies software tool available on the webpage <http://jspecies.ribohost.com/jspeciesws/> [25]. Additionally, digital DNA-DNA hybridization between the selected strains was performed by the genome-to-genome distance (GGDC) method. GGDC was calculated using a web service (<http://ggdc.dsmz.de>) [17] and the recommended BLAST method. The presented GGDC results are based on the recommended formula 2. The type strain genomes server pipeline (TYGGS, <https://tygs.dsmz.de/>) was used to compare the genomes of the strains P9^T, P42^T, P141, and CCUG67011 with the genomes of the type strains deposited in the DSMZ data base [8,18].

All genomes were annotated with the PROKKA software tool for comparison purposes [30]. The protein amino acid sequences obtained were compared using the GET_HOMOLOGUES software [5], using the criterion of 70% similarity over 50% of coverage alignment. A functional analysis of the protein-encoding genes was also performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>)

Results and discussion

Isolation

Ten strains were isolated from asymptomatic trouts in the Kayseri city in different years. One strain, P117, was isolated from symptomatic fish displaying loss of appetite, anaemia, and dorsal fin erosion with no accompanying microbiota. Two isolates, P139 and P141, were associated to dead fish with accompanying flora (*Flavobacterium* sp.) (Table S1). The strain CCUG 67011 was isolated from a human specimen.

Phylogenetic analysis

The 16S rRNA sequences were identical in all 14 isolated strains and the phylogenetic analysis clustered them into the *P. koreensis* SG (Fig. 1), with similarity percentages of 99.3% to the type strain of *P. baetica*, and 99.4% with the type strain of *P. helmanticensis*, (Table S2). To improve the accuracy of the identification at species level, the 4-genes concatenated nucleotide sequences (MLSA) were compared to the *P. koreensis* SG type strains as shown in the dendrogram of Fig. 2. The corresponding matrix values are given in Table S2. The intraspecies similarity values ranged from 99.9 to 100% among the 3 strains: P9^T, P25 and P141 strains and from 99.2% to 100% among the strains P42^T, P1, P2, P10, P24a, P27, P30, P117, P139, P152 and CCUG67011. The highest species type strain similarity values were determined as 95.5% and 95.2% for *P. baetica* with the P9^T cluster of strains with the P42^T cluster of strains, respectively (Table S2). The accepted MLSA species cut-off value for species differentiation is 97% [13,18]. The concatenated 81-genes sequences (obtained with the autoMLST method)

were also analyzed with the same strains, such that the same two clusters were observed as shown in Fig. 3 and all branches in the tree were supported by high bootstrap values. The results obtained in the present study suggested that these strains could belong to a two novel species in the genus *Pseudomonas*.

Phenotypic characteristics

The 14 strains were motile Gram negative-stained rods. The colonies were round, flat, opaque and beige colored, with regular and translucent margins and reached an approximate diameter of 3–6 mm after growth for 2 days at 30 °C on LB plates. Electron microscopic examination revealed the presence of one monopolar inserted flagellum in the strains P9^T and P42^T as shown in Tables 2A and B and Fig. S1.

The phenotypic characteristics that differentiated the 14 strains from their closest phylogenetic neighbours are given in Table 1. The complete list of phenotypic characteristics is given in Table 2 and Table S3. The proposals of new species are supported frequently by the description of a single strain. In this study we present 14 strains for these two novel species which increases the phenotypic variability within species. As a consequence, few phenotypic characteristics permits the differentiation of the two close novel species. This situation is frequent in the genus *Pseudomonas*. For instance, classical subdivision in biotypes that differentiate biovars A and B in the species *P. putida* are not supported by phylogenetic analyses. This fact is much more complex among species in the *P. fluorescens* phylogenetic group. As stated by Palleroni in the last version of the Bergey's Manual: "It is quite possible that isolation of more strains of each of the different biovars may result in the future in the proposal of new species with the general properties of *P. fluorescens*, but circumscribed based on genomic differences [24]." Potential phenotypic differential characteristics can be deduced in the comparative genomic analysis as stated below.

