The diversity of *Pseudomonas* species isolated from fish farms in Turkey

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A B S T R A C T

The prevalence of *Pseudomonas* species in freshwater fish and water from aquaculture farms in Turkey was determined on a monthly basis, in the period covering 2013–2017 and two seasons of 2018. The farms included in the study were located in six different regions of Turkey. A total of 90 *Pseudomonas* strains were isolated from water and diseased and healthy fish, and were classified under 20 species. The phenotypic characterization of the strains was based on oxidase and catalase activities, haemolysis, tolerance to temperature and sodium chloride, the production of fluorescent pigments and antibiotic resistance spectra. The phylogenetic identification of the 90 isolates was performed by a 4-gene multilocus sequence analysis, based on the partial sequencing of the 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes. This is the first report on the isolation of several *Pseudomonas* species, namely, *P. brenneri, P. defensor, P. haemolytica, P. lactis, P. lundensis, P. lurida, P. mandelii, P. meridiana, P. migulae, P. proteolytica, P. simiae,* and *P. weihenstephanensis,* from freshwater salmonid fish. Two of these species*, P. haemolytica* and *P. lactis* have been isolated for the first time from fish farms, an environment quite different from their original isolation source, raw milk. Furthermore, seven putative new *Pseudomonas* species were isolated from water and farmed rainbow trout. During the 2013 to 2018 period, several *Pseudomonas* species were detected to have spread from the Aegean and Central Anatolia regions to the Eastern Anatolia and Black Sea regions.

1. Introduction

A large number of *Pseudomonas* species are pathogenic for humans, animals and aquatic organisms (Nixon et al., 2001)). *Pseudomonas* species have been described as one of the most common bacterial infectious agents of cultured fish and have been reported to cause stress-related diseases in freshwater fish, especially under farming conditions (López et al., 2012; Derome et al., 2016). Several studies have reported almost 100% mortality due to infection with *Pseudomonas* spp. in rainbow trout, sea bream, sea bass and ayu in farm settings (López et al., 2012; Pridgeon, 2012; Thomas et al., 2014; Derome et al., 2016). Although *Pseudomonas* spp. have been described as opportunistic pathogens, many species have also been identified as the primary pathogen of several diseases in farmed fish, including *P. aeruginosa, P. anguilliseptica, P. baetica, P. chlororaphis, P. fluorescens, P. koreensis, P. luteola, P. plecoglossicida, P. pseudoalcaligenes* and *P. putida* (Altinok et al., 2006; Altinok

et al., 2007; López et al., 2012; Thomas et al., 2014; Austin and Austin, 2016; Derome et al., 2016). Recently, a new species, named *P. tructae*, was isolated from rainbow trout kidney (Oh et al., 2019). The pathogenicity of many other species, including among others *P. asiatica, P. brenneri, P. lactis, P. lundensis, P. lurida, P. mandelii, P. meridiana, P. migulae, P. proteolytica, P. simiae* and *P. weihenstephanensis*, for farmed fish has not yet been established (Austin and Austin, 2016). In fact, noninformation on the exact cause of disease hinders taking preventative measures and using disease control agents. To date, only two formalin-inactivated vaccines have been developed against *P. anguillaseptica* and *P. plecoglossicida* (Austin and Austin, 2016). On the other hand, *Pseudomonas* species have common use as probiotic organisms in aquaculture, because they can suppress major mycological and bacterial agents, such as *Saprolegnia* and aeromonads, and probably other emerging diseases (Das et al., 2006; Liu et al., 2015).

The identification of *Pseudomonas* species, and in particular their

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differentiation from other closest genera, require the performance of multiple laboratory tests and a detailed molecular analysis (Mulet et al., 2012; Palleroni, 2015). The main phenotypic characteristics of the genus are pigment production, such as fluorescein or pyocyanin, and oxidase-positivity, but the identification of non-pigmented and/or oxidase-negative species by biochemical methods alone is difficult (Palleroni, 2015). Molecular methods used for the identification of *Pseudomonas* species include 16S rRNA sequencing for genus identification and multilocus sequence analysis (MLSA) for species affiliation (Baumann et al., 1983; Ait Tayeb et al., 2005; Mulet et al., 2009, 2012; Gomila et al., 2015).

In the present study, with an aim to determine the prevalence of *Pseudomonas* species, salmonid freshwater fish farmed in six different regions of Turkey were surveyed on a monthly basis in a period covering 2013–2017 and two seasons of 2018. Bacteriological examinations were performed on both seemingly healthy fish and symptomatic or moribund fish. In total, 90 *Pseudomonas* strains were isolated and taxonomically characterized by extensive phenotypic analyses and a multilocus sequencing approach, based on the analyses of partial sequences of the 16S rRNA, *gyrB*, *rpoB* and *rpoD* genes. To our knowledge, this the first time that *P. brenneri, P. haemolytica, P. lactis, P. lundensis, P. lurida, P. mandelii, P. meridiana, P. migulae, P. proteolytica, P. simiae,* and *P. weihenstephanensis* have been recovered from freshwater salmonid fish. In addition, strains of at least seven putative novel species were isolated and taxonomically characterized using a polyphasic approach. This study provides an update of the known diversity, abundance and specific habitat (farm) distribution of *Pseudomonas* species.

2. Material and methods

2.1. Bacterial isolation

Aquaculture farms with high production capacities, using spring water, stream water and dammed lake water, and located in six different regions of Turkey (Marmara, Aegean, Central Anatolia, Black Sea, Mediterranean, and Eastern Anatolia), were included in this study. Sampling was done mainly from rainbow trouts (*Oncorhynchus mykiss*, Walbaum, 1792) and from other trout species, including the Black sea trout, Brook trout, and Brown trout (*Salmo trutta labrax,* Pallas, 1814; *Salvelinus fontinalis,* Mitchill, 1814; and *Salmo trutta magrostigma,* Dumeril, 1858; respectively). Samples were taken from fish, eggs or milt in the spawning period, and also from alevins. As part of a health surveillance study, 100 rainbow trouts, which were either subclinically infected, clinically infected or moribund, were randomly collected on a monthly basis, during a period covering 2013–2017 and two seasons in 2018. Fish farms located in the Black Sea region produce rainbow trout and local trout species. The local trout species (*Salmo trutta* group) farmed in the Black Sea region were sampled twice a year, during the period from 2013 to 2017, such that a total of 1000 *Salmo trutta* were sampled throughout the surveillance period.

More than four thousand fish were analysed during the five-year surveillance period (2013–2017). Bacteria were isolated from both seemingly healthy fish (based on farmer observations, and external and internal examinations) and from diseased fish, either symptomatic or moribund. In farms with only seemingly healthy fish, sampling involved fish of all weights, from egg to brood stock, regardless of their health situation and the farmers' suggestions.

Furthermore, eggs or milt were also sampled in the spawning period, which covered the period from November to March in breeding stocks farmed under a natural light-dark regime and also the summer season in stocks exposed to photoperiod manipulations.

Alevin or yolk-sac larvae were sampled twice a year, such that a hundred were sampled each time. From 2013 to 2017, a total of 200 fish (alevin or yolk-sac larvae) were sampled each year. The samples were collected aseptically from the yolk-sac of alevin or larvae with a sterile loop. When the yolk-sac was not apparent in small fish weighing around

0.5 g, samples were taken from the surface and abdominal cavity.

Fish were classified according to health status, region, cohort, weight, and month. Samples from diseased fish were taken aseptically with sterile loops and swabs from skin lesions, and the liver, kidney, spleen, yolk sac, ovary and eggs. Samples were plated on tryptic soy agar (Merck, 105,458, TSA), and blood agar (BA; with 5% sheep blood). In the event of the occurrence of mass fish death on a farm, in addition to the fish samples, water samples were also collected from pond inlets, outlets, and benthic zones to monitor the dissemination of the agents. Water samples were taken from fish farms three times (from ten different ponds) in 2014, twice (from six different ponds and also one benthic zone) in 2015, and three times (from six different ponds) in 2016 and 2017. In total, 60 pond water samples were analysed for the detection of fish pathogens from 2014 to 2017. In 2018, the sampling in Sivas was repeated in April, due to a disease case, and another health control sampling was performed in December in Mugla.

According to the health surveillance program, fish samples were also used for investigating the presence of *Aeromonas* and *Flavobacterium* species. Isolation and identification were performed by following the protocols described by Loch et al. (2013) for *Flavobacterium* spp. Special attention was paid to the hatcheries, where these genera had been detected previously (Duman et al., 2017, 2018; Saticioglu et al., 2018).

Samplings were carried out according to the guidelines for the diagnosis of fish diseases and in compliance with the international guidelines for animal welfare and guidelines for aquatic animal health surveillance (OIE-Office International Des E, 2000; Austin and Newaj-Fyzul, 2017). Water samples were collected into sterile bottles and transported to the laboratory on ice. Samples were serially diluted and concentrated on nitrocellulose membrane filters (0.45-μm pore size; Millipore) by passing 100 ml of each dilution through the filter using the membrane filtration technique. The filters were then placed onto Pseudomonas F agar plates (Merck, 110,989). The spread plate technique was also employed by spreading 100 μl of pond water samples onto tryptic soy agar (TSA), blood agar (BA) and Pseudomonas F agar, and bacterial colonies were selected by fluorescent pigment production, morphology, and phenotypical tests (Khan et al., 2010; Palleroni, 2015).

