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Description of a Novel Fish Pathogen, *Plesiomonas shigelloides* subsp. *oncorhynchi*, Isolated from Rainbow Trout (*Oncorhynchus mykiss*): First Genome Analysis and Comparative Genomics

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Abstract: *Plesiomonas shigelloides* is the only species in its genus, and has zoonotic importance due to the serious implications resulting from the consumption of contaminated seafood. This is the first report on the genomic features of the whole-genome sequence (WGS) of *P. shigelloides* strain V-78, recovered from diseased rainbow trout, *Oncorhynchus mykiss*. The genome of *P. shigelloides* V-78 consists of 4,478,098 base pairs (bp), which encode 3730 proteins, and has a G + C content of 51.1%. The bioinformatics analysis of WGS of V-78 confirmed the presence of 121 tRNA genes and 42 rRNA genes (15 genes for 5S rRNA, 13 genes for 16S rRNA, and 14 genes for 23S rRNA). Comprehensive genome analyses revealed that the strain encodes for secondary metabolites, antimicrobial resistance, and virulence genes. The strain V-78 has 31 known antibiotic resistance models, which encode many antimicrobial resistances. In addition, strain V-78 has 42 different virulence genes, such as adhesion, secretion system, and motility. The digital DNA–DNA hybridization value against *P. shigelloides* NCTC 10360 was 74.2%, while the average nucleotide identity value was 97.1%. Based on the scrutinized analysis of genomic data, strain V-78 should be considered a novel subspecies of *P. shigelloides*, for which *Plesiomonas shigelloides* subsp. *oncorhynchi* is proposed.

Keywords: whole-genome sequence; *Plesiomonas* genus; Plesiomoniasis; aquaculture

Key Contribution: A novel member of *Plesiomonas* genus, *P. shigelloides* subsp. *oncorhynchi* was described which could be a novel pathogenic agent for rainbow trout.

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1. Introduction

Plesiomonas shigelloides, formerly known as *Pseudomonas shigelloides*, has a debatable taxonomic history and is currently classified in the family *Enterobacteriaceae* as the only member of the genus *Plesiomonas* [1]. This bacterium naturally inhabits freshwater and marine environments, as well as animals dwelling in those ecosystems, including fish, amphibia, and insects [2]. The natural habitat of *P. shigelloides* is associated with its transmission to the human body, since this agent enters the body through contact with either contaminated water or seafood. Asymptomatic carriage of the agent is common among freshwater and marine species such as oysters, shrimp, trout, sea bass, and sea bream.

Natural disasters may lead to infectious disease outbreaks when they result in substantial population displacement and exacerbate synergic risk factors (changes in the environment, fish conditions, and vulnerability to existing pathogens) for disease transmission. The commensals and symbionts could become pathogenic through a process called the Rasputin Effect, which focuses on those instances when benign and even beneficial relationships between microbes and their hosts opportunistically change and become detrimental toward the host [3]. *Plesiomonas* has not yet been associated with post-natural disaster infections (45). However, indirect evidence suggests that plesiomonads could be involved in illnesses after major natural aquatic disasters [4]. A large number of freshwater plesiomonad strains have commonly been reported in rainbow trout (*Oncorhynchus mykiss*) [5,6], carp (*Ctenopharyngodon idellus*) [7], and tilapia (*Oreochromis Niloticus*) [4,8–10]. High mortality rates in trout due to *P. shigelloides* alone [11] along with other bacteria, such as *Flavobacterium* spp. and *Aeromonas hydrophila,* were also reported [12]. This pathogen was also identified as one of the main pathogens in various fish [7,10,13], providing more evidence that *P. shigelloides* might be highly pathogenic for farmed fishes. Besides farmed species, a 100% mortality rate was recorded in cichlid ornamental fish due to infection caused by *P. shigelloides* [14].

While the agent is assumed to be a member of the microbiota or to be responsible for disease, especially in co-infection reports with other fish pathogens, there is very limited information about the genomic characteristics of *P. shigelloides*. Almost all published data strongly suggest that the genus and species designation *P. shigelloides* is composed of a collection of homogeneous bacteria at the phenotypic and molecular levels [4]. A population study of a diverse collection of 77 *P. shigelloides* strains from different geographic as well as environmental settings, indicated by a monophyletic clade nested within the *Enterobacteriaceae* [15,16]. The complete genome analyses have noted *P. shigelloides* strains isolated from humans, animals, and fish, but not from rainbow trout [4,17,18]. This lack of information causes a gap in the relation of the agent isolated from fish to humans in zoonotic potency, transmission routes, and virulence. Successful bacterial identification reports by 16S rRNA or 23S rRNA gene analysis indicate the prevalence of *P. shigelloides* in fish [19,20], but the paucity of genomic information limits our knowledge of its exact role in terms of virulence characteristics and antimicrobial resistance gene transfer.

To the best of our knowledge, there have only been seven reports on the genome analysis of *P. shigelloides* isolates recovered from different sources, but there have been no genome reports recovered from rainbow trout [17,18,21]. We aimed to characterize *P. shigelloides* strains isolated from fish by genome analysis and to annotate the first genome of strain *P. shigelloides* V-78 as a novel subspecies. In addition, there are no reports about the virulence and antimicrobial characteristics of *P. shigelloides* for rainbow trout. Thus, we detected virulence and antimicrobial resistance genes in the genomes of strains, which provided a better understanding of the pathogenicity of this agent in fish.

2. Materials and Methods

2.1. Bacteria Isolation and Phenotypic Characterization

Isolates were collected from rainbow trout (*Oncorhynchus mykiss*) farms using conventional flow-through systems with non-disinfected surface water and spring water in the Central Anatolia Aegean region of Turkey. Besides rainbow trout isolates, another group of strains was also collected from ornamental freshwater fish which were transferred to the fish disease laboratory between 2018–2021. The bacterial isolates were collected during a fish health surveillance program from the internal organs of diseased fish (showing darkening in color, exophthalmia, and ulceration on skin and fins) of different weights, such as 0.5–1 g and 200 g rainbow trout, and from species of freshwater ornamental fish. Two strains, designated as V-63 and V-78 and isolated from the samples

collected from the Aegean region in 2018 and Central Anatolia in 2013, respectively, were selected for comprehensive genome analyses.

The isolates were phenotypically characterized by Gram-staining, motility, oxidase and catalase reaction tests, and glucose fermentation in O/F medium (Merck 103865). Hemolytic characteristic was tested on a 5% sheep blood-added agar, and sensitivity to Vibriostatic agent was also tested (Oxoid DD0014 and DD0015). Growth in the presence of NaCl was tested in tryptic soy broth (TSB, Merck 105459) supplemented with 0–3% NaCl (*w*/*v*). The optimum growth temperature range was tested in the range of 4–50 °C on TSB medium. In addition, the ability to grow on Thiosulfate Citrate Bile Sucrose (TCBS) agar (Merck, 103854) and to hydrolyze gelatin were determined by following previously published methods [22].