Chemotaxonomic analysis: protein profile and fatty acid analysis

The MALDI-TOF-MS profiles of each strain showed that all strains of the putative novel species were closely related to each other with similarity values higher than 95%, and clustered in the same branch with the type strains of *P. helmanticensis*, *P. baetica* and *P. koreensis*. Other strains with no close relation, including *P. granadensis* or *P. kribbensis*, with no close relation were also outside this cluster. The protein dendrogram is presented in Fig. S2. In this case, the m/z protein profiles were not discriminative enough for the identification of these close related species.

Table 3 shows the fatty acids methyl esters analysis results for 4 of the selected isolates (P9^T, P42^T, P141 and CCUG 67011) and two of their closest related type strains *P. helmanticensis* and *P. baetica*, together with *P. koreensis* as representative of the *P. koreensis* SG. The characteristic C_{16:0} fatty acid of the *Pseudomonas* genus was present in all strains, together with other major fatty acids (C_{10:0} 3-OH, C_{12:0} 2-OH and C_{12:0} 3-OH) [24]. *P. anatoliensis* and *P. iridis* can be differentiated phenotypically by chemotaxonomy data, using fatty acids methyl ester analysis. While the content of summed feature 3 ranged between 36–38% for *P. anatoliensis* and 29–30% for *P. iridis*, the percentage of C_{17:0} cyclo was traces–2.16% for *P. anatoliensis* and 7.48–6.40% for *P. iridis*. Furthermore, the range of summed feature 8 was 8.54–7.59% for *P. anatoliensis* and 9.87–10.53% for *P. iridis*. These characteristics are valuable tools for the differentiation of the proposed two novel species.

General taxonomic genome features

The P9^T draft genome was assembled in 18 contigs with an average coverage of 78X. The main characteristics of the whole-

Fig. 1. Phylogenetic tree based on 16S rRNA gene of *P. anatoliensis*, *P. iridis* and phylogenetically close members of *Pseudomonas*. Distance matrices were calculated by the Jukes-Cantor method. Dendrograms were generated by the neighbour-joining method. *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and maximum parsimony methods. GenBank accession numbers are given in parentheses. Accession numbers indicated in bold are for sequences determined in this study.

genome sequence of strain P9^T are presented in Table S4a. For the P141 genome, 18 contigs with an average 308X coverage were obtained. All genome relatedness values of strain P9^T with its closest relatives in the *P. koreensis* SG calculated with the algorithms ANIb, ANIm and GGDC were clearly below the established cut-off values for each algorithm (ANIb: 95%, ANIm: 95% and GGDC: 70%), as shown in Table S5. This confirmed that strain P9^T represents a novel genomic species in the group. The similarity to the

closest relative, P42^T was 94.02% for ANIb, 95.06% for ANIm and 58.60% for GGDC. The P9^T and P141 strains displayed an ANIb percentage of 98.31%, an ANIm percentage of 98.66% and a GGDC percentage of 87.60% between them (Table S5). These high similarity values indicate that these two strains belonged to a unique genomic species.

The P42^T draft genome was assembled in 18 contigs with an average coverage of 477X. The main characteristics of the whole-

Fig. 2. Phylogenetic tree based on concatenated sequences of the 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes of *P. anatoliensis*, *P. iridis* and phylogenetically close members of *Pseudomonas*. Genetic matrices were calculated by the Jukes-Cantor method. Dendrograms were generated by the neighbour-joining method. *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and maximum parsimony methods. GenBank accession numbers are given in parentheses in the following order: 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes. Accession numbers indicated in bold are for sequences determined in this study.

genome sequence of strain P42^T are presented in Table S4b. For the CCUG 67011 genome, 46 contigs with an average 560X coverage were obtained. All genome relatedness values of strain P42^T with its closest relatives in the *P. koreensis* subgroup calculated by using the algorithms ANIb, ANIm and GGDC were clearly below the established cut-off values for each algorithm as shown in Table S5, confirming that strain P42^T represents a novel genomic species in the group. The similarity to the closest relative, *P. anatoliensis* P9^T was 94.02% for ANIb, 95.07% for ANIm and 58.06% for GGDC. The P42^T and CCUG 67011 strains showed an ANIb percentages of 96.36%, an ANIm percentages of 97.33% and a GGDC percentage of 75.60% (Table S5). These high similarity values indicate that these two strains belonged to a unique genomic species. The genomes of P9^T, P141, P42^T and CCUG67011 were also com-

pared to the genomes of the type strains in the *P. koreensis* subgroup available on the Type (strain) Genome Server (TYGS). The corresponding obtained dendrogram is shown in Fig. S3. The results clearly indicated that the four strains represent a two putative novel species.