All isolates were sub-cultured on Pseudomonas F medium for two days at 28 °C to ensure purity. Furthermore, all isolates were cultured on tryptic soy broth (TSB) (Merck, 105,459) at 28 °C for 24-48 h, and pure cultures were supplemented with 20% glycerol and kept at −80 °C for long-term storage. The strains used in this study, and their origins are listed in Table 1.

2.2. Physiological and biochemical characteristics

The biochemical characteristics of the isolates were determined using conventional microbial tests, including the assessment of colony morphology, Gram staining, oxidase and catalase activities, growth on different media, and to different temperature conditions (Palleroni, 2015; Austin and Austin, 2016). Bacterial motility was observed by the hanging drop technique and checked on sulfide-indole-motility (SIM) medium (Merck, 1,054,700,500) (Austin and Austin, 2016). The pellicle formation ability of the *Pseudomonas* isolates was detected as described by Palleroni (2015), and haemolytic characteristics were assessed by plating on blood agar containing 5% sheep blood. Growth temperatures (4, 25, 37, 42, and 45 °C) were determined in TSB medium, and growth in the presence of NaCl (0–10% *w/v*) was tested in TSB medium containing different concentrations of NaCl. Proteinase production (hydrolysis) was tested by plating on nutrient agar containing 2.5% skimmed milk for 72–96 h at 28 °C. Proteolytic activity was estimated based on the size of the clear zone around the colony (Vazquez et al., 1995). The bacteria were cultured routinely on Pseudomonas F agar medium at 28 °C. The production of fluorescent pigments was tested on King B medium (Lamichhane and Varvaro, 2013). Plates were viewed on an ultraviolet (UV) transilluminator, and luminescence was determined for each culture at a wavelength of 360 nm. Luminescence strength was

Table 1
Strains used in this study and their origins.

Strains	Fish species	Fish weight (g)	Isolation year	Isolation month	Town	City	Region	Fish health status*	Accompanying species
P1	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P2	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P3a	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P3b	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P5	Oncorhynchus mykiss	0.4	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P7	Oncorhynchus mykiss	0.4	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P9	Oncorhynchus mykiss	0.5	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P10	Oncorhynchus mykiss	0.5	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P11	water	water	2014	December	Uzunyayla	Kayseri	Central Anatolia		
P16	Oncorhynchus mykiss	30-40	2014	December	Uzunyayla	Kayseri	Central Anatolia		
P21	Oncorhynchus mykiss	10	2013	November	Ören	Mugla	Aegean		
P24-1	Oncorhynchus mykiss	0.5	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P24-2	Oncorhynchus mykiss	0.5	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P25	Oncorhynchus mykiss	0.5	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P27	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P29	Oncorhynchus mykiss	0.4	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P30	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P31	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia		
P34	Oncorhynchus mykiss	10	2013	December	Uzunyayla	Kayseri	Central Anatolia	Dorsal lesion	
P42	Oncorhynchus mykiss	0.1-0.5	2014	March	Uzunyayla	Kayseri	Central Anatolia		Flavobacterium psychrophilum[b]/ branchiarum[a]
P43	Oncorhynchus mykiss	0.5-1	2014	March	Uzunyayla	Kayseri	Central Anatolia		Flavobacterium psychrophilum[a]/ branchiarum[a]
P44	Oncorhynchus mykiss	3-5	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P45	Oncorhynchus mykiss	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P46	Oncorhynchus mykiss	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P47	Oncorhynchus mykiss	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P48	Oncorhynchus mykiss	20	2013	July	Su Şehri	Sivas	Central Anatolia	Dorsal lesion	
P49	Oncorhynchus mykiss	0.2-0.5	2013	June	Uzunyayla	Kayseri	Central Anatolia	Dorsal lesion	
P50	Oncorhynchus mykiss	0.2-0.5	2013	June	Uzunyayla	Kayseri	Central Anatolia	Dorsal lesion	
P51	Oncorhynchus mykiss	3000	2013	July	Su Şehri	Sivas	Central Anatolia	Dorsal lesion	
P52	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P54	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P55	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P56	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P57	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P58	Oncorhynchus mykiss	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P59		3000	2013	July	Uzunyayla	Kayseri		Moribund fish	

Table 1 (*continued*)

Strains	Fish species	Fish weight (g)	Isolation year	Isolation month	Town	City	Region	Fish health status*	Accompanying species
	Oncorhynchus mykiss						Central Anatolia		
P66	*Oncorhynchus mykiss*	0.3	2014	February	Ören	Mugla	Aegean		
P67	*Oncorhynchus mykiss*	0.3	2014	February	Ören	Mugla	Aegean		
P68b	*Oncorhynchus mykiss*	0.3	2014	February	Ören	Mugla	Aegean		
P72	water	water	2014	January	Su Şehri	sivas	Central Anatolia		
P73	water	water	2014	January	Uzunyayla	Kayseri	Central Anatolia		
P91	*Oncorhynchus mykiss*	3-5	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P93	*Oncorhynchus mykiss*	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P94	*Oncorhynchus mykiss*	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P95	*Oncorhynchus mykiss*	8	2013	July	Uzunyayla	Kayseri	Central Anatolia		
P97	*Oncorhynchus mykiss*	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P99	*Oncorhynchus mykiss*	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P100	*Oncorhynchus mykiss*	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P101	*Oncorhynchus mykiss*	3000	2013	July	Uzunyayla	Kayseri	Central Anatolia	Moribund fish	
P102	*Oncorhynchus mykiss*	20	2013	July	Su Şehri	Sivas	Central Anatolia	Dorsal lesion	
P106	*Oncorhynchus mykiss*	20	2013	July	Su Şehri	Sivas	Central Anatolia	Dorsal lesion	
P109	*Oncorhynchus mykiss*	60-70	2013	December	Ören	Mugla	Aegean		
P114	*Oncorhynchus mykiss*	0.5-1	2018	December	Ören	Mugla	Aegean		
P115	*Oncorhynchus mykiss*	0.5-1	2018	April	Su Şehri	Sivas	Central Anatolia	Loss of appetite, anemia, dorsal fin erosion	
P116	*Oncorhynchus mykiss*	0.5-1	2018	April	Su Şehri	Sivas	Central Anatolia	Loss of appetite, anemia, dorsal fin erosion	
P117	*Oncorhynchus mykiss*	0.5-1	2018	April	Su Şehri	Sivas	Central Anatolia	Loss of appetite, anemia, dorsal fin erosion	
P121	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean		*Flavobacterium frigidimaris*[a]
P122	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean		*Flavobacterium frigidimaris*[a]
P123	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean		*Flavobacterium frigidimaris*[a]
P124	*Oncorhynchus mykiss*	egg	2017	January	Ören	Mugla	Aegean		*Flavobacterium oncorhynchi*[a]*/glaciei*[a]
P131	*Oncorhynchus mykiss*	15-20	2017	January	Dalaman	Mugla	Aegean		
P132	*Oncorhynchus mykiss*	0.2-0.3	2017	January	Dalaman	Mugla	Aegean		*Flavobacterium psychrophilum*[b]*/ branchiarum*[a]
P135	*Oncorhynchus mykiss*	0.1	2017	January	Dalaman	Mugla	Aegean		*Flavobacterium tructae*[a]*/ tiangeerense*[a]
P137	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean		*Flavobacterium* spp.[a]
P138	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean	High mortality	*Flavobacterium* spp.[a]
P139	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean	High mortality	*Flavobacterium* spp.[a]
P141	*Oncorhynchus mykiss*	0.1	2017	January	Ören	Mugla	Aegean	High mortality	*Flavobacterium* spp.[a]
P147	*Oncorhynchus mykiss*	0.1	2017	January	Uzunyayla	Kayseri	Central Anatolia		*Flavobacterium* spp.[a]
P152	*Oncorhynchus mykiss*	yolk sac	2017	January	Uzunyayla	Kayseri	Central Anatolia		
P154a	*Oncorhynchus mykiss*	0.1	2017	January	Çağlayan	Rize	Black Sea region		*Flavobacterium collinsii*[a]
P154b	*Oncorhynchus mykiss*	0.1	2017	January	Çağlayan	Rize	Black Sea region		*Flavobacterium collinsii*[a]
P155	*Oncorhynchus mykiss*	0.1	2017	January	Çağlayan	Rize	Black Sea region	Abnormal swiming	*Flavobacterium collinsii*[a]

Table 1 (*continued*)