2.2. DNA Extraction, PCR and Sequence Analysis

The genomic DNA of the isolates was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount and purity of the DNA in each sample were measured at 260 nm and 260/280 nm wavelengths with a spectrophotometer (Multiskan Go, Thermo, Waltham, MA, United States).

The isolates were identified by MLSA analysis using 16S rRNA, *atp*A (ATP synthase α subunit), *gyr*B (DNA gyrase β subunit), *pry*H (uridylate kinase), and *rec*A (recombinase A) gene sequences [22,23]. A polymerase chain reaction (PCR) was performed as described in previous works [22]. All PCR products were sequenced by Macrogen Korea (Republic of Korea). The DNA fragments used for the analysis were 1400 bp for 16S rRNA, 570 bp for *gyr*B, 501 bp for *pyr*H, 462 bp for *rec*A, and 489 bp for *atp*A.

2.3. Genome Sequencing, Assembly and Identification

A comparison between isolates was made based on the 16S rRNA gene sequence. The evolutionary history was inferred using the neighbor-joining method [24]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) is shown next to the branches [25]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [26], and are in the units of the number of base substitutions per site. This analysis involved 8 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1546 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [27].

For comprehensive genome-based analyses, the whole-genome sequence of V-78 was obtained as described previously [17]. The genome of strain V-78 was sequenced with the Oxford Nanopore MinION system (Oxford Nanopore Technologies, Oxford, UK). The high-quality reads of the genome were assembled into contigs by de novo assembly using the Canu v. 1.7.1 for strain V-78. The draft genome sequence data were submitted to GenBank. The annotation of contigs longer than 1000 bp was achieved by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [28]. In addition, the genome of strain V-78 was annotated on the Rapid Annotations Using Subsystems Technology (RAST) server (https://rast.nmpdr.org/ (Accessed on 24 March 2023)) [29] using the RASTtk pipeline [30]. The type strain genomes server pipeline (TYGS, https://tygs.dsmz. de/ (Accessed on 24 March 2023)) was employed to perform phylogenomic analysis and determine the digital DNA–DNA hybridization (dDDH) values between the genome of strain V-78 and those of the type strains deposited in the DSMZ database.

2.4. Comparative Genome Analysis

The publicly available genomes of *P. shigelloides* in the NCBI GenBank database were retrieved for comparative genome analysis. From 37 genomes, a total of 19 *P. shigelloides* genomes with known isolation sources were selected for further analyses. The Codon Tree method selects single-copy genes from a determined set of genomes using Bacterial and Viral Bioinformatic resource center (BV-BRC) programs, and analyzes aligned proteins and coding DNA from single-copy genes using the program RAxML (https://www.bv-brc.org/app/PhylogeneticTree (accessed on 24 March 2023)) [31]. For comprehensive genomic characterization, secondary metabolite biosynthetic gene clusters and antibiotic resistance models encoded by the genome of strain V-78 were determined using antiSMASH version 6.1.1 (https://antismash.secondarymetabolites.org/#!/start (Accessed on 24 March 2023)) [32] and Antibiotic Resistant Target Seeker version 2 (ARTS) (https://arts.ziemertlab.com/ (accessed on 24 March 2023)) [33], respectively. To determine the virulence genes of the strains, the VFDB (virulence factor of pathogenic bacteria) online database (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi (accessed on 24 March 2023)) was used. The GenBank files of *P. shigelloides* genomes were subjected to BLASTn against the VFDB with default settings. In addition, the genomic islands, i.e., the clusters of genes of probable horizontal transfer origin, were predicted by IslandCompare software, which is available at https://islandcompare.ca/ (accessed on 24 March 2023)[34]. The compilation of taxonomic, functional, and ecological features of strain V-78 was acquired by the Protologger web server, which is available at http://protologger.de/ (accessed on 24 March 2023)[35].

2.5. Antimicrobial Susceptibility and Resistance Gene Characteristics

The isolates were tested for their susceptibility to antimicrobials by an agar disk diffusion method, as essentially described in the Clinical and Laboratory Standards Institute (CLSI) guideline M42-A [36], using Mueller–Hinton Agar (MHA, BBL-Becton Dickinson, Franklin Lakes, NJ, USA). The antibacterial susceptibility patterns of the isolates were determined using disks containing the following antibacterial agents: trimethoprim/sulfamethoxazole (SXT, 25 μg), amoxicillin (AML, 25 μg), doxycycline (DO, 30 μg), tetracycline (TE, 30 μg), oxytetracycline (OT, 30 μg), oxolinic acid (OA, 2 μg), enrofloxacin (ENR, 5 μg), ciprofloxacin (CIP, 5 μg), flumequine (UB, 30 μg), cefalexin (CN, 10 μg), ampicillin (AMP, 10 μg), erythromycin (E, 15 μg), lincomycin (MY, 15 μg), and florfenicol (FFC, 30 μg). All disks were obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). The selected antimicrobial agents were those commonly used and reported for aquaculture [37–39]. The bacterial isolates were suspended in sterile 0.85% saline to obtain a 0.5 McFarland (bioMerieux S.A.) turbidity, and were then seeded on the test media. The plates were incubated for 24–28 h at 28 °C. The control strains *A. salmonicida* subsp. *salmonicida* ATCC 33658 and *E. coli* ATCC 25922 were incubated at 28 °C for 24–28 h [36].

3. Results

3.1. Phenotypic Characterization

The strains were Gram-negative, with short bacilli. They were also motile, oxidase and catalase positive, glucose fermentative, non-hemolytic on sheep blood, and sensitive to Vibriostatic agent (O/129 150 μg). The tolerance tests for growth in the presence of NaCl and at a range of temperatures showed that the strains were able to grow in the presence of up to 1.5% NaCl and at a temperature range of 4–45 °C. Unlike Vibrios, the isolates V-63 and V-78 did not grow on TCBS medium and could not hydrolyze gelatin.