Genome comparison between *P. anatoliensis* and *P. iridis* strains

In order to identify the proteins that differentiate the two species, a comparative analysis was made between the genomes of the four strains studied. As shown in the Venn diagram (Fig. 4) both species share 4198 genes, representing between 71% and 77% of their respective protein coding genes. The species delineation threshold proposed by Goris and collaborators is 85% of conserved

Fig. 3. Phylogenetic tree based on concatenated sequences of 81 selected housekeeping genes of *P. anatoliensis* strains, *P. iridis* strains and phylogenetically close members of *Pseudomonas*. Dendrograms were generated by the maximum likelihood method. *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes. GenBank accession numbers are given in parentheses.

proteins [10]. *P. anatoliensis* strains showed 89% conserved proteins and *P. iridis* strains showed 86% conserved proteins. Two-hundred and seventy-six genes were exclusively present in *P. anatoliensis* but not in *P. iridis*.

In the final orthologous table in the M1CROB1AL1Z3R web server 5376 orthologous ORFs were obtained in the analysis of the four genomes under study. ORFs were distributed as follows: 5109 in *P. anatoliensis* P141; 5105 in *P. anatoliensis* P9T; 4798 in *P. iridis* P42T and 4716 in *P. iridis* CCUG 67011. The core proteome of the four genomes contained 4314 genes. When *P. baetica* type strain was included in the analysis as outgroup, the core proteome was reduced to 4010 genes, and 5490 orthologous ORFs were found. Fig. 5 shows the phylogenetic tree based on the concatenated nucleotide sequences of the core genes (Fig. 5A) and the dendrogram corresponding to the genes shared by each pair of strains (Fig. 5B). In both figures *P. anatoliensis* and *P. iridis* strains are separated. It is worth to highlight that *P. anatoliensis* strains were closer to *P. baetica* than to *P. iridis* strains in the number of shared ORFs. Fig. 5 demonstrates that *P. anatoliensis* and *P. baetica* not only shared a high number of genes, but also that they have a common ancestor in the analysis of the core genome. The ANIb values of *P. baetica* ranged from 87.59 to 88.2% with the four strains under study, clearly below the species threshold. As a consequence, *P. anatoliensis*, *P. baetica* and *P. iridis* should be considered three different species.

A function was assigned in the PROKKA analysis to 107 of these proteins, whereas 169 were hypothetical proteins. One-hundred and thirty-one genes were exclusively present in *P. iridis* and not in *P. anatoliensis*. A function was assigned to 58 of these proteins, whereas 73 were hypothetical proteins. Supplemental Table S6 shows the complete list of species-specific genes.

The proteins with function prediction were deeply analyzed to identify potential additional phenotypic traits that could be useful in the differentiation of the two novel species. Exclusive genes coding for two interesting properties were found in *P. anatoliensis*. Both strains of this species possess two toxin/antitoxin systems, one of type I (SmyE/SmyR) and the other of type II (HigBA-2 related to the system RelE/ParE). These systems are considered metabolism regulators that act in response to environmental stress conditions and are involved in biofilms and bacterial virulence [32] (A third interesting differential gene encodes a colicin-like protein containing an S2 type pyocin domain. Colicins mainly act intraspecifically and have an ecological role [26].

Only the HigBA type II toxin/antitoxin system was detected in *P. iridis* strains. This system differs from the HigBA-2 system found in *P. anatoliensis* strains. Three other genes related to environmental factors were predicted in the *P. iridis* strains: (1) Three copies of the universal stress protein UspE, known as involved in protecting the cell against DNA damage. *uspE* is induced in the stationary phase and by a variety of stresses causing the growth arrest of cells.