Strains	Fish species	Fish weight (g)	Isolation year	Isolation month	Town	City	Region	Fish health status*	Accompanying species
P156	*Salvelinus fontinalis*	egg	2017	January	Çağlayan	Rize	Black Sea region		*Flavobacterium collinsii[a]*
P158	*Oncorhynchus mykiss*	0.1	2017	January	Keban	Elazığ	Eastern region		
P162	*Oncorhynchus mykiss*	yolk-sac	2017	January	Keban	Elazıg	Eastern region		
P163	*Salmo trutta magrostigma*	yolk-sac	2017	January	Çamlıhemşin	Rize	Black Sea region		*Flavobacterium collinsii[a]*
P164	*Salmo trutta magrostigma*	egg	2017	January	Çamlıhemşin	Rize	Black Sea region		
P166	*Salvelinus. fontinalis*	0.2	2017	January	Çamlıhemşin	Rize	Black Sea region		
P167	*Salmo trutta labrax*	0.2	2017	January	Çamlıhemşin	Rize	Black Sea region		*Flavobacterium piscis[a]*
P168	*Salvelinus fontinalis*	1500	2017	January	Çamlıhemşin	Rize	Black Sea region		*Flavobacterium psychrophilum[b]/ aquidurense[a]*
P169	*Oncorhynchus mykiss*	egg	2017	January	Çağlayan	Rize	Black Sea region		*Flavobacterium hydatis[a]*
V3	DSM 7189	soil	1992	July	California	ABD			
V8	DSM 50090	pre-filter tanks	1990	August	Northern Ireland	United Kingdom			
V34	*Oncorhynchus mykiss*	200	2013	July	Uzunyayla	Kayseri	Central Anatolia	Darkening in color and exopthalmia	
V35	*Oncorhynchus mykiss*	200	2013	July	Uzunyayla	Kayseri	Central Anatolia	Darkening in color and exopthalmia	
V36	*Oncorhynchus mykiss*	200	2013	July	Uzunyayla	Kayseri	Central Anatolia	Darkening in color and exopthalmia	
V37	*Oncorhynchus mykiss*	200	2013	July	Uzunyayla	Kayseri	Central Anatolia	Darkening in color and exopthalmia	
V38	*Oncorhynchus mykiss*	200	2013	July	Uzunyayla	Kayseri	Central Anatolia	Darkening in color and exopthalmia	
V42	*Oncorhynchus mykiss*	200	2013	June	Su Şehri	Sivas	Central Anatolia	Darkening in color and exopthalmia	*Aeromonas media (MG310171)[c]*
V43	*Oncorhynchus mykiss*	200	2013	June	Su Şehri	Sivas	Central Anatolia	Darkening in color and exopthalmia	*Aeromonas media (MG310171)[c]*
V45	*Oncorhynchus mykiss*	200	2013	June	Su Şehri	Sivas	Central Anatolia	Darkening in color and exopthalmia	*Aeromonas media (MG310171)[c]*
V81	*Oncorhynchus mykiss*	200	2013	July	Su Şehri	Sivas	Central Anatolia	Darkening in color and exopthalmia	*Aeromonas media (MG310171)[c]*

* If not indicated in the fish health status column means that the fish were subclinically infected with loss of appetite, darkening in color or slow movement.a: Indicates data from this study; b, corresponds to reference Saticioglu et al. 2018; and c, to reference Duman et al., 2018. V3, *P. putida* DSM 12535 and V8, *P. putida* DSM 50090 are bacterial controls.

calculated according to the intensity of bacterial fluorescence, ranging from poor (+) to very rich (++++), and compared among the bacteria by UV transillumination as described by Lamichhane and Varvaro (2013). The reference strains *Pseudomonas fluorescens* DSM 50090 and *Pseudomonas putida* DSM 12735 were used as controls to validate the biochemical tests.

The bacteria were also tested for survival in sterile distilled water and sterile tap water alone. Distilled water (conductivity, 3mS/cm) and tap water (conductivity, 343 mS/cm) were sterilized in an autoclave and distributed into 24 well-plates. Bacterial suspensions prepared from pure and fresh cultures were adjusted to 0.5 McFarland turbidity, and 100-µl aliquots of the bacterial suspensions were added to the plate wells. After the plates were incubated at 28 °C for 24 and 48 h, optical density was measured at a wavelength of 595 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA), and survival was defined in comparison to the control sample (without bacteria) (CLSI, 2017).

2.3. Antibiotic resistance

Resistance to antibiotics of different groups was determined using the disc diffusion method by complying with the performance standards for the antimicrobial susceptibility testing of bacteria isolated from aquatic animals (Clinical and Laboratory Standards Institute, CLSI guidelines M42-A) (CLSI - Clinical and Laboratory Standards Institute,

2006). The antibiotics used in the susceptibility tests included sulfon-amides (trimethoprim/sulfamethoxazole, SXT, 1/19), aminopenicillins (amoxicillin, AML), tetracyclines (doxycycline, DO; tetracycline, TE; oxytetracycline, OT), fluoroquinolones (enrofloxacin, ENR; ciprofloxa-cin, CIP; flumequine, UB), aminoglycosides (gentamicin, CN), penicil-lins (ampicillin, AMP), macrolides (erythromycin, E), clindamycin (lincomycin, MY), and chloramphenicol (florfenicol, FFC). Antibiotic discs were placed on Mueller-Hinton agar (MHA) plates previously inoculated with a pure culture of the strains. After incubation at 28 °C (24–28 h), inhibition zones were measured in mm and compared with the critical values of the European Committee on Antimicrobial Sus-ceptibility Testing (EUCAST-European Committee on Antimicrobial Susceptibility Testing, 2020, www.eucast.org) to evaluate whether a strain was sensitive or resistant. *E. coli* ATCC 25922 was used as the quality control (QC) strain, and the acceptable ranges set by the CLSI for this strain were applied (CLSI - Clinical and Laboratory Standards Institute, 2014).

2.4. DNA extraction, PCR amplification, and DNA sequencing conditions

The genomic DNA of the bacterial isolates was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNA samples were optimized according to concentration and purity before molecular analysis. PCR amplifica-tion, primer selection, purification of the amplified products, DNA

sequencing, and the sequence analysis procedures were performed according to previous descriptions (Mulet et al., 2017).

2.5. Phylogenetic analysis

Individual trees were generated from the isolates, and multilocus sequence analyses (MLSA) were carried out based on the concatenated partial sequences of the 16S rRNA (1263 nt), *gyr*B (RNA gyrase subunit B, 810 nt), *rpo*D (RNA polymerase subunit D, 694 nt), and *rpo*B (RNA polymerase beta subunit, 923 nt) genes, totalling 3690 nt (Mulet et al., 2017). The EZ Bio Cloud Database, https://www.ezbiocloud.net/, was used to affiliate the 16S rRNA sequences to the genus *Pseudomonas* (Yoon et al., 2017). The Jukes-Cantor (JC), maximum likelihood (ML) (Jukes and Cantor, 1969; Felsenstein, 1981), and maximum parsimony (MP) (Nei and Kumar, 2000) algorithms were used for comparisons.

2.6. Statistical analysis

Analyses of variance, standard deviation calculations and regression analyses were performed using the Big Data analysis program with conventional standard deviation analysis (Analytics, 2013). Multiple regression analysis was used to analyse the results in relation to *Pseudomonas* species count and species score, the number of cases associated with the organ of isolation, water temperature, fish length, month of isolation and mixed isolation status. Graphics of the statistical results were constructed with Excel from the Tableau database (Analytics, 2013). Isolates, for which information was incomplete (*i.e.* isolation date, water temperature), were not included in the statistical analysis.

2.7. Ethical statements

This research was supported by the Scientific and Technological Research Council of Turkey and approved by the Local Ethics Commission (report 2012-14-04).

3. Results

3.1. Bacterial isolation

A total of 90 *Pseudomonas* strains were isolated, including 81 from rainbow trouts (*Onchorhynchus mykiss*, OM), three from pond water, three from brook trouts (*Salvelinus fontinalis,* SF), two from brown trouts (*Salmo trutta macrostigma,* STM) and one from the Black sea trout (*Salmo trutta labrax,* STL). Origins varied from egg to brood stock fish (3000 g) or yolk-sac and pond water. Most strains were recovered from fish of a weight below 10 g (Table 1, supplementary Fig. S1). Thirty-eight percent of the fish presented with disease symptoms or lesions, such as anaemia, darkening in colour, dorsal fin erosion, exophthalmia, high mortality, loss of appetite, and abnormal swimming, and some fish were moribund as indicated in Table 1. *Pseudomonas* spp. were isolated in four different regions of Turkey and were detected at higher rates in the Central Anatolia and Aegean regions. No isolates were recovered from the samples taken in the Marmara and Mediterranean regions. The distribution of the isolates is presented in Fig. 1 (and in the following interactive link together with GPS data and information on farm location, *Pseudomonas* species, town, province and region https://www.google.com/maps/d/viewer?hl=en&mid=1lE1b5mFWsZeKwNwpAwj4ItdO3FUz2rWd&ll=39.54525767883057%2C35.11440467765806&z=5). The numbers of the *Pseudomonas* species identified and their distribution by provinces are shown in Fig. 2.