3.2. PCR and Sequence Analysis

The strains were identified as *Plesiomonas* spp. by 16S rRNA gene sequence analysis. The corresponding phylogenetic tree is represented in Figures S2 and S3 demonstrating

that strains V-63 and V-78 belonged to the genus *Plesiomonas*. For better identification, the following genes, as suggested in the PubMLST database for *Vibrio* spp., were also sequenced: *gyr*B, *pry*H, *rec*A, and *atp*A. These genes were analyzed by Blast in the NCBI database, and the closest matches were *P. shigelloides* strains. The sequences were accessed from the PubMLST database, and the following alleles for the genes *gyr*B, *pry*H, *rec*A, and *atp*A were determined: 133, 103, 127, and 93 for V-63 and 136, 104, 128, and 95 for V-78, respectively. An ST number was given for the new allele profiles—177 and 178 for V-63 and V-78, respectively—in the PubMLST database due to the similarity to the *Vibrioneaceae* genus. The strains are closely related to the *P. shigelloides* reference strain, sharing at least 99% similarity in the sequences of the mentioned genes.

3.3. Phylogenomics

Genome-based identification of the V-78 strain was performed by the TYGS web server, and it was identified as *P. shigelloides* (Figure 1). The phylogenetic results indicated that strain V-78 was very closely related to *P. shigelloides* NCTC 10360T (Figure 1), with a dDDH value of 74.2%.

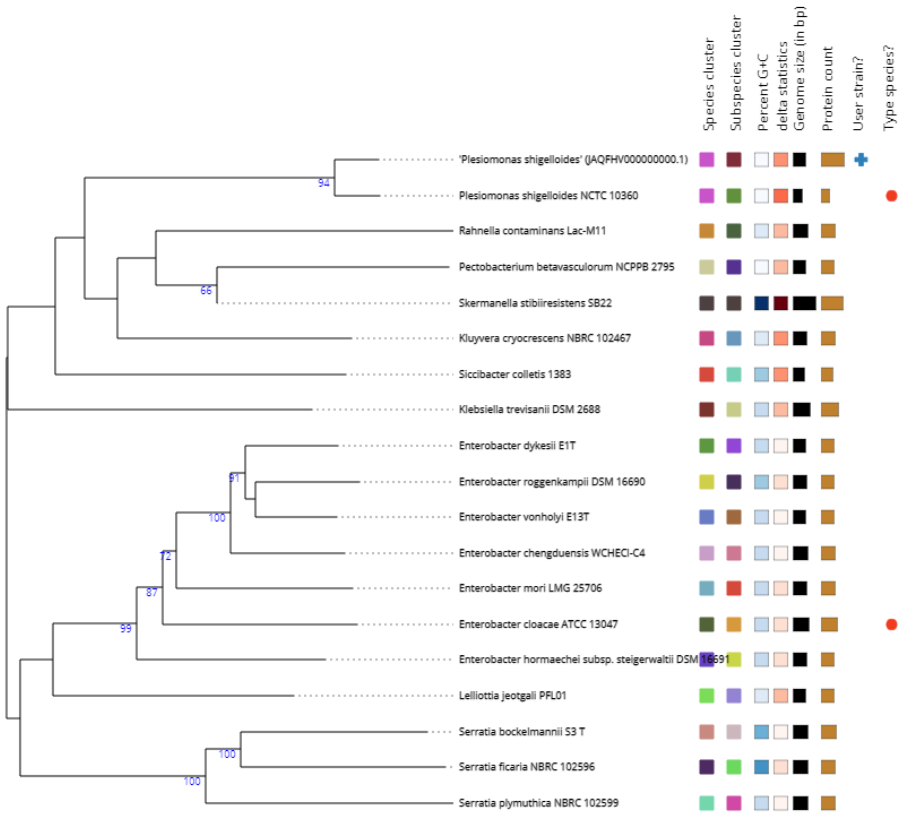

Figure 1. Tree inferred with FastME 2.1.6.1 with GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of the GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values, >60% from 100 replications, with an average branch support of 65.1%. The tree was rooted at the midpoint.

At the time of writing the manuscript (February 2023), a total of 37 *Plesiomonas shigelloides* genomes were available in the GenBank database. However, six of these genomes were assembled from the metagenomics data, two genomes lacked information regarding the isolation source, and three genomes did not have information on the country of origin. Therefore, eight genomes were selected among the isolates obtained from various fish species, including rainbow trout (Table 1). The genome size of strain V-

78 was significantly greater than that of the other *Plesiomonas* genomes. The codon tree was built including 19 genomes. The codon tree reveals phylogenomic heterogeneity within the genus *Plesiomonas,* inferred from the alignment of coding DNA from 931 single-copy genes and amino acid sequences. The strain V-78 was located on a distinct branch of the tree (Figure 2).