Table 1

Characteristics differentiating of *Pseudomonas anatoliensis* and *Pseudomonas iridis* from the type strains of the most related species. +, Positive; –, negative; w, weak. Strains: 1, *Pseudomonas anatoliensis* P9^T; 2, *Pseudomonas iridis* P42^T; 3, *Pseudomonas baetica* CECT 7720^T; 4, *Pseudomonas helmanticensis* CECT 8548^T; 5, *Pseudomonas koreensis* LMG 21318^T; 6, *Pseudomonas fluorescens* ATCC 13525^T.

Characteristics	1	2	3	4	5	6
Temperature (°C)	4–37	4–37	4–30	4–30	4–30	4–37
NaCl (%) (w/v)	0–8	0–9	0–6	0–5	0–6	0–8
pH	7–10	7–9	6–9	5–11	5–9	5–9
API 20 NE test:						
Presence of arginine dihydrolase	–	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	–	–	–
Assimilation of:						
Phenylacetate	+	+	–	–	–	–
BIOLOG GENIII test:						
Carbon source utilization assays						
D-Sorbitol	–	–	–	–	–	+
Tween 40	+	w	w	+	+	+
glycyl-L-proline	–	–	–	+	+	+
D-Galacturonic Acid	+	+	–	–	–	+
Methyl Pyruvate	w	w	+	+	+	w
D-Galactonic Acid lactone	–	+	w	w	–	w
alfa-Hydroxy Butyric Acid	w	–	–	–	w	+
D-Trehalose	+	+	–	–	–	+
myo-Inositol	–	–	–	+	–	+
D-Glucuronic Acid	+	+	+	–	–	+
alfa-Keto-Butyric Acid	w	–	–	–	w	+
D-Fucose	–	+	+	+	+	+
Glucuronamide	w	+	+	+	+	+
Sucrose	–	–	–	–	–	+
Mucic acid	+	+	w	+	+	+
D-Malic Acid	–	–	w	+	+	+
L-Rhamnose	–	–	–	–	–	+
D-Serine	–	–	+	+	+	+
Bromo-Succinic Acid	–	–	w	+	+	+
Formic Acid	–	–	–	+	+	+
Chemical sensitivity assays						
Lithium Chloride	–	+	–	+	+	+
Sodium Butyrate	–	–	–	–	w	+
Minocycline	+	+	+	–	+	+
Sodium Bromate	+	+	+	w	+	+

Most organisms have multiple paralogs of USPs, where the number of copies depends on the organism. (2) A gene coding for an antiseptic resistance protein transporter that belongs to a group of secondary transporters. (3) A gene coding for aquaporin Z (P42_00343 and CCUG67011_00252). The AqpZ channel is selectively permeable to water, although other functions are not excluded. AqpZ has a role in both the short- and the long-term osmoregulatory response and is required by rapidly growing cells. AqpZ-like proteins seem to be necessary for the virulence expressed by some pathogenic bacteria [4].

Overall, these results indicate that it is of fundamental importance to clarify the taxonomic position of the novel isolates at the species level. *P. aeruginosa*, *P. fluorescens*, *P. putida* and *P. syringae* are well known species commonly encountered in the aquaculture sector [2]. Today the *Pseudomonas* genus has become complex and comprises by more than 240 recognized species in the list of prokaryotic names with standing in nomenclature (LPSN DSMZ, <https://www.lpsn.dsmz.de>). The growing number of species provides a more comprehensive insight into the *Pseudomonas* genus [15]. Six different species are being proposed as novel species from fish farms in Turkey (manuscript in press [7]). We have demonstrated in this study the presence of strains of the same species of completely different origins, fish or human, and from diverse geographic locations, like Turkey and Sweden. This raises intriguing questions for further genetic, evolutionary and ecological research. This is not the first report of such a case, as a recent publication on *P. sivasensis*, described a similar situation, where strains of different origins were isolated from fish farms, human speci-

mens and river water, and *P. haemolytica* originally described as having been isolated from raw milk was also found in fish farms [6]. It is important to determine the prevalence of these strains in this habitat through time (as shown by the different year of isolation in the same aquaculture farm), for a better understanding of any specific role they may play and their correlation with fish and other microorganisms.