In 56 fish without any symptoms, 23 *Flavobacterium* strains were isolated together with *Pseudomonas* strains. The *Flavobacterium* species identified include *F. aquidurense, F. branchiarum, F. collinsii, F. frigidimaris, F. glaciei, F. hydatis, F. oncorhynchi, F. piscis, F. psychrophilum,*

Fig. 1. Map showing the distribution of *Pseudomonas* species in fish farms in Turkey.

Fig. 2. Distribution and number of *Pseudomonas* species in fish farms in Turkey.

F. tiangeerense and *F. tructae*. In six cases, more than one species of *Flavobacterium* were recovered. From the samples taken from diseased fish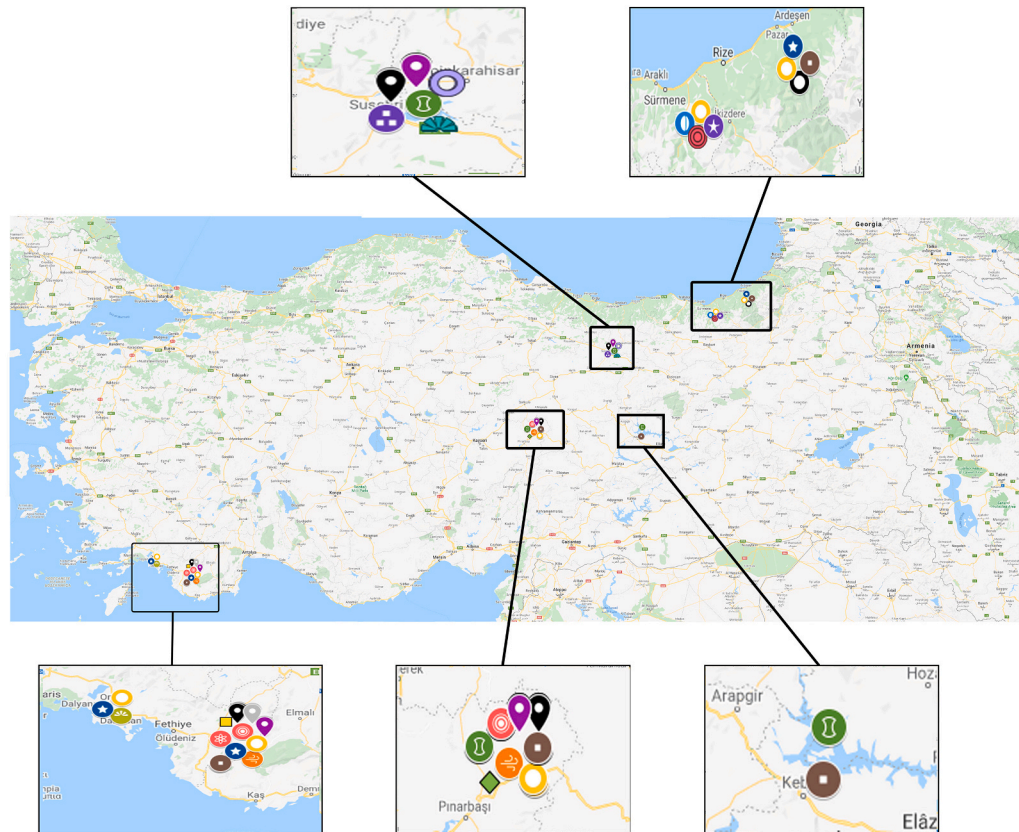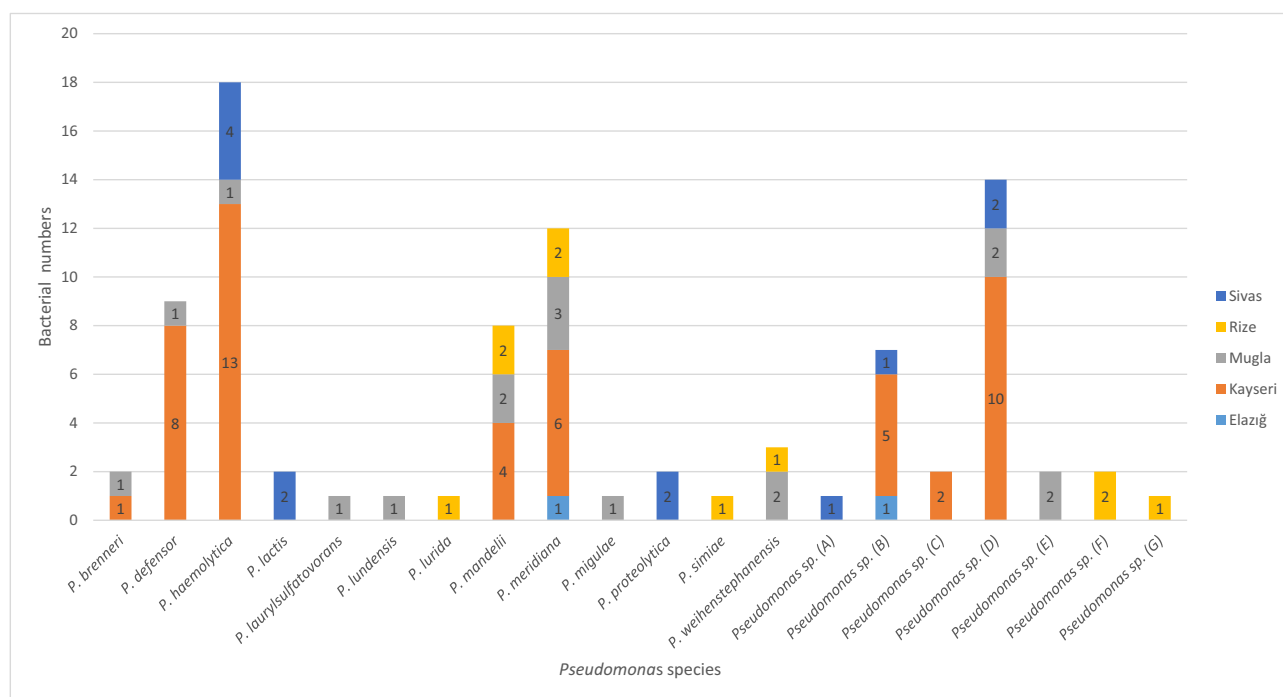, apart from 34 *Pseudomonas* strains, 8 strains of motile *Aeromonas* and *Flavobacterium* were recovered and identified as *Aeromonas media, F. collinsii,* and *Flavobacterium* spp. (Table 1).

3.2. Phylogenetic identification

The phylogenetic assignation of the *Pseudomonas* strains at species level is summarized in Fig. 3. An initial analysis of the 16S rRNA sequences affiliated the isolates to the genus *Pseudomonas*, as shown in Supplementary Table 1, but the low species differentiation power of this gene did not allow identification at species level. Eighty-eight of the 90 strains belonged to the *Pseudomonas fluorescens* group and its six subgroups: *P. fluorescens* SG (39 strains), *P. gessardii* SG (16 strains), *P. fragi* SG (4 strains), *P. mandellii* SG (13 strains), *P. koreensis* SG (15 strains) and *P. jessenii* SG (1 strain). Only two strains were classified under the *P. putida* group (*Pseudomonas* sp. E). For a precise identification at species level, the *rpoD* gene sequences were analysed for all strains. At a cutoff value of 95%, 61 strains were identified at species level and affiliated to 14 different *Pseudomonas* species, the most prevalent of which were *P. haemolytica* (18 strains), *P. meridiana* (12 strains), *P. defensor* (9 strains) and *P. mandelii* (8 strains). The remaining species were represented by 1 to 3 strains, as shown in Table 2, supplementary Table 1, and Fig. 3, and are detailed in supplementary Figs. 2 and 3.

To further phylogenetically characterize the remaining 29 strains (32%), a MLSA based on the concatenated sequences of the *gyrB, rpoB, rpoD* and 16S rRNA genes was performed. The results are shown in Fig. 3, supplementary Fig. 3 and supplementary Table 1. The cut-off value to assign a strain to a species is 97% in the 4-gene MLSA. Seven presumptive new species, previously not described, could be delineated and were labelled with capital letters as follows: *Pseudomonas* sp. (A), 1 strain; *Pseudomonas* sp. (B), 7 strains; *Pseudomonas* sp. (C), 2 strains; *Pseudomonas* sp. (D), 14 strains; *Pseudomonas* sp. (E), 2 strains; *Pseudomonas* sp. (F), 2 strains and *Pseudomonas* sp. (G), 1 strain (supplementary Table 1). *Pseudomonas* sp. (F) and *Pseudomonas* sp. (G) were 97.1% similar to known species type strains, but could not be assigned to any of them in the phylogenetic tree. *Pseudomonas* sp. (B) and (D) were isolated

most frequently in the Kayseri province, and *P. meridiana* and *Pseudomonas* sp. (B) were isolated in Elazig. Supplementary Table 2 shows the GenBank nucleotide accession numbers of the sequences used in this study. The accession numbers indicated in bold are for the sequences determined in this study.

