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Population genetic and evolution analysis of *Vibrio* isolated from Turkish fish farms

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ABSTRACT

The genus *Vibrio* includes important pathogenic species for human and aquatic organisms such as *Vibrio cholera*, *V. parahaemolyticus*, *V. vulnificus, V. anguillarum* or *V. harveyi*. At present, Vibrionaceae family consists of >190 described species, classified into nine genera. Vibrio are widespread in shellfish, finfish and marine ecosystems and show resourceful ecologies, which recognized as one of the most diverse bacterial groups for illuminating the genome evolution. In the present study, to clarify the relationship among aquatic species in the genus, a multilocus sequence analysis (MLSA) and typing (MLST) approach was applied to characterize 51 *Vibrio* isolates from Turkish fish farms, 146 strains deposited in the PubMLST database and 59 type strains from GenBank. For all studied isolates (n = 256), diversity analysis, population structure, determination of recombination, demographic history and gene flow were performed based on the MLST scheme. *Vibrio* isolates, subjected to the study, showed a high diversity within the *Vibrio* population and also genetic interactions into the genus. We found 17 new described sequence types by MLST analysis that were isolated from rainbow trout, sea bream and sea bass in Turkish fish farms, which clearly indicate that the genes underwent recombination frequently. While predominant sequence types were found in the presented study, differences of genotypes need to be evaluated in a disease situation or preventing measurements. The findings about genetic recombination possibly helps to understand differences of *Vibrio* infections in fish. Furthermore, elucidating of genetic variability within species shed light on providing effective measurements in aquaculture by vaccine production and drug applications.

1. Introduction

Vibrio genus comprises ubiquitous bacteria in the aquatic environment, including essential zoonotic pathogens such as *Vibrio cholera, V. parahaemolyticus*, and *V. vulnificus* (Farmer et al., 2015; Johnson et al., 2012). In addition to described zoonotic species, the genus *Vibrio* includes about 145 species and some of them cause devastating mortalities in numerous cultured fish species, primarily *V. anguillarum, V. harveyi, V. ordalii, V. alginolyticus* (Farmer et al., 2015; Parte et al., 2020; Woo and Bruno, 2011). Besides the known pathogenic species of *Vibrio*, there are also numerous newly characterized species even though their zoonotic and pathogenic role are still unknown.

In many countries, fish are vaccinated each year for protection against pathogenic agents, including *V. anguillarum, V. salmonicida, V. alginolyticus* and *V. vulnificus;* some of them are licensed vaccines (Colquhoun and Lillehaug, 2014); still, there are numerous mortality reports in cultured fish species because of *Vibrio* whether fish were vaccinated or not (Mechri et al., 2017; Sharma and Dube, 2017; Woo and Bruno, 2011). The success of vaccine development deals with obstacles such as highly variable species, highly close but different pathogenic species or misidentified bacteria (Castiblanco and Anaya, 2015). Thus, the accurate identification and exact characterization of the *Vibrio* spp. play a

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key role in protecting fish. DNA-based molecular methods have become more attractive and widely acceptable because of high discriminatory power (Mulet et al., 2010) and one of the most used molecular methods is 16S rRNA gene sequencing for identification for an agent. Together with molecular identification, genetic typing and determination of genetic variabilities lead up effective monitoring pathogen transmission especially Repetitive Extragenic Palindromic sequence (REP-PCR), Random Amplified Polymorphic DNA (RAPD), DNA-DNA hybridization technique (dDDH) and Multilocus sequence typing (MLST) (Mulet et al., 2010; Saticioglu et al., 2018).

MLST method allows discrimination of the species of the genus and determines low intraspecific genetic variations by using different housekeeping genes at the same time. To better understand genetic variations in bacteria, a public database (PubMLST), was launched and about 739,319 isolates and >20 million alleles were added recently (PubMLST, 2014). Previously, *Vibrio* spp. MLST database was constructed with different *Vibrio* spp., which were isolated from live marine animals collected from Italy in 2007 and reference strains, including 160 Sequence type (ST) and >440 alleles (https://pubmlst.org/organisms/*Vibrio*-spp) but the evolutionary relationship among the strains was not studied.

One of the MLST advantages is the possibility of progressively enriching the database by adding new isolates; therefore, we added 51 new *Vibrio* isolates from different hosts isolated between 2014 and 2018 in Turkey. The aims of this study are to clarify the identification of 51 Turkish *Vibrio* isolates by phylogenetic analysis and to elucidate the evolution of genus *Vibrio* focusing on the clades *Anguillarum, Cholera, Harveyi, Mediterranei, Orientalis, Ponticus, Scophtalmi, Splendidus* and *Vulnificus*.