Conclusion

The 16S rRNA nucleotide sequences of the studied strains, showed that they belonged to the genus *Pseudomonas*. The phenotypic data, MALDI-TOF MS protein profiles and fatty acids methyl esters analysis results revealed that these strains constituted two different groups from the closest type strains of the *P. koreensis* subgroup. Multilocus sequence analyses also located these species in a cluster with their similarity values to the closest type strains lower than the cut-off values established for the species. Finally, the ANIb and GGDC indices confirmed that the representative strains, P9^T and P42^T, showed similarity percentages ranging between 88.20–87.99% and 37.4–36.70%, respectively, to their closest related known species indicating that they conform not one, but two novel species.

Based on all the phenotypic, chemotaxonomic, and phylogenetic and genomic results obtained in the present study, we conclude the strains P9^T and P25 and P141, represent a novel species for which the name of *Pseudomonas anatoliensis* sp. nov. is pro-

Table 2ADescription of *Pseudomonas anatoliensis* sp. nov.

Species name	<i>Pseudomonas anatoliensis</i>
Genus name	<i>Pseudomonas</i>
Specific epithet	<i>Anatoliensis</i>
Species status	sp. nov.
Species etymology	a.na.to.li.en.sis. L. fem. adj. anatoliensis, from Anatolia where the strain was isolated
Designation of the type strain	P9
Strain collection numbers	CECT 30172, CCUG 74755
16S rRNA gene accession number	LR797545
Alternative housekeeping genes	<i>gyrB</i> gene [LR797676], <i>rpoD</i> gene [LR797698] and <i>rpoB</i> gene [LR797612]
Genome accession number	JACOPT000000000
Genome status	Draft
Genome size (pb)	6,453,902
GC mol %	58.7
Country of origin	Turkey
Region of origin	Central Anatolia
Other	Kayseri
Date of isolation	December 2013
Source of isolation	<i>Oncorhynchus mykiss</i> (abdominal cavity)
Sampling date	December 2013
Geographic location	Kayseri
Latitude	38°57'57.4"N
Longitude	36°40'07.2"E
Depth	0
Altitude (alti)	0
Number of strains in study	3
Source of isolation of non-type strains	<i>Oncorhynchus mykiss</i> (abdominal cavity)
Growth medium, incubation conditions used for standard cultivation	Luria-Bertani medium (LB) at 30 °C
Gram stain	NEGATIVE
Cell shape	rod
Cell size (length or diameter)	3.7–3.1 µm long and 0.9–1.1 µm wide
Motility	Motile
If motile	flagellar
If flagellated	one polar flagellum
Sporulation (resting cells)	none
Colony morphology	Round, flat, opaque and beige coloured, with regular margins and translucent margins (3–6 mm in diameter) and approximately 3–6 mm in diameter after growth for 2 days at 30 °C on LB plates.
Temperature range	4–37
Lowest temperature for growth	4
Highest temperature for growth	37
Temperature optimum	30
Lowest pH for growth	7
Highest pH for growth	10