3.3. Physiological and biochemical characterization

All strains shared the essential phenotypic characteristics of members of the genus *Pseudomonas*. They were Gram-negative, motile, rod-shaped, oxidase- and catalase-positive, non-fermentative bacteria with a strictly oxidative metabolism, and 84% of them produced diffusible fluorescent pigments. These were the primary strain selection criteria for this study, and the 90 strains underwent detailed phenotypic characterization to assess their fitness and probable role in the aquaculture setting. Results are shown in Table 2. All strains were able to grow at NaCl concentrations of 0–4.5%, but most of them did not tolerate higher concentrations of NaCl, such that 20% of the strains did not grow on 6% NaCl, 61% did not grow on 7% NaCl, 73% did not grow on 8% NaCl and 91% did not grow on 9% NaCl. The only species that tolerated 9% NaCl were *P. haemolytica* (P45, P91 and P93), *P. lundensis* (P131), *P. proteolytica* (P102), *P. weihenstephanensis* (P132), and *Pseudomonas* sp. (D) (P10 and P42).

In order to determine the potential environmental and public health risks posed by the isolates, their survival in sterile distilled water and tap water was tested, and all isolates were observed to survive throughout the test period. All of the isolates grew at 4 °C, 25 °C, and 37 °C with the exception of *P. defensor* (P57) and *Pseudomonas* sp. (C) (P50), which did not grow at 4 °C, and *P. defensor* (P101), *P. haemolytica* (P16, P95), *P. mandelii* (P5), *P. proteolytica* (P102), *Pseudomonas* sp. (B) (P29), and *Pseudomonas* sp. (D) (P25), which did not grow at 37 °C. *P. haemolytica* (V81) displayed the highest growth temperature, which was 45 °C. As indicated in Table 2, some strains produced a pellicle in the liquid-air interphase under different salinity and temperature conditions. The analysis of the proteolytic activity of the isolates demonstrated that 91% tested positive for this activity, as shown in Table 2. Haemolytic activity was detected in *P. haemolytica, P. lactis, P. lurida, P. meridiana* (only P167, P168, V37 and V38), *Pseudomonas* sp. (B) (excluding P7),
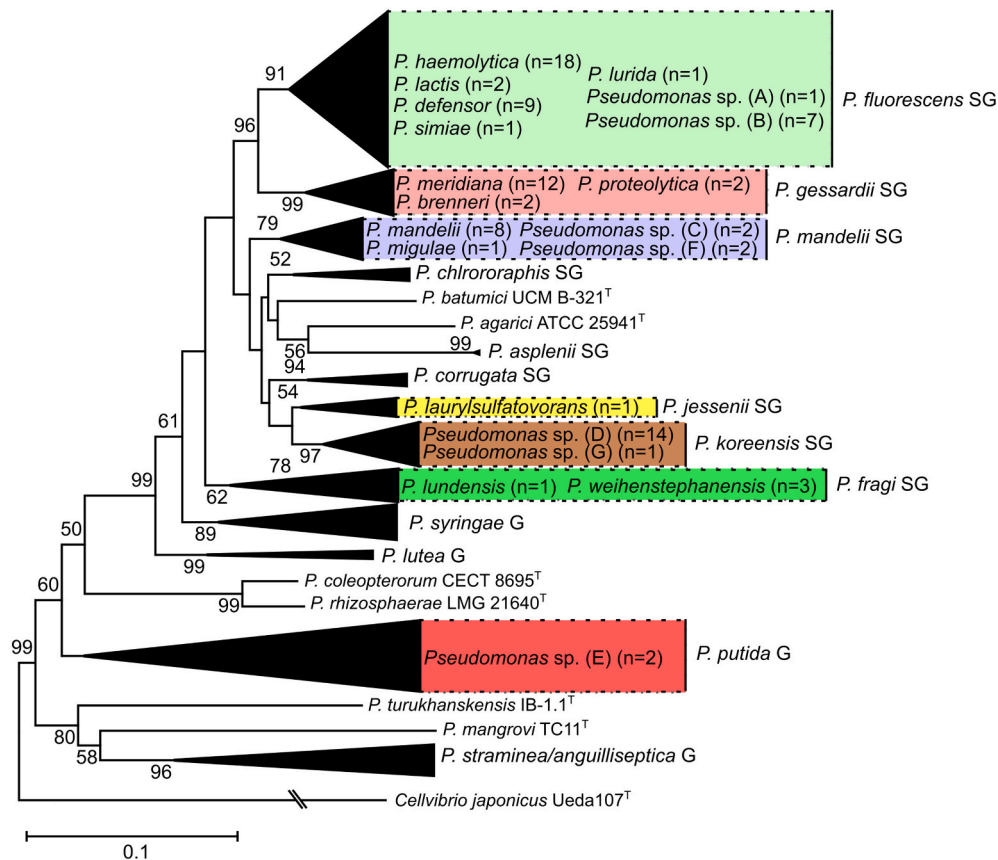
Fig. 3. Phylogenetic tree of the strains isolated in this study and *Pseudomonas* species type strains of the *Pseudomonas fluorescens* lineage, based on the partial sequences of the *rpoD* gene. Isolates from this study are highlighted in colour and "n" indicates the number of strains. *Cellvibrio japonicus* Ueda107 was used as an outgroup. The bar indicates sequence divergence. Bootstrap values higher than 50% (from 1000 replicates) are indicated at the nodes.

Pseudomonas sp. (C), *Pseudomonas* sp. (D) (P1, P24–1, P42, and P141), and *Pseudomonas* sp. (G). The remaining strains were non-haemolytic.

3.4. Antibiotic susceptibility

Antibiotic breakpoints are not available in the CLSI database for the genus *Pseudomonas,* with the exception of *P. aeruginosa*. The bacterial isolates were evaluated for their antibiotic sensitivity to nine different groups of antibiotics, but only ciprofloxacin (CIP) susceptibility values were given in the EUCAST updated breakpoint table (EUCAST-European Committee on Antimicrobial Susceptibility Testing, Version 10.0, valid from 2020 to 01-01). According to EUCAST data (2020), *Pseudomonas* spp. are susceptible if the breakpoint is higher than 50 mm, and isolates are assumed to be intermediately resistant when breakpoints are between 50 and 26 mm. Accordingly, while *P. lurida* and *Pseudomonas* sp. (E) were determined to be resistant (<26 mm) to ciprofloxacin, the remaining isolates were assumed to be intermediately resistant. All isolates were fully resistant to lincomycin (MY), and the majority were resistant to trimethoprim/sulfamethoxazole (SXT, 1/19), amoxicillin (AML), ampicillin (AMP), erythromycin (E), and florfenicol (FFC) with no growth zone (0 mm) in the disc diffusion test. Most *Pseudomonas* species showed an inhibition zone for doxycycline (DO), tetracycline (TE), oxytetracycline (OT), enrofloxacin (ENR), ciprofloxacin (CIP), flumequine (UB), and gentamicin (CN), but were not evaluated for antibiotic susceptibility or resistance. *Pseudomonas* sp. (D), (E), (F), *P. defensor*, and *P. meridiana* were the most resistant species among all identified *Pseudomonas* with no inhibition zone (0 mm) as indicated in supplementary Table S3.

3.5. Statistical results

All data were compared by using logistic regression analysis tests and were analysed by an iteratively reweighted least squares methodology. The number of strains isolated from healthy fish was 56, while 34 species were detected in fish displaying various symptoms. The number of accompanying species was 31, and in 6 asymptomatic cases, more than one species was isolated in association with *Pseudomonas* spp. The species most frequently isolated from rainbow trouts were *P. haemolytica*, *Pseudomonas* sp. (D), *P. meridiana*, *P. defensor* and *P. mandelii* (Fig. 1, http s://www.google.com/maps/d/viewer?hl=en&mid=1lE1b5mFWsZe KwNwpAwj4ItdO3FUz2rWd&ll=39.54525767883057%2C35.1144046 7765806&z=5, and Fig. 2). All species, excluding *P. simiae* and *P. lurida*, were isolated from rainbow trouts, while *Pseudomonas* sp. (B) was the only one recovered from pond water (supplementary Fig. S4). *Pseudomonas* species were isolated from different regions of Turkey, more frequently from Kayseri, Mugla and Sivas, and less frequently from Rize and Elazig (Figs. 1 and 2). Statistical analyses demonstrated a correlation between species and the month of isolation. *P. haemolytica* was frequently isolated in July, *P. meridiana* in January, and *Pseudomonas* sp. (D) in December. None of the *Pseudomonas* spp. were isolated in May, September and October. A heterogenic species distribution was detected during the 2010–2015 and 2015–2020 periods. After 2015, *Pseudomonas* were frequently detected in January, April and September (supplementary Fig. S5). Ten species were isolated from injured fish with symptoms, such as anaemia, darkening in colour, fin erosion, exophthalmia and abnormal swimming. These symptoms were mainly attributed to a possible infection with *P. haemolytica, P. meridiana* and *P. defensor* (Fig. 4, Table 1). *P. migulae* presented the highest growth temperature, and *P. proteolytica* presented with the lowest growth

Table 2
The identification, and physiological and biochemical characteristics of the strains.