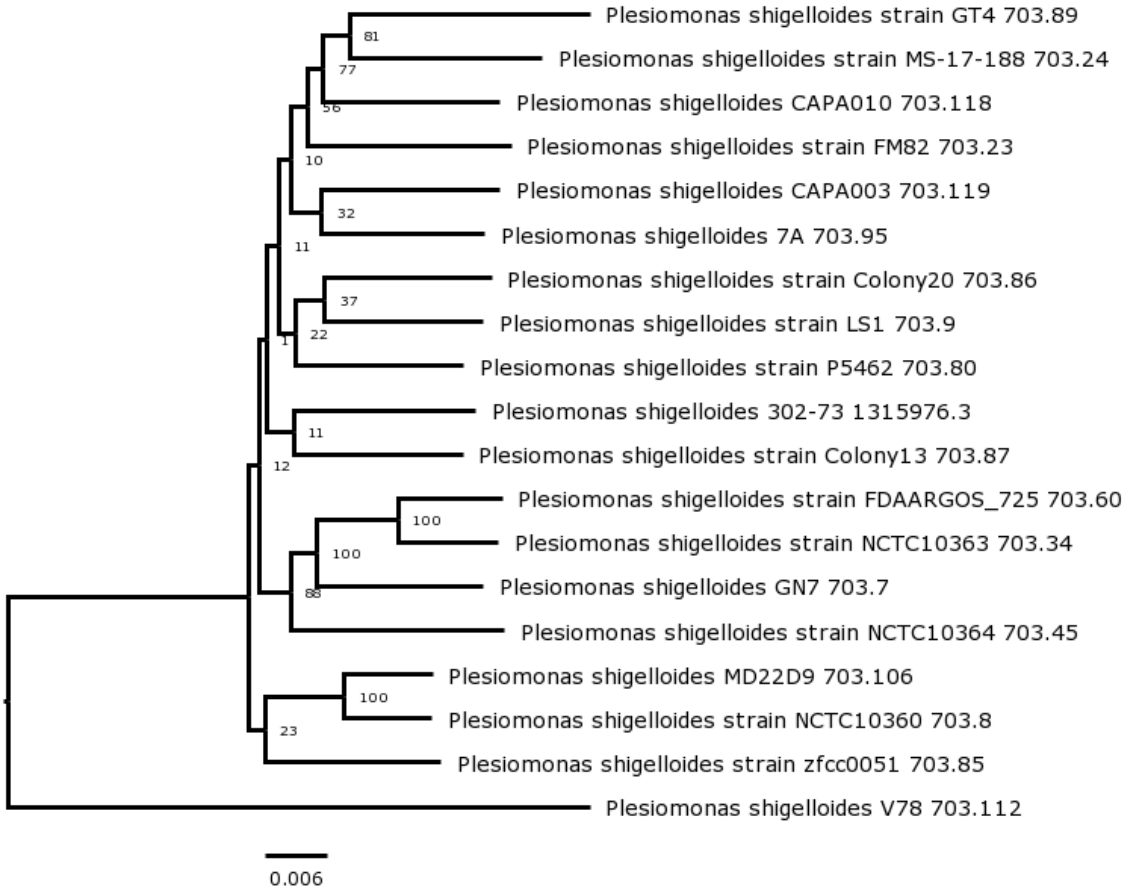

Figure 2. The codon tree based on 931 single-copy genes found in the genomes of strain V-78 and its close relatives within the species *P. shigelloides,* built using the BV-BRC server (https://www.bv-brc.org/app/PhylogeneticTree (accessed on 24 March 2023)).

Table 1. *P. shigelloides* strains compared in this study.

Strain Name	Host	Country	Total Genes	Housekeeping Genes	Known Resistance	Virulence Genes
zfcc0051 *	Zebra fish	USA	3818	596	31	41
V-78 *	Rainbow trout	Turkey	5454	297	19	41
P5462	Gentoo penguin	Hong Kong	3287	596	33	40
NCTC10364	Human	Unknown	3098	584	32	42
NCTC10363	Human	Unknown	3264	599	30	40
NCTC10360	Dog	Unknown	2886	583	28	40
MS-17-188 *	Catfish	USA	3426	595	32	42
MD22D9	Macrobdella decora	USA	3190	595	32	40
LS1 *	*Percocypris pingi*	China	3484	590	33	43
GT4 *	*Oreochromis niloticus*	Brazil	3134	600	34	43

GN7	Water	Malaysia	3434	596	33	42
FM82 *	Fish	Brazil	3233	593	32	41
FDAARGOS_725	Human	USA	3250	597	32	40
Colony20	Food	Thailand	2598	576	28	43
Colony13	Human	Thailand	2648	580	28	39
CAPA010 *	*Oreochromis niloticus*	Peru	3176	599	33	42
7A	River	Sweden	3298	597	32	41
302-73	Human	Japan	3398	592	32	40
CAPA003 *	*Oreochromis niloticus*	Peru	3185	596	33	41

* The *Plesiomonas* isolates from fish

The genome of strain V-78 harbors 13 gene sequences coding for 16S rRNA, the pairwise identity levels of which, along with that of *P. shigelloides* NCTC 10360^T, ranged from 99.38% to 99.93%. These rRNA gene sequences also differed by length, ranging from 1534 bp to 1541 bp, and by intra-strain similarity levels of 99.28–100%.

The genomes of closely related species were retrieved from the Genome Taxonomy Database (GTDB) by the Protologger to perform pairwise analyses for the average nucleotide identity (ANI) and percentage of conserved proteins (POCP). The ANI values were calculated by the FastANI (v1.2) algorithm [37], while the POCP values were acquired by custom Python scripts available at the GitHub repository by the Protologger (Table S1). The genome of strain V-78 shared 97.1% ANI value with the type strain of *P. shigelloides*, while that value, along with the other closely related taxa, was found to be lower than 80%, implying that the genus exhibits considerable distance from the other genera (Figure S1). The highest POCP value of 68.1% was calculated between the genome of strain V-78 and that of the type strain of *P. shigelloides*, as expected. In addition to the genome-based phylogenetic analysis, a 16S rRNA-based phylogenetic tree is also provided in Figures S2 and S3. The other POCP values were below the threshold of 50% for genus demarcation [38], proving the distinctness of the genus *Plesiomonas* (Table 2).

Table 2. Percentage of conserved proteins (POCP) of strain V-78 from related type strains, as calculated by the Protologger.

Strain	Type Strain	POCP (%)
V-78	*Plesiomonas shigelloides*	68.1
	Yersinia entomophaga	43.2
	Obesumbacterium proteus	42.4
	Hafnia paralvei	42.1
	Buttiauxella agrestis	41.8
	Citrobacter werkmanii	41.8
	Buttiauxella noackiae	41.8
	Citrobacter pasteurii	41.7
	Salmonella enterica	41.7
	Buttiauxella brennerae	41.6
	Citrobacter freundii	41.5
	Enterobacter soli	41.2
	Serratia marcescens	40.9
	Serratia grimesii	40.8
	Pectobacterium carotovorum	40.8
	Serratia proteamaculans	40.6
	Buttiauxella ferragutiae	40.6

Serratia nematodiphila	40.5
Citrobacter braakii	40.5
Serratia odorifera	40.3
Chania multitudinisentens	40.3
Serratia quinivorans	40.2
Yersinia pestis	40.2
Serratia fonticola	40.2
Buttiauxella gaviniae	40.1
Enterobacter hormaechei	40.0
Proteus hauseri	39.9
Serratia ficaria	39.9
Serratia plymuthica	39.9
Cronobacter sakazakii	39.8
Citrobacter amalonaticus	39.7
Pectobacterium parmentieri	39.5
Enterobacter cloacae	39.4
Dickeya chrysanthemi	39.4
Raoultella planticola	39.0
Klebsiella pneumoniae	38.9
Raoultella ornithinolytica	38.8
Lonsdalea quercina	38.3
Pantoea agglomerans	38.1
Enterobacter ludwigii	38.0
Rouxiella chamberiensis	38.0
Pantoea allii	37.2
Rouxiella silvae	37.2
Rouxiella badensis	37.0
Sodalis praecaptivus	36.3
Pantoea cypripedii	35.3

3.4. Functional and Ecological Analyses

Functional and ecological features of strain V-78 were predicted by Protologger software. Of the 5454 identified coding sequences, 235 genes were anticipated to encode for transporter proteins, while 50 genes were thought to be responsible for secretion. The number of unique enzymes encoded by the genome of V-78 was determined to be 920. Arbutin, glucose, salicin, sucrose, and trehalose were predicted to be utilized by the strain as carbon sources. In addition, strain V-78 could produce acetate from acetyl-CoA (EC:2.3.1.8, 2.7.2.1) and propionate from propanoyl-CoA (EC:2.3.1.8, 2.7.2.1), as predicted by the Protologger. The flagellar proteins identified in the genome were *Flh*A, *Flh*B, *Flg*B, *Flg*C, *Flg*D, *Flg*E, *Flg*F, *Flg*G, *Flg*J, *Flg*K, *Flg*L, *Fli*D, *Fli*F, *Fli*G, *Fli*M, *Mot*A, and *Mot*B. The strain was able to produce L-cysteine and acetate by utilizing sulfide and L-serine (EC:2.3.1.30, 2.5.1.47). The genome also has pathways for L-glutamate production from ammonia via L-glutamine (EC:6.3.1.2, 1.4.1.-) and biosynthetic genes for biotin, folate, and riboflavin synthesis. A total of 157 carbohydrate-active enzymes (CAZymes), mainly in glycoside hydrolase (GH) families, were identified within the genome of strain V-78. The glycoside transferase (GT), carbohydrate esterase (CE), polysaccharide lyase (PL), and carbohydrate-binding module (CBM) families were also predicted in the genome.