Lowest NaCl concentration for growth	0
Highest NaCl concentration for growth	8
Salinity category	moderate halophile (optimum 7–15% NaCl)
Positive tests with BIOLOG	The strains have the ability to oxidize: alfa-D-glucose, D-mannose, D-galacturonic acid, gamma-amino-butyric acid, D-fructose, L-alanine, D-galactose, L-arginine, D-gluconic acid, L-lactic acid, beta-hydroxy-D,L-butyric acid, glycogen, L-aspartic acid, D-glucuronic acid, citric acid, N-acetyl-D-glucosamine, L-glutamic acid, alfa-keto glutaric acid, L-histidine, mucic acid, propionic acid, L-pyroglyutamic acid, quinic acid, L-malic acid, acetic acid, inosine, L-serine and D-saccharic acid. Strains were able to grow at 1% NaCl, 4% NaCl, 1% sodium lactate, troleandomycin, lincomycin, vancomycin, nalidixic acid, aztreonam, pH 5, pH 6, fusidic acid, rifamycin SV, guanidine HCl, tetrazolium violet, D-serine, minocycline, nioproof 4, tetrazolium blue, potassium tellurite and sodium bromate
Negative tests with BIOLOG	The strains were unable to oxidize: D-raffinose, D-sorbitol, gelatin, pectin, p-hydroxy-phenylacetic acid, dextrin, alfa-D-lactose, glycyl-L-proline, D-maltose, D-melibiose, D-lactic acid methyl ester, beta-methyl-D-glucoside, myo-inositol, D-cellobiose, D-salicin, 3-methyl glucose, gentiobiose, D-glucose-6-PO4, acetoacetic acid, sucrose, N-acetyl-beta-D-mannosamine, L-fucose, D-fructose-6-PO4, D-malic acid, turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, stachyose, N-acetyl-neuraminic acid, D-serine, formic acid. Strains were unable to grow at lithium chloride and sodium butyrate
Variable tests with BIOLOG	Tween 40, D-mannitol, methyl pyruvate, D-arabitol, D-galactonic acid lactone, D-trehalose, D-fucose, glucuronamide and bromo-succinic acid
Positive tests with API	hydrolysis of gelatin and the assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-D-glucosamine, gluconate, caprate, malate and citrate
Negative tests with API (APIN)	Reduction of nitrate to nitrite and nitrite to N ₂ , indole production, glucose fermentation, the urease, hydrolysis of aesculin, the beta-galactosidase and the assimilation of maltose and adipate
Variable tests with API (APIV)	presence of arginine dihydrolase and assimilation of phenylacetate
Commercial kits used	BIOLOG GENIII, API 20NE
Energy metabolism	chemoorganotroph
Oxidase	positive
Catalase	positive
Negative tests	Pigment on King A agar
Major fatty acids	Sum In Feature 3 (C _{16:1} ω7c and/or C _{16:1} ω6c, 38.64–36.81%), C _{16:0} (36.91–35.16%), Sum In Feature 8 (C _{18:1} ω6c and/or C _{18:1} ω7c, 8.54–7.59%), C _{12:0} 2OH (5.20–5.06%), C _{10:0} 3OH (4.43–3.69%), C _{12:0} 3OH (4.21–4.16%) and C _{12:0} (2.16–2.08%)
Biosafety level	1
Habitat	fish farm (http://purl.obolibrary.org/obo/ENVO_00000294)
Biotic relationship	free-living
Known pathogenicity	None