Strain	Bacteria	Fluorescence	Pellicle formation	Haemolysis on blood agar	Motility	Oxidase	Growth with NaCl (%)					Survival		Growth temperature (°C)					Proteolytic activity
							[b]0-4.5	6	7	8	9	Destilled water (3µm)	Tap water (343µm)	4	25	37	42	45	
P34	P. brenneri	-	-	-	+	+	+	+	-	-	-	+	+++	±	+	+	-	-	+
P114	P. brenneri	+++	-	-	+	+	+	-	-	-	-	++	++++	+	+	+	-	-	+
P21	P. defensor	-	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P47	P. defensor	+++	+	-	+	+	+	+	+	+	-	++	++	+	+	+	-	-	+
P54	P. defensor	++	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P55	P. defensor	-	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P57	P. defensor	++	-	-	+	+	+	+	+	+	-	++	+++	-	+	+	-	-	+
P58	P. defensor	+	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P99	P. defensor	+++	-	-	+	+	+	+	+	+	-	++	++++	+	+	+	-	-	+
P100	P. defensor	+++	+	-	+	+	+	+	+	+	-	++	++++	+	+	+	-	-	+
P101	P. defensor	++	+	-	+	+	+	+	+	+	-	++	++++	+	+	-	-	-	+
P16	P. haemolytica	+	-	-	+	+	+	+	-	-	-	++	+++	+	+	-	-	-	+
P43	P. haemolytica	++++	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P44	P. haemolytica	+++	-	β	+	+	+	+	+	-	-	++	+++	+	+	+	-	-	+
P45	P. haemolytica	+++	+	β	+	+	+	+	+	+	+	++	+++	+	+	+	-	-	+
P46	P. haemolytica	+++	+	β	+	+	+	+	+	+	-	++	+++	+	+	+	-	-	+
P52	P. haemolytica	++++	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P56	P. haemolytica	+	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P59	P. haemolytica	++++	-	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P68b	P. haemolytica	++	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P91	P. haemolytica	+++	+	β	+	+	+	+	+	+	+	++	+++	+	+	+	-	-	+
P93	P. haemolytica	+++	+	β	+	+	+	+	+	+	+	++	++++	+	+	+	-	-	+
P94	P. haemolytica	+++	+	β	+	+	+	+	+	-	-	++	++++	+	+	+	-	-	+
P95	P. haemolytica	+++	+	β	+	+	+	+	+	+	-	++	++++	+	+	-	-	-	+
P97	P. haemolytica	+++	+	β	+	+	+	+	-	-	-	++	++++	+	+	+	-	-	+
V42	P. haemolytica	+	+	β	+	+	+	+	+	-	-	++	+++	+	+	+	-	-	+
V43	P. haemolytica	+	+	β	+	+	+	+	+	-	-	++	+++	+	+	+	-	-	+
V45	P. haemolytica	+	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
V81	P. haemolytica	++	+	β	+	+	+	+	+	-	-	++	+++	+	+	+	+	+	+
P48	P. lactis	+++	+	β	+	+	+	+	+	+	-	++	+++	+	+	+	-	-	+
P106	P. lactis	+++	+	β	+	+	+	+	+	+	-	++	++++	+	+	+	-	-	+
P138	P. laurylsulfatovorans	-	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P131	P. lundensis	-	-	-	+	+	+	+	+	+	+	++	+++	+	+	+	-	-	+
P163	P. lurida	++++	-	β	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P3a	P. mandelii	++	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P3b	P. mandelii	++	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P5	P. mandelii	-	-	-	+	+	+	+	-	-	-	++	+++	+	+	-	-	-	+
P31	P. mandelii	+	-	-	+	+	+	+	-	-	-	++	+++	±	+	+	+	-	+
P124	P. mandelii	-	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P135	P. mandelii	-	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	-
P164	P. mandelii	-	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P169	P. mandelii	-	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P121	P. meridiana	++++	-	-	+	+	+	+	+	+	-	++	+++	+	+	+	-	-	+
P123	P. meridiana	-	-	-	+	+	+	+	+	-	-	++	+++	+	+	+	-	-	+
P137	P. meridiana	++++	-	-	+	+	+	+	-	-	-	++	+++	+	+	-	-	-	+
P147	P. meridiana	++++	-	-	+	+	+	+	-	-	-	++	++++	+	+	+	-	-	+
P162	P. meridiana	++	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P167	P. meridiana	-	-	β	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P168	P. meridiana	+++	-	β	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
V34	P. meridiana	++	+	-	+	+	+	+	+	-	-	++	++++	+	+	+	-	-	+
V35	P. meridiana	++	+	-	+	+	+	+	+	-	-	++	++++	+	+	+	+	-	+

Table 2 (*continued*)

| Strain	Bacteria	Fluorescence	Pellicle formation	Haemolysis on blood agar	Motility	Oxidase	Growth with NaCl (%)					Survival		Growth temperature (°C)					Proteolytic activity
							[b]0-4.5	6	7	8	9	Destilled water (3μm)	Tap water (343μm)	4	25	37	42	45	
V36	*P. meridiana*	++	+	-	+	+	+	+	+	-	-	++	++++	+	+	+	+	-	+
V37	*P. meridiana*	++	+	β	+	+	+	+	-	-	-	++	++++	+	+	+	-	-	+
V38	*P. meridiana*	++	+	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P109	*P. migulae*	+++	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	+	-	+
P51	*P. proteolytica*	++++	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P102	*P. proteolytica*	++	+	-	+	+	+	+	+	+	+	++	+++	+	+	-	-	-	+
P156	*P. simiae*	++++	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P122	*P. weihenstephanensis*	-	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	-
P132	*P. weihenstephanensis*	++	-	-	+	+	+	+	+	+	+	++	+++	+	+	+	-	-	+
P166	*P. weihenstephanensis*	-	-	-	+	+	+	-	-	-	-	++	+++	±	+	+	-	-	+
P115	*Pseudomonas* sp. (A)	++++	-	-	+	+	+	+	-	-	-	+	+++	+	+	+	-	-	+
P7	*Pseudomonas* sp. (B)	++	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P11	*Pseudomonas* sp. (B)	++++	-	β	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P24-2	*Pseudomonas* sp. (B)	++++	-	β	+	+	+	+	-	-	-	++	+++	±	+	+	+	-	+
P29	*Pseudomonas* sp. (B)	+++	-	β	+	+	+	+	-	-	-	++	+++	+	+	-	-	-	+
P72	*Pseudomonas* sp. (B)	+++	+	β	+	+	+	+	+	+	-	++	++++	+	+	+	-	-	+
P73	*Pseudomonas* sp. (B)	+++	-	β	+	+	+	+	+	+	-	++	++++	+	+	+	-	-	+
P158	*Pseudomonas* sp. (B)	++++	-	β	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P49	*Pseudomonas* sp. (C)	+++	-	α	+	+	+	-	-	-	-	++	++++	+	+	+	-	-	-
P50	*Pseudomonas* sp. (C)	+	-	α	+	+	+	+	-	-	-	++	+++	-	+	+	-	-	-
P1	*Pseudomonas* sp. (D)	+	-	α	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P2	*Pseudomonas* sp. (D)	+	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P9	*Pseudomonas* sp. (D)	++++	-	-	+	+	+	+	+	+	-	++	+++	+	+	+	-	-	+
P10	*Pseudomonas* sp. (D)	+	-	-	+	+	+	+	+	+	+	++	+++	+	+	+	-	-	+
P24-1	*Pseudomonas* sp. (D)	+	-	α	+	+	+	+	-	-	+	++	+++	+	+	+	-	-	+
P25	*Pseudomonas* sp. (D)	++++	-	-	+	+	+	+	-	-	-	++	+++	+	+	-	-	-	+
P27	*Pseudomonas* sp. (D)	+	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P30	*Pseudomonas* sp. (D)	+	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P42	*Pseudomonas* sp. (D)	+	+	β	+	+	+	+	+	+	+	++	++++	+	+	+	-	-	+
P116	*Pseudomonas* sp. (D)	+	-	-	+	+	+	+	+	+	-	++	+++	+	+	+	-	-	-
P117	*Pseudomonas* sp. (D)	+	-	-	+	+	+	+	+	-	-	++	+++	+	+	+	-	-	+
P139	*Pseudomonas* sp. (D)	+	-	-	+	+	+	+	-	-	-	++	++++	+	+	+	-	-	+
P141	*Pseudomonas* sp. (D)	+++	-	β	+	+	+	+	+	-	-	++	++++	+	+	+	-	-	+
P152	*Pseudomonas* sp. (D)	++	-	-	+	+	+	+	-	-	-	++	++++	+	+	+	-	-	-
P66	*Pseudomonas* sp. (E)	+	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	-
P67	*Pseudomonas* sp. (E)	+	+	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P154a	*Pseudomonas* sp. (F)	+	-	-	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
P154b	*Pseudomonas* sp. (F)	+++	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	+
P155	*Pseudomonas* sp. (G)	+	-	β	+	+	+	-	-	-	-	++	+++	+	+	+	-	-	+
DSM 12735[a]	*P. putida*	-	-	α	+	+	+	+	-	-	-	++	+++	-	+	+	-	-	-
DSM 50090[a]	*P. fluorescens*	+	-	-	+	+	+	+	-	-	-	++	+++	+	+	+	-	-	-
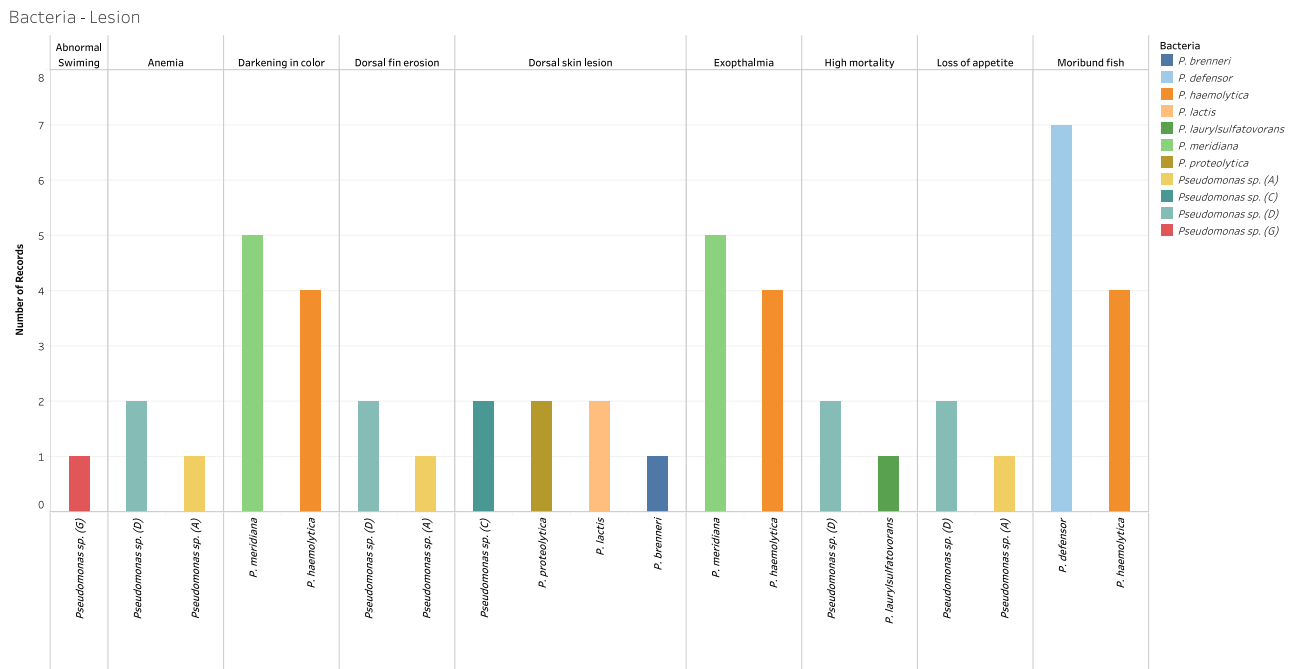
Fig. 4. Fish lesions, symptoms and *Pseudomonas* species correlation.