For an ecological analysis, both the 16S rRNA gene and whole-genome sequences were submitted to the Protologger [35]. The genome of strain V-78 was compared with a database of 49,094 metagenome-assembled genomes (MAGs) collected from various habitats as described by Hitch et al. [35]. Briefly, the comparison was made using MASH

[39], and the results were filtered using a distance threshold of <0.05. In addition, the 16S rRNA gene was employed to perform a comparative analysis involving operational taxonomic units (OTUs) generated from 19,000 amplicon datasets from 19 different habitats obtained from the IMNGS database [40]. Only one MAG (MAG-ID: HeQ_2017__SZAXPI029476-50__bin_9.fa), collected from an adult human stool [41], clustered with the genome of strain V-78. Habitat distribution and preference analysis based on comparing 16S rRNA gene amplicons revealed that strain V-78 was detected in all 19 environments in the IMNGS database, with a total of 38,163,501 OTUs (Figure 3). The highest detection ratio was observed within the rhizosphere amplicons, while the mean relative abundance was the highest in human gut amplicons compared to other environments (Table 3).

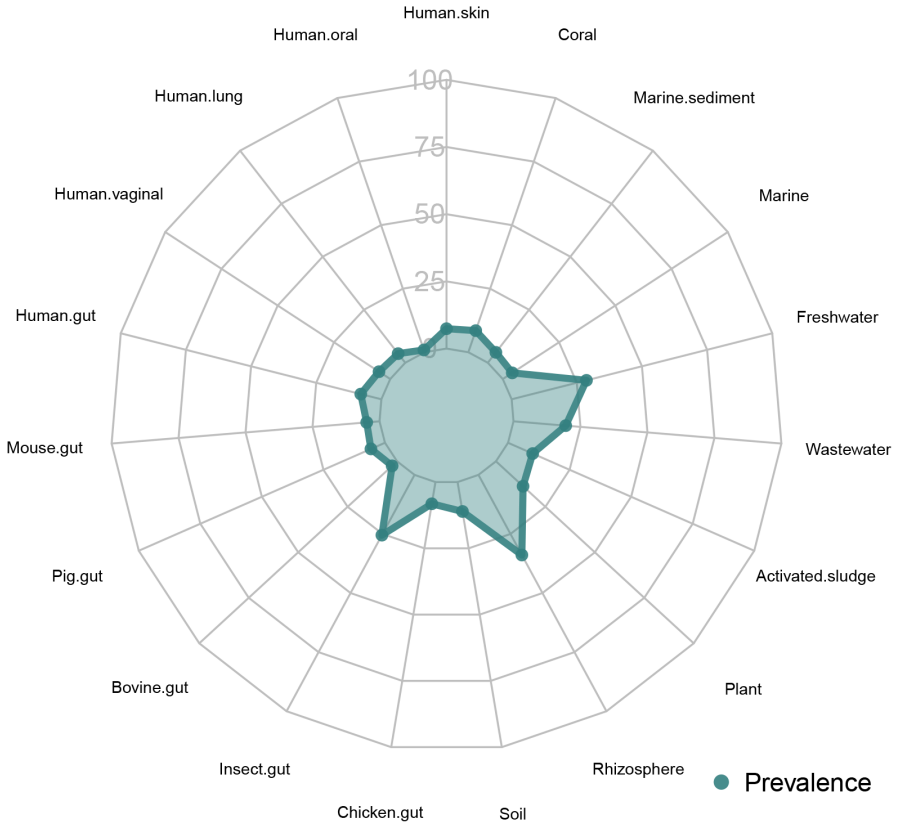

Figure 3. Habitat distribution and preference analysis comparing the 16S rRNA gene sequence of strain V-78 with a database of 16S rRNA gene amplicons obtained from 19 different environments by the Protologger.

Table 3. Habitat preference and distribution analysis of strain V-78 based on the 16S rRNA gene amplicons obtained from the IMNGS database by the Protologger software.

Environment	Detection Ratio (%)	Mean Relative Abundance (%)	Standard Deviation (%)
Rhizosphere	33.9	0.71	3.47
Freshwater	28.7	3.29	12.49
Insect gut	25.5	4.42	16.15
Wastewater	19.5	0.20	0.55
Plant	13.7	3.26	11.07
Soil	11.1	0.20	1.37
Activated sludge	10.0	0.02	0.05
Coral	8.5	0.38	0.66
Chicken gut	8.2	0.23	1.17

Human gut	7.9	6.17	16.59
Human skin	7.4	0.22	0.67
Pig gut	5.7	0.04	0.08
Human vaginal	5.0	0.02	0.04
Marine sediment	4.9	0.10	0.21
Mouse gut	4.8	0.15	0.33
Human lung	4.3	0.38	1.61
Marine	4.2	0.05	0.22
Bovine gut	2.5	0.03	0.05
Human oral	0.9	0.20	0.36

3.5. Secondary Metabolites

Secondary metabolite biosynthesis gene clusters encoded by the genome of strain V-78 were identified by the antiSMASH server. The strain has two gene clusters coding for a betalactone and a thiopeptide. The betalactone gene cluster shared 2% similarity with the gene cluster of gausemycin A/B, a lipoglycopeptide antibiotic, while the thiopeptide gene cluster showed 33% similarity to the menaquinone biosynthetic gene cluster. In addition, RAST annotation revealed that the genome of strain V-78 encodes genes for anthranilate phosphoribosyltransferase, phosphoribosylanthranilate isomerase, and tryptophan synthase alpha and beta chains, which are responsible for synthesis of auxin-type plant hormones.