Table 2BDescription of *Pseudomonas iridis* sp. nov.

Species name	<i>Pseudomonas iridis</i>
Genus name	<i>Pseudomonas</i>
Specific epithet	<i>iridis</i>
Species status	sp. nov.
Species etymology	i'ri.dis. L. gen. n. iridis, of Iris, the Greek goddess of the rainbow
Designation of the type strain	P42
Strain collection numbers	CECT 30174, CCUG 74870
16S rRNA gene accession number	LR797554
Alternative housekeeping genes	<i>gyrB</i> gene [LR797682], <i>rpoD</i> gene [LR797704] and <i>rpoB</i> gene [LR797621]
Genome accession number	JACOPU000000000
Genome status	Draft
Genome size pb	6,141,407
GC mol %	58,8
Country of origin	Turkey
Region of origin	Central Anatolia
Other	Kayseri
Date of isolation	March 2014
Source of isolation	<i>Oncorhynchus mykiss</i> (abdominal cavity)
Sampling date	March 2014
Geographic location	Kayseri
Latitude	38°57'57.4"N
Longitude	36°40'07.2"E
Depth	0
Altitude (alti)	0
Number of strains in study	11
Source of isolation of non-type strains	<i>Oncorhynchus mykiss</i> (abdominal cavity)
Growth medium, incubation conditions used for standard cultivation	Luria-Bertani medium (LB) at 30 °C
Gram stain	NEGATIVE
Cell shape	Rod
Cell size (length or diameter)	0.8–1.8 µm long and 0.4–0.7 µm wide
Motility	Motile
If motile	flagellar
If flagellated	one polar flagellum
Sporulation (resting cells)	none
Colony morphology	Round, flat, opaque and beige coloured, with regular margins and translucent margins (3–6 mm in diameter) and approximately 3–6 mm in diameter after growth for 2 days at 30 °C on LB plates.
Temperature range	4–37
Lowest temperature for growth	4
Highest temperature for growth	37
Temperature optimum	30
Lowest pH for growth	7
Highest pH for growth	9
Lowest NaCl concentration for growth	0
Highest NaCl concentration for growth	8
Salinity category	moderate halophile (optimum 7–15% NaCl)
Positive tests with BIOLOG	The strains have the ability to oxidize: alfa-D-glucose, D-mannose, D-mannitol, D-galacturonic acid, gamma-amino-butyric acid, D-fructose, D-arabitol, L-alanine, D-falactose, L-arginine, D-gluconic acid, L-lactic acid, beta-hydroxy-D, L-butyric acid, glycogen, L-aspartic acid, D-glucuronic acid, citric acid, L-glutamic acid, glucuronamide, alfa-keto-glutaric acid, L-histidine, mucic acid, propionic acid, L-pyroglyutamic acid, quinic acid, L-malic acid, acetic acid, inosine, L-serine, D-saccharic acid. Strains were unable to grow at 1% NaCl, 4% NaCl, pH 5, pH 6, 1% sodium lactate, troleandomycin, lincomycin, vancomycin, nalidixic acid, aztreonam, fusidic acid, rifamycin SV, guanidine HCl, tetrazolium violet, D-serine, minocycline, Nioproof 4, tetrazolium blue, potassium tellurite, sodium bromate
Negative tests with BIOLOG	The strains were unable to oxidize: D-raffinose, D-sorbitol, gelatin, pectin, p-hydroxy-phenylacetic acid, dextrin, alfa-D-lactose, glycy-L-proline, D-maltose, D-melibiose, D-lactic acid methyl ester, alfa-hydroxy butyric acid, beta-methyl-D-glucoside, myo-inositol, D-cellobiose, D-salicin, 3-methyl glucose, alfa-keto-butyric acid, gentiobiose, D-glucose-6-PO4, acetoacetic acid, sucrose, N-acetyl-beta-D-mannosamine, L-fucose, D-fructose-6-PO4, turanose, N-acetyl-D-galactosamine, L-rhamnose, D-aspartic acid, stachyose, N-acetyl-neuraminic acid
Variable tests with BIOLOG	D-malic acid, D-serine, bromo-succinic acid, formic acid and sodium butyrate
Positive tests with API	The assimilation of glucose, arabinose, mannose, mannitol, gluconate, caprate, malate and citrate
Negative tests with API (APIN)	Reduction of nitrate to nitrite and nitrite to N ₂ , glucose fermentation, the urease, the beta-galactosidase and the assimilation of maltose and adipate
Variable tests with API (APIV)	Indole production, presence of arginine dihydrolase, hydrolysis of aesculin and gelatin, N-acetyl-D-glucosamine and phenylacetate
Commercial kits used?	BIOLOG GENIII, API 20NE
Energy metabolism	Chemoorganotroph
Oxidase	Positive
Catalase	positive
Negative tests	Pigment on King A agar
Major fatty acids	C _{16:0} (36.09–35.97%), Sum In Feature 3 (C _{16:1} ω7c and/or C _{16:1} ω6c, 30.66–29.86%), Sum In Feature 8 (C _{18:1} ω6c and/or C _{18:1} ω7c, 10.53–9.87%), C _{17:0} cyclo (7.48–6.40%), C _{12:0} 2OH (5.36–4.85%), C _{12:0} 3OH (4.28–4.20%), C _{10:0} 3OH (4.16–3.58%) and C _{12:0} (2.26–1.70%)
Biosafety level	1
Habitat	fish farm (http://purl.obolibrary.org/obo/ENVO_00000294)
Biotic relationship	free-living
Known pathogenicity	none

Table 3
Cellular fatty composition (%) derived from FAME analysis of *Pseudomonas anatoliensis*, *Pseudomonas iridis* and the type strains of closely related *Pseudomonas* species.