temperature (average data). *P. lundensis* had the highest tolerance to salinity, whilst *P. migulae* and *P. laurylsulfatovorans* had the lowest salt tolerance (average data) (supplementary Table 4 and supplementary Fig. 6). Also, a regression analysis was performed to determine the correlation between *Pseudomonas* species and fish weight. *P. defensor, P. proteolytica*, and *P. haemolytica* were isolated only from brood stock fish with a weight up to 3000 g, whilst the other species were commonly isolated from small fish, from about yolk-sac up to 60 g (supplementary Table 3 and supplementary Fig. 1). The weights of the sampled fish are presented in supplementary Table 3. Fish weight was indicated as "0" if the weight of the sampled fish was between yolk-sac to 1 g of fry.

As shown in Table 1 and supplementary Fig. 7; *Pseudomonas* spp. were accompanied by *A. media* and *Flavobacteria* spp., such that *P. haemolytica* and *P. meridiana* were commonly detected with other bacteria species. *P. haemolytica* was frequently isolated together with *Aeromonas media* and *F. aquidurense, F. branchiarum, F. collinsii, F. frigidimaris, F. glaciei, F. hydatis, F. oncorhynchi, F. piscis, F. psychrophilum, F. tiangeerense, F. tructae,* and *Flavobacterium* sp. Furthermore, *P. meridiana* was accompanied by the following *Flavobacterium* species: *F. frigidimaris, F. piscis, F. psychrophilum, F. aquidurense* and *Flavobacterium* sp. (Duman et al., 2018; Saticioglu et al., 2018; and unpublished data). *P. brenneri, P. defensor, P. inefficax, P. lactis, P. lundensis, P. migulae, P. proteolytica, Pseudomonas* sp. (A), *Pseudomonas* sp. (B), *Pseudomonas* sp. (C) and *Pseudomonas* sp. (E) were isolated alone with no accompanying bacteria species.

4. Discussion

Aquaculture farms are specific habitats, in which bacteria define economic sustainability. Bacterial water quality is a prerequisite for aquaculture, due to the strong links between the environment, fish, and humans as the ultimate consumers. The present study, aimed at performing an extensive survey of fish farms in Turkey, mainly focussed on the genus *Pseudomonas*, which is a very complex genus that occupies many habitats and has important functional roles in aquaculture. *Pseudomonas* species may show effect in many different ways: i) The presence of the pathogenic *P. aeruginosa* is an indicator of low water quality (Mena and Gerba, 2009); ii) Several *Pseudomonas* species have been described as fresh water fish pathogens, which are associated either with

no symptom or with haemorrhage or skin and eye lesions; iii) the intensive use of antibiotics on fish farms can lead to the environmental dissemination of antibiotic-resistant *Pseudomonas* spp., which are of growing concern; and iv) some *Pseudomonas* species can be used as biocontrol or anti-pathogen agents against fungal infections (Bergan, 1981; De Bentzmann and Plésiat, 2011; Osman et al., 2012; Liu et al., 2015; Austin and Austin, 2016). To study *Pseudomonas* spp. in relation to these four aspects, and to establish preventive measures for the containment of potential disease outbreaks, this study was aimed at precise identification at species level.

4.1. Species diversity

In this study, 20 different *Pseudomonas* species were isolated from water, and new species were described in fish. These species are being reported for the first time in aquaculture farms in Turkey. To our knowledge, strains of the following *Pseudomonas* species have been isolated for the first time from freshwater salmonid fish: *P. brenneri, P. lactis, P. lundensis, P. lurida, P. mandelii, P. migulae, P. meridiana, P. proteolytica, P. simiae* and *P. weihenstephanensis*. One third of the isolates were identified as one of the 7 putative new *Pseudomonas* species, which require further taxonomic analysis: *Pseudomonas* sp. (A), (B), (C), (D), (E), (F) and (G).

Thirty eight percent of the identified *Pseudomonas* species (*P. brenneri, P. lactis, P. proteolytica, P. haemolytica, P. defensor, P. laurylsulfatovorans, P. meridiana, Pseudomonas* sp. (A), (C), (D) and (G)) were isolated from fish with disease symptoms and/or lesions. Therefore, they are considered as potential pathogens for cultured freshwater fish. The pathogenicity of these species needs to be confirmed by fulfilling Koch's postulates in fish and conducting genomic studies.

P. haemolytica was the species with the highest number of isolates, appearing in a higher number of aquaculture farms (Fig. 2) in different years (supplementary Fig. S5), and in both healthy and symptomatic fish of different weights (Fig. 4, supplementary Table 4). *P. haemolytica* strains were isolated alone or in association with other species (*A. media*) (Table 1). Recently, *P. haemolytica* has been proposed as a new species, based on the characterization of strains isolated from raw milk (Hofmann et al., 2020). The present study describes, for the first time, its presence in a different habitat, farmed fish. Overall, these data indicate

the presence, adaptation and persistence of this bacterium in the environment (see above).

Pseudomonas sp. (B) is the only species found in farm water and also constitutes the first group of strains selected for taxonomic polyphasic study so as to be proposed as a new species (work in progress). The newly described *Pseudomonas* sp. (G) caused abnormal swimming (upside-down and spirally) and disease symptoms completely different from those observed with the other identified species.

This is the first step in monitoring the presence and controlling the spread of these bacteria in this particular environment.

4.2. Geographical distribution of the species

The most common *Pseudomonas* species reported from fish farms in the Black sea region of Turkey are *P. putida, P. fluorescens, P. luteola,* and other Pseudomonas not identified at species level. Furthermore, *P. aeruginosa* and *P. stutzeri* have been isolated in the northeast of the Mediterranean region (Matyar et al., 2010; Öztürk and Altinok, 2014; Ture et al., 2018). None of these species were isolated in the present study. Most of the *Pseudomonas* species were isolated from the two biggest rainbow trout hatcheries in the Aegean (Mugla) and Central Anatolia (Kayseri) regions. While *P. brenneri, P. defensor, P. haemolytica,* and *Pseudomonas* sp. (D) were isolated only from these two hatcheries (Mugla, Kayseri), *P. lactis, P. laurylsulfatovorans, P. lundensis, P. migulae,* and *Pseudomonas* sp. (A), (C), and (E) were isolated from only one fish farm (Sivas, Kayseri or Mugla).