3.6. Antimicrobial Susceptibility

The strains were fully resistant to lincomycin, and full susceptibilities were tested for sulfamethoxazole + trimetprim and ciprofloxacin. The quality control strains, *A. salmonicida* subps. *salmonicida* and *E. coli*, had the suggested values in the CLSI (Table 4). However, cut-off values are not presented for *Plesiomonas* or *Enterobacterales* in EUCAST [42] for amoxicillin, doxycycline, tetracycline, oxytetracycline, oxolinic acid, enrofloxacin, flumequine, cefalexin, ampicillin, erythromycin, lincomycin, and florfenicol; therefore, the strains could not be evaluated to be susceptible or resistant (Table 4). Data on the evaluated breakpoints for SXT, CIP, CN, AMP, and MY are presented in Table 4.

Table 4. The inhibition zones (mm) of antimicrobial agents for quality control and *P. shigelloides* strains.

Family	**Antibiotics**	**Disk Content (μg)**	***A. salmonicida* subps. *salmonicida* *ATCC 33658***	***E. coli* ATCC 25922**	***P. shigelloides***		
					V-63	**V-78**	**AF160**
Sulfonamides	Trimethoprim/Sulfamethoxazole (SXT)	25	23	27	25 (S)	25 (S)	21 (S)
Aminopenicillins	Amoxicillin (AML) *	25	32	23	12	13	25
Tetracyclines	Doxycycline (DO) *	30	31	25	22	20	15
	Tetracycline (TE) *	30	35	30	15	13	12
	Oxytetracycline (OT) *	30	30	29	10	0 (R)	0 (R)
Quinolone	Oxolinic Acid (OA) *	2	35	28	24	25	10
Fluoroquinolone	Enrofloxacin (ENR) *	5	40	37	31	35	25
	Ciprofloxacin (CIP)	5	50	40	33 (S)	33 (S)	25 (S)
	Flumequine (UB) *	30	40	35	26	30	28
Cephalosporins	Cefalexin (CN) *	10	25	25	20 (S) **	20 (S) **	21 (S) **
Penicillins	Ampicillin (AMP) *	10	32	20	11 (R) **	12 (R) **	10 (R) **
Macrolides	Erythromycin (E) *	15	22	8	16	11	10

Clindamycin	Lincomycin (MY) *	15	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Chloramphenicol	Florfenicol (FFC) *	30	40	24	11	12	12

* Cut-off values are not available for the *Plesiomonas* or *Enterobacterales* species on EUCAST. ** Evaluation was performed for *Enterobacterales* on EUCAST.

3.7. Antimicrobial Resistance Genes and Virulence

A total of 24 antimicrobial resistance models and targets were determined by the ARTS server across 19 genomes. The detected resistance models include ATP-binding cassette (ABC) antibiotic efflux pump, CARB-PSE beta-lactamases (Class a), chloramphenicol acetyltransferase (CAT), ABC transporter, membrane fusion proteins of the MexEF-OprN and MexGHI-OpmD multidrug efflux complexes, major facilitator superfamily (MFS) antibiotic efflux pump, and resistance–nodulation–cell division (RND) antibiotic efflux pump. Of the 24 resistance models and targets, Class A beta-lactamase (RF0053) and tetracycline resistance MFS efflux pump were encoded only in the genome of strain V-78. The former is responsible for resistance through hydrolysis of beta-lactam antibiotics such as penicillins, cephalosporins, and carbapenems, while the latter enables the bacteria to pump out tetracycline or tetracycline derivatives selectively (Table S2).

Identifying the genomic islands encoding mainly virulence factors, antimicrobial resistance genes, and other adaptation elements is a useful strategy for adopting a population-based approach to genomic epidemiology and characterization [34]. The IslandCompare tool was used to identify and compare the genomic islands residing in the genomes of *P. shigelloides* strains. All strains differed in terms of size, number, location, and identity of the genomic islands encoded in their genomes (Figure 4).

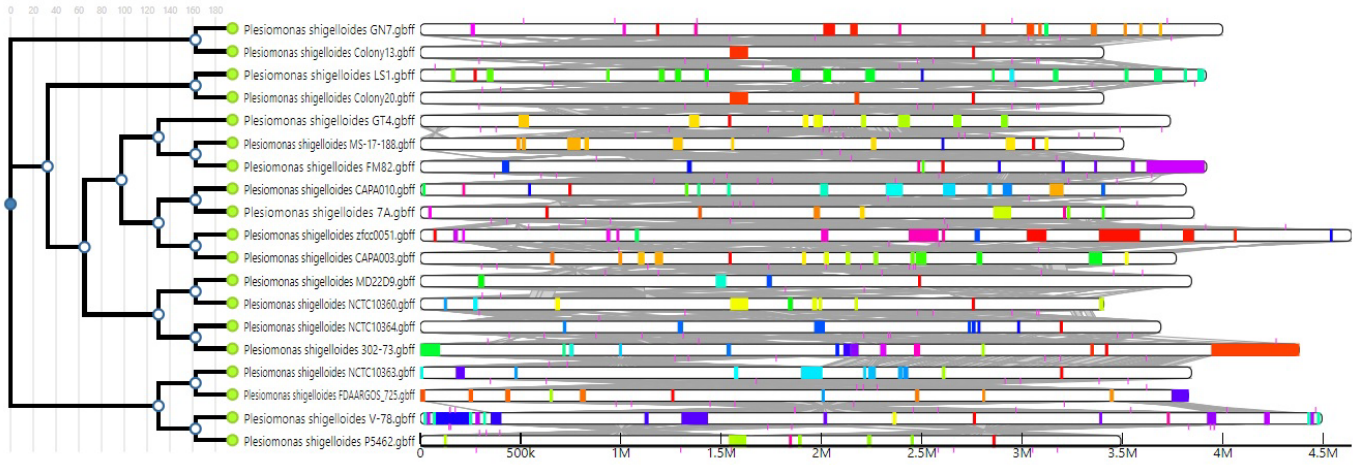

Figure 4. Genomic islands encoded in 19 *Plesiomonas* genomes.

A total of 83 virulence genes were detected in the *P. shigelloides* genomes obtained from the GenBank database, including our isolate, V-78. Of the 83 virulence genes, only 20 virulence genes were commonly found in all strains encoding motility, immune modulation, T2SS (Type II secretion system), adherence, immunogenic lipoprotein A, stress survival, biofilm formation, stress protein, and early escape from the phagosomes of macrophages, toxins and enzymes secretion. The virulence genes, according to each analyzed isolate, are presented in Supplementary Table S3. Different from other *P. shigelloides* strains, the *tviB* gene was detected only in the strains V-78 and MS-17-188 (isolated from catfish). The *tvi*B gene is relevant because it might prevent antibody-mediated opsonization and increase resistance to host peroxide, complement activation by the alternate pathway, and complement-mediated lysis. The *lgt*F and *flgG* genes, encoding for immune modulation and motility, respectively, were detected only in the genomes of strain V-78 and strain CAPA003, isolated from *Oreochromis niloticus*.