2. Materials and methods

2.1. Bacterial isolates and phenotypic characterization

A total of 51 *Vibrio* isolates (Table 1) were isolated from mainly seabream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) cultures in offshore farms. Additionally, isolates were collected from rainbow trout (*Oncorhynchus mykiss*) farms using conventional flow-through systems with non-disinfected surface water and spring water located in five different regions of Turkey. The farms were selected due to their high production capacity (the farms that provided samples supplied a total of at least 80% of the production capacity of Turkey). The bacterial isolates were collected during a fish health surveillance program from internal organs of healthy (no external lesions) and diseased fish (moribund and with external lesions) of different species, weight, age and regions. Samples from external lesions, internal organs such as kidney, liver, spleen, and ascites fluid were collected monthly over four years from at least 30 farms (2014–2018).

Samples were cultured on blood agar (BA; 5% sheep blood added), brain heart infusion agar (BHIA, Merck, USA), tryptic soy agar (TSA, Merck, USA) and marine agar (MA, Merck, USA). Sampling was carried out following the guidelines for the diagnosis of fish diseases and in consideration of international and national guidelines for animal welfare (OIE, 2015).

The biochemical characteristics of all isolates were determined using conventional microbial tests such as colony morphology, color upon Gram staining, bacterial and swarming motility, oxidase and catalase activities, oxidative fermentation (O/F), growth on Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Merck, USA), susceptibility to Vibriostatic agent (0/129, 10 μg and 150 μg) different temperature and salinities, hemolysis on blood agar (5% sheep blood added), lysis on gelatin, and Tween 20 and 80 (Farmer et al., 2015).

Moreover, the sequences of 146 strains deposited in PubMLST (https://pubmlst.org/organisms/vibrio-spp), as well as the sequences of type strains ($n = 59$) housed in the GenBank database, representative of the clades identified, were used (Supplementary Table S1). It is interesting to note that some strains deposited in the database were excluded for different reasons: i) It is not *Vibrio* (ES114, ATCC 43305, V63 and V78), ii) It has an extra codon and some software does not work with spaces (Vi_20, Vi_51, Vi_54, Vi_60, Vi_62, Vi_73, Vi_9a and Vi_16a), iii) we used only type strains to known species (40B, 775, ATCC 43996, LMG 23867, NCTC 11218, ATCC 39315, RIMD 2210633 and VM603) and iv) It is duplicated (BAA-1116 and LMG 19703 T).

2.2. DNA extraction, PCR and sequence analysis

DNA was extracted with spin column filtration kits according to the manufacturer's instructions (QIAamp DNA mini kit, 51,306, Hilden, Germany). The amount and purity of DNA in each sample were measured at wavelengths of 260 nm and 260/280 nm with a spectrophotometer (Multiskan Go, Thermo, Vantaa, Finland).

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. All Turkish isolates included in this study were previously identified as *Vibrio* sp. by 16S rRNA gene sequencing (data not shown). Following the previous MLST database, the housekeeping genes used were *atp*A (ATP synthase α subunit), *gyr*B (DNA gyrase β subunit), *pry*H (Uridylate kinase) and *rec*A (Recombinase A) (https://pubmlst.org/organisms/vibrio-spp; Jäckel et al., 2020; Preheim et al., 2011; Rahman et al., 2014; Schwartz et al., 2017). Polymerase chain reaction (PCR) was performed in a total volume of 25 μl containing 2.5 μl 10× PCR Buffer, 5 μl 5× Q-Solution, 0.5 μl 0.2 mM dNTP mix (10 mM of each), 37.5 pmol of each primer (Supplementary Table S2), 1 μl 25 mM $MgCl_2$, 0.125 μl HotStarTaq DNA Polymerase (Qiagen, Venlo, Netherlands), 10 ng DNA template, and DNase/RNase-Free Distilled Water. All PCR products were sent for sequence analysis to Macrogen Korea (Republic of Korea). Sequences obtained for *atp*A, *gyr*B, *pyr*H and *rec*A gene regions of 51 *Vibrio* isolates were deposited in the pubmlst database (https://pubmlst.org/organisms/vibrio-spp), allele numbers and sequence types (STs) were generated by pubmlst database.

2.3. Diversity analyses and population structure

Both forward and reverse sequences for each strain were trimmed, aligned and analyzed using BioNumerics software (version 7.6, Applied Maths, Sint-Martens-Latem, Belgium). For each MLST locus, the sequences obtained for all isolates were compared, and each unique sequence (differing from the others at one or more nucleotides) was assigned with a new allele number. Each isolate was unambiguously defined by an allele profile or sequence type (ST) derived from the combination of numbers corresponding to the alleles at each of the loci analyzed. The same ST was assigned to multiple strains when they shared the same allelic profile. Allelic profiles were used for subsequent analysis.

A descriptive diversity analysis was performed for the individual genes and concatenated sequences (*atp*A, *gyr*B, *pyr*