P. mandelii, P. meridiana, and *P. weihenstephanensis* were detected in July and December in 2013, in the Aegean and Central Anatolia regions. In January 2017, *P. mandelii, P. meridiana,* and *P. weihenstephanensis* were isolated not only in the Aegean and Central Anatolia regions, but also in the Black sea region. This was attributed to the fish farms in the Black Sea region receiving stocks mostly from the Aegean and Central Anatolia regions. A similar situation was observed for bacteria isolated from fish farms located in Eastern Anatolia, such that *P. meridiana* and *Pseudomonas* sp. (B) were first isolated in the Aegean and Central Anatolia regions, indicating their spread to the Eastern Anatolia and Black sea regions.

4.3. Accompanying species

Throughout the surveillance study, in addition to the 90 *Pseudomonas* strains from the different regions of Turkey, we also isolated 98 motile *Aeromonas* (Duman et al., 2018), 137 *Yersinia ruckeri* (Duman et al., 2017), 25 *F. psychrophilum* (Saticioglu et al., 2018), 84 *Flavobacterium* spp. (unpublished data), and 137 *Lactococcus garvieae* (Duman et al., 2020) strains. The share of *Pseudomonas* strains among the isolated species was 15.7%, and *Pseudomonas* was the least isolated genus of all the aquatic pathogens investigated between 2013 and 2017 (data not shown). The isolation rate of *Pseudomonas* compared to other species suggests an opportunistic or primarily invasive character for this genus in fish because *P. haemolytica, P. laurylsulfatovorans, P. lurida, P. mandelii, P. meridiana, P. simiae, P. weihenstephanensis,* and *Pseudomonas* sp. (D), (F) and (G) were isolated in association with *Flavobacterium* species and *A. media.* Pathogenicity has not been reported for *Flavobacterium* species (except *for F. psychrophilum)* (Austin and Austin, 2016). Many *Pseudomonas* species have been reported as opportunistic or primarily invasive pathogens (Austin and Austin, 2016). Accordingly, *Pseudomonas* spp. could be primary pathogens accompanied by opportunistic pathogens or *vice versa.* None *Pseudomonas* species found in this study has been described previously as fish pathogen, but 26 strains identified under 9 species have been isolated from symptomatic fishes without any accompanying microbiota (Table 1).

4.4. Determinants of bacterial fitness

Several phenotypic traits of the isolates were studied to demonstrate

their ability to thrive in the study environment. Iron uptake systems, biofilm formation and haemolysis were three relevant pathogenicity traits detected in most of the species identified. Fluorescent pigments, which act as siderophores, were a selective criterion for isolation. Half of the isolates were able to form pellicles that could confer survival advantages, such as nutrient availability through metabolic cooperation, acquisition of new genetic traits, and protection from the environment (Kolter and Greenberg, 2006; Karatan and Watnick, 2009). None of the pellicle-forming species detected in the present study have been previously reported to have this ability. In the present study, haemolysis was detected as a virulence characteristic in 7 species. *P. haemolytica* was isolated, for the first time, from rainbow trout and all strains were strongly β-haemolytic. This species has been reported to have been isolated from raw milk and it is difficult to explain its presence in fish farms. It is considered that this could be related to the water sources being used for the provision of water to cattle, sheep, and goats, and in some regions, cattle having access to the water sources before the water reached the fish farm. These propositions could explain why *P. haemolytica* was detected in both dairy products and farmed fish as well as the detection of *P. lactis,* another species originally isolated from bovine raw milk (Von Neubeck et al., 2017). However, in addition to *P. haemolytica* and *P. lactis,* other species, including *P. lurida, P. meridiana, P. weihenstephanensis,* and *Pseudomonas* sp. (B), (C), (D) and (G) were also haemolytic, which could explain the haemorrhagic symptoms displayed by the fish they had colonized.

Pseudomonas species are known to display varying levels of tolerance to salinity, temperature and pH, and to persist in the environment, even in the absence of nutrients (Kiran et al., 2004; Kumar et al., 2008; LaBauve and Wargo, 2012; Liu et al., 2015; Palleroni, 2015; Moradali et al., 2017). In the present study, all isolates tolerated 4.5% NaCl, and some were highly tolerant, up to 9% NaCl (some strains of *P. haemolytica, P. lundensis, P. proteolytica, P. weihenstephanensis* and *Pseudomonas* sp. (D)). While almost all isolates could grow at an incubation temperature of 37 °C, *P. mandelii* (P31), *P. meridiana* (V35 and V36), *P. migulae* and *Pseudomonas* sp. (B) tolerated 42 °C, and *P. haemolytica* (V81) was heat-tolerant up to 45 °C. Furthermore, it was determined that all of the isolates were able to survive in sterile distilled water and tap water without any additives. Several of these phenotypic findings point out to the possibility of these strains being major zoonotic agents and potential pathogens for humans and terrestrial animals.

Pseudomonas spp. have been recognized as primarily invasive or opportunistic pathogens for many organisms and, furthermore, this genus has also gained importance in terms of antimicrobial resistance. Even in cases of mass death, when veterinarians prefer to use broadspectrum antimicrobials regardless of the etiological agent, it is important to review the antibiogram of the isolated strains. Several researchers have evaluated the antimicrobial sensitivity of *Pseudomonas* species isolated from fish, and have reported them as multidrug-resistant bacteria, based on their resistance to ampicillin, trimethoprim+sulfamethoxazole, cefotaxime, aztreonam, nitrofurantoin and other groups of antimicrobials (Matyar et al., 2008; Devarajan et al., 2017). A limited number of antimicrobial agents are licensed for the treatment of fish diseases and those that have found common use in Turkey are trimethoprim/sulfamethoxazole, doxycycline, tetracycline, enrofloxacin, and florfenicol. In the present study, we tested all *Pseudomonas* strains against the antimicrobials commonly used in fish farms in Turkey, and determined that almost all species were resistant to sulfonamides (SXT), aminopenicillins (AML), penicillins (AMP), macrolides (E), clindamycin (MY) and chloramphenicol (FFC). In contrast to previously published results, we found that the isolates were frequently susceptible to tetracyclines (DO, TE, and OT), quinolones (ENR, CIP, UB), and an aminoglycoside (CN). As the antimicrobial resistance of the large majority of these species have not been described before, a literature-based comparison of the isolates was able to be made only to a very limited level. The comparison of the isolates showed that *P. defensor, P. meridiana,* and *Pseudomonas* sp. (E) were the most resistant

species.

An important result of this study is the isolation of a high number of *Pseudomonas* species from an environment previously not described for these species. *P. aeruginosa*, an important health indicator, and the fish pathogens *P. anguilliseptica, P. chlororaphis, P. putida* and *P. luteola* have been detected neither in fish nor in water, indicating a good microbial quality of water. Only one new *Pseudomonas* species, namely, *Pseudomonas* sp. (B), was found exclusively in water and not in fish. None of the *Pseudomonas* species isolated in this study were considered as fish pathogens until now. *Pseudomonas* sp. (D) is phylogenetically close to *P. baetica*, which is considered a fish pathogen and, consequently should be tested for pathogenicity. Either the spread of the strains from one region to another has been confirmed (*P. mandelii, P. meridiana, P. weihenstephanensis),* or the appearance of some bacteria, such as *P. haemolytica* and *P. lactis,* has been described for the first time in fish farms, which represent a totally different environment from the original source of isolation. The newly identified species were highly resistant to the antibiotics commonly used in aquaculture, and this fact should be carefully addressed in the context of the environmental dissemination of antibiotic resistance. The phenotypic and genotypic characteristics of these isolates could contribute to a better understanding of their future role, and their fitness in spreading to other regions.

5. Conclusion

The multilocus analyses presented is a precise method for the identification at the species level of *Pseudomonas* associated with water and fish in aquaculture farms. It demonstrates the high diversity of species present in this particular habitat:12 Pseudomonas species are reported for the first time associated with freshwater salmonid fish and seven not yet described putative new *Pseudomonas* species have been also found. The newly identified species were highly resistant to the antibiotics used in aquaculture. These data highlight the importance of continuous monitoring of the presence and spreading of microorganisms in aquaculture farms.

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CRediT authorship contribution statement

Muhammed Duman: Methodology, Software, Data curation, Writing - original draft, Editing. **Magdalena Mulet**: Methodology, Software. **Soner Altun**: Methodology, Software, Writing - original draft. **Izzet Burcin Saticioglu**: Methodology, Software, Writing - original draft. **Burak Ozdemir**: Software. **Nihed Ajmi**: Software. **Jorge Lalucat**: Data curation, Writing - original draft. **Elena García-Valdés**: Data curation, Writing - original draft, Supervision, Editing.

Declaration of Competing Interest

The authors declare no competing or financial interests.

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