Functioning as a tripartite multidrug efflux pump essential for resistance to β-lactams (penicillin G and nafcillin), macrolides (erythromycin), host-derived compounds (peptide LL-37), and progesterone, and essential for the growth of gonococci in the lower genital tracts of experimentally infected female mice, the *mtrD* gene was found in V-78, Zfcc051 (isolated from zebra fish), and CAPA003. In addition, the *fbpC* gene, which encodes for a periplasmic-binding protein-dependent iron transport system necessary for the utilization of iron bound to transferrin or iron chelates, was found only in the genome of strain V-78.

4. Discussion

Plesiomonas shigelloides is a unique, Gram-negative, polar-flagellated pathogenic bacterium that is naturally found in freshwater ecosystems, such as rivers, lakes, surface waters, and marine estuaries in tropical and temperate climates [43]. Unlike other aquatic pathogens, the isolation of *P. shigelloides* might be enhanced by an incubation temperature of 42–44 °C, since most aeromonads or other psychrophilic microorganisms cannot tolerate this temperature range. The temperature tolerance of bacteria, especially in hot conditions (42–44 °C), causes high mortality in freshwater ornamental fish species living in climate habitats, aside from cold-water fishes such as rainbow trout [44]. In our study, the isolation of *P. shigelloides* from rainbow trout and freshwater ornamental fish species demonstrated its broad host range and temperature-tolerant characteristics. However, there are reports about the pathogenicity of *P. shigelloides* for fish species, especially for rainbow trout. This is the first study providing a description (see Appendix A) of a novel *P. shigelloides* subspecies; thus, the exact pathogenicity of the species should be examined. Because *P. shigelloides* is also a member of the microbiota of rainbow trout, an experimental challenge test should be provided for the described *P. shigelloides* subspecies using pathogen-free fish. Although *P. shigelloides* is grouped in *Enterobacteriaceae*, the agent is oxidase- and catalase-positive. It also shares high similarity with the genera *Vibrio* and *Pseudomonas*, especially regarding sensitivity for the Vibriostatic agent, which we detected in our study. Besides susceptibility to the Vibriostatic agent, we identified our isolates by *gyr*B, *pry*H, *rec*A, and *atp*A, which were specifically characterized for the *Vibrio* species. Previously, the species were placed chronologically into the genera *Pseudomonas*, *Aeromonas*, and *Vibrio* [45]. Habs and Schubert [46] initially established the genus *Plesiomonas* in the family *Vibrionaceae* based on a number of its unique features and its similarity to the genus *Aeromonas* ("plesio", neighbor, "monas", *Aeromonas*). The genus *Plesiomonas* has several characteristics in common with members of the family *Vibrionaceae*, such as being polarly flagellated, facultatively anaerobic, and cytochrome oxidase-positive. The similarity between our study and the taxonomical history of *P. shigelloides* enable the misidentification of this species as *Vibrio* sp. As it is commonly reported that *P. shigelloides* is the only member of the *Plesiomonas* genus, the strains have a genetic homology and a close relationship with each other. Unlike the close phylogenetic relationships among *P. shigelloides* strains, Gu et al. [47] reported that the strains had significant variability based on isolation source, as was revealed by Random Amplified Polymorphic DNA (RAPD) analysis. In our study, the strains obtained from the GenBank database were clustered in a phylogenetic group; however, our strain was separated from all genomes uploaded in the GenBank database. Although the genome of strain V-78 encodes 5454 genes, a higher number than the genomes of the other strains analyzed, it has the lowest number of core genes among the strains, which implies its flexibility for horizontal gene transfer events. The genomic island comparison also approved the versatility of the *P. shigelloides* genomes, since each genome has various genomic islands scattered across it.

The members of *P. shigelloides* naturally occur in freshwater and marine habitats. However, the comprehensive analysis of taxonomic, functional, and ecological features revealed that strain V-78 mostly matched with the 16S rRNA gene amplicons obtained from rhizosphere metagenomes followed by freshwater and insect gut metagenomes.

Both CAZymes and secondary metabolites, such as plant hormones produced by *P. shigelloides* strains, may contribute to their adaptation to various environments, from the plant rhizosphere to the insect gut.

From a taxonomical point of view, strain V-78 is certainly a member of the genus *Plesiomonas*. However, the overall genome-relatedness indices, i.e., dDDH, ANI, and POCP, revealed that strain V-78 might be differentiated from the type strain and other strains of *P. shigelloides*. The dDDH, ANI, and POCP values between strain V-78 and the type strain of *P. shigelloides* were calculated as 74.2%, 97.1%, and 68.1%, respectively. These relatively low values imply that strain V-78 must be considered a subspecies of *P. shigelloides*. For validation of the novel subspecies presented in the manuscript, we also followed the International Committee on Systematics of Prokaryotes and the Bacteriology and Applied Microbiology Division of the International Union of Microbiological Societies. In this respect, genome sequence, general genomic information, ANI and dDDH values, 16S rRNA gene accession numbers, isolation, habitat and sample description, morphology and growth conditions, physiology, functional and ecological analysis, and species descriptions were also provided. We also intend to deposit our strains in two or more public culture collections located in different countries for final validation of the V-78 strain as a subspecies of *P. shigelloides*, as ruled by the International Committee on Systematics of Prokaryotes.

In humans, most enteric *Plesiomonas* infections are self-limiting and do not need specific antimicrobial treatment. Some severe dysenteric and chronic intestinal infections, however, benefit from antimicrobial therapy. In these cases, fluoroquinolones and trimethoprim are generally considered to be the best oral agents because they have been shown to shorten the clinical course of diarrhea caused by *Plesiomonas* [48,49]. For fish, because *P. shigelloides* is an agent that seems to be an opportunistic pathogen or host of microbiota, veterinarians do not need an antimicrobial treatment when the mortality rate is low. However, empiric treatments have been used in high-mortality cases due to lack of identification or the fact that this species was ignored. Unfortunately, CLSI and EUCAST do not publish a breakpoint for antimicrobials commonly used in fish health other than ciprofloxacin and trimethoprim/sulfamethoxazole. We tested our strains on fourteen different antimicrobial agents belonging to nine groups; unfortunately, we determined our isolates to be susceptible or resistant to only two agents. The isolates are commonly tested and reported as susceptible or not for antimicrobials used in humans, but few reports have suggested antimicrobials for fish isolates [50]. Due to the ubiquitous characteristics of *P. shigelloides*, we do not suggest empirical treatment or antimicrobial usage for fish strains because the strains could easily gain antimicrobial resistance, as revealed by the genomic island analysis. We also suggest that epidemiological cut-off values (ECVs) should be established to determine the antimicrobial susceptibility of *P. shigelloides* strains. While there is a significant gap in the antimicrobial susceptibility values of *P. shigelloides*, we detected 19 to 34 antimicrobial resistance genes in the genomes of the *P. shigelloides* strains which we employed. Unlike other strains obtained from the GenBank database, the genome of strain V-78 harbors a relatively low number of antimicrobial resistance genes.