Fatty acids (%)	1	2	3	4	5	6	7
C _{10:0} 3OH	4.43	3.69	4.16	3.58	3.63	2.4	4.3
C _{12:0}	2.16	2.08	1.70	2.26	2.09	2.0	3.8
C _{12:0} 2OH	5.06	5.20	5.36	4.85	4.97	4.9	6.4
C _{12:0} 3OH	4.16	4.21	4.28	4.20	3.77	2.9	4.9
C _{14:0}	TR	TR	TR	TR	TR	TR	TR
Sum In Feature 3	38.64	36.81	29.86	30.66	34.70	32.9	23.8
C _{16:0}	35.16	36.91	35.97	36.09	37.50	31.9	27.9
C _{17:0} cyclo	TR	2.16	7.48	6.40	3.42	5.1	16.0
Sum In Feature 8	8.54	7.59	9.87	10.53	8.62	15.6	11.4
C _{18:0}	TR	TR	TR	TR	TR	TR	-

All strains were cultured on tryptone soy agar (TSA) and incubated at 28 °C for 48 h. Strains: *P. anatoliensis* (1. P9^T and 2. P141); *P. iridis* (3. P42^T and 4. CCUG 67011), 5. *P. baetica* CECT 7720^T; 6. *P. helmanticensis* CCUG 51519^T and 7. *P. koreensis* LMG 21316^T. Summed feature represents a set of more than one CFA that could not be resolved: summed feature 3 contained C_{16:1} ω7c and/or C_{16:1} ω6c and summed feature 8 contained C_{18:1} ω6c and/or C_{18:1} ω7c; -, not detectable; TR, trace amount (<1%).

Fig. 4. Venn diagram of the common and differential genes shared by *P. anatoliensis* and *P. iridis* strains.

posed, with P9^T as a type strain (=CCUG 74755^T = and CECT 3172^T). The isolates P1, P2, P10, P27, P30, P24a, P42^T, P117, P139, P141, P152 and CCUG 67011 represent another novel species for which the name *Pseudomonas iridis* sp. nov. is proposed, with P42^T as a type strain (=CCUG 74870^T = and CECT 3174^T). These species are predominantly found in aquatic habitats.

Nucleotide sequence accession numbers: The GenBank/EMBL/ DDBJ accession numbers of the nucleotide sequences reported in this study are as follows: LR797545, LR797549 and LR797586 (strains *Pseudomonas anatoliensis* P9^T, P25 and P141) and LR797540-LR797541, LR797546, LR797548, LR797550-LR797551, LR797554-LR797555, LR797585 and LR797588 (strains *Pseudomonas iridis* P1, P2, P10, P24a, P27, P30, P42^T, P117, P139 and P152) for the 16S rRNA gene; LR797676, LR797679 and LR797691 (strains *Pseudomonas anatoliensis* P9^T, P25 and P141) and LR797674-LR797675, LR797677- LR797678, LR797680-LR797682, LR797689-LR797690 and LR797692 (strains *Pseudomonas iridis* P1, P2, P10, P24a, P27, P30, P42^T, P117, P139 and P152) for the *gyrB* gene; LR797698, LR797701 and LR797713 (strains *Pseudomonas anatoliensis* P9^T, P25 and P141) and LR797696-LR797697, LR797699-LR797700, LR797702-LR797704, LR797711-LR797712 and LR797714 (strains *Pseudomonas iridis* P1, P2, P10, P24a, P27, P30, P42^T, P117, P139 and P152) for the *rpoB* gene; LR797612, LR797616 and LR797653 (strains *Pseudomonas anatoliensis* P9^T, P25 and P141) and LR797607-LR797608, LR797613, LR797615, LR797617-LR797618, LR797621, LR797642, LR797652 and LR797655 strains *Pseudomonas iridis* P1, P2, P10, P24a, P27, P30, P42^T, P117, P139 and P152) for the *rpoD* gene.

The accession numbers for the genome sequences of strains P9^T and P42^T, P141 and CCUG67011 are: JACOPT000000000,

Fig. 5. (A) Phylogenetic maximum likelihood tree based on the nucleotide core proteome of the genomes under study. Bar indicates sequence divergence. (B) UPGMA dendrogram of the orthologous protein coding genes among the strains under study. Scale bar indicates distance measurement.

JACOPU000000000, JACVHC000000000, and JACXXO000000000 respectively. The version described in this paper is the first version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.syapm.2021.126198>.

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