As it is an important opportunistic pathogen, the pathogenesis of *P. shigelloides* can be correlated to multiple virulence genes which encode secreted proteins and toxins. A variety of virulence factors have been associated with the pathogenesis of this microorganism, including β-hemolysins [51–53], enterotoxins [54], cholera-like toxins [55], and possible endotoxins [56]. Among them, *ast* and *act* encode enterotoxins in many bacteria, which damage intestinal epithelial cells; lyse red blood cells; and cause gastroenteritis, water-like diarrhea, and death [57,58]. In our studies, the *P. shigelloides* strains which we analyzed harbored 20 common virulence genes, including *che*D, *flh*A, *fli*I, *fli*N, *fli*p, *gmh*A/*lpc*A, *gsp*D, *gsp*E, *gsp*G, *htp*B, *Ilp*A, *kat*B, *kds*A, *lpx*C, *lpx*D, *lux*S, *mgt*B, *clp*C, *rfa*D, and *xcp*R, which may be related to pathogenicity in humans, animals, and fish.

5. Conclusions

Although various virulence gene determinants were assessed in *P. shigelloides* strains that originated from different sources, this is the first comprehensive report presenting virulence genes in detail. We also present all virulence genes, totaling 83, found in the genomes of all employed strains. Our strain, V-78, showed a difference from the antimicrobial resistance genes of other employed strains, but all strains had a similar count of virulence genes, ranging between 39 and 43. These findings regarding virulence genes suggest that *P. shigelloides* poses an important threat to all living organisms by means of different virulence genes, yet with similar counts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8040179/s1. Table S1: Average nucleotide identity values between the genome of strain V-78 and closely related type strains, inferred by the Protologger using FastANI (v1.2) algorithm; Table S2: Antibiotic resistance models detected in the genomes of Plesiomonas shigelloides strains by the ARTS server; Table S3: Common and strain-based virulence genes detected in the analyzed genomes; Figure S1: The genome-based tree was created in the Protologger software after the genome was checked for completeness and contamination using CheckM (v1.0.12) [59]. Then, a concatenated protein sequence tree approach employing multiple marker genes was applied by the Protologger using GTDB-Tk (r89) (v1.2.0) for placement within the GTDB taxonomy system via recognition of marker genes to assign a domain [60]. Figure S2: *Plesiomonas shigelloides* strain V-63 tree based on 16S rRNA sequence. Figure S3 *Plesiomonas shigelloides* strain V-78 tree based on 16S rRNA sequence.

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Data Availability Statement: The data presented in this study are available with manuscript file and supplementary files. The sequence data were deposited in the GenBank with accession number for the whole genome is JAQFHV000000000 and also accession numbers for the 16S rRNA genes are OQ683920 and OQ683923 for strain V-63 and V-78, respectively.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Protologue: Emended description of *Plesiomonas shigelloides* (Bader 1954) Habs and Schubert 1962.

N.L. fem. dim. n. Shigella, a generic name; L. adj. suff. -oides, ressembling, similar; from Gr. neut. adj. suff. -eides, resembling, similar; from Gr. neut. n. eîdos, that which is seen, form, shape, figure; N.L. adj. shigelloides, Shigella-like

The description is the same as reported by Janda (2015), with the following modifications:

The genome size of the type strain is 3.4 Mb and genomic G + C content is 52.0%. Type strain: ATCC 14029; CCUG 410; CIP 63.5; DSM 8224; LMG 4242; NCCB 80007; NCTC 10360.

cvof *Plesiomonas shigelloides* subsp *shigelloides* subsp. nov.

N.L. fem. dim. n. *Shigella*, a generic name; L. adj. suff. *-oides*, ressembling, similar; from Gr. neut. adj. suff. *-eides*, resembling, similar; from Gr. neut. n. *eîdos*, that which is seen, form, shape, figure; N.L. adj. *shigelloides*, Shigella-like

The description is as given by Janda (2015), with the following modifications: The genome size of the type strain is 3.4 Mb and genomic G + C content is 52.0%. Genome size of strains in the subspecies ranges from 3.0 to 4.08. Genomic GC content ranges from 50.9 to 52.4%. Type strain: ATCC 14029; CCUG 410; CIP 63.5; DSM 8224; LMG 4242; NCCB 80007; NCTC 10360.

Description of *Plesiomonas shigelloides* subsp. *oncorhynchi* subsp. nov.

on.co.rhyn'chi. N.L. gen. masc. n. *oncorhynchi*, of *Oncorhynchus*, named after the rainbow trout, *Oncorhynchus mykiss*, from which the type strain was isolated

Gram-negative, short bacilli, motile, oxidase and catalase positive, facultatively aerobic, glucose fermentative, non-hemolytic on sheep blood, grown on Mac Conkey agar but not on thiosulfate-citrate-bile salts-sucrose agar (TCBS). Able to tolerate up to 1.5% NaCl and grow at a temperature range of 4–45 °C. Negative for gelatin, Tween 20, and Tween 80 hydrolysis. Susceptible to Vibriostatic agent at 10 μg and 150 μg. The isolate V-63 was collected from rainbow trout weighing 0.5–1 g from farms located in Kütahya (latitude: 39°46′16.5″N, longitude: 29°38′32.5″E), and the sampling date was 01.04.2018. The isolate V-78 was collected from rainbow trout weighing 200 g from farms located in Kayseri (Latitude: 39°00′15.1″N, Longitude: 36°38′32.5″E), and the sampling date was 01.09.2013. AF160 was isolated from goldfish kept in an aquarium producer in Istanbul (latitude: 40°58′41.4″N, longitude: 28°47′15.1″E), Turkey. The sampling date for AF160 was 2021.

The genome size of the type strain was 4.4 Mb, and the genomic G + C content was 51.1%. The GenBank accession number for the whole genome is JAQFHV000000000. The GenBank accession numbers for the 16S rRNA genes are OQ683920 and OQ683923 for strain V-63 and V-78, respectively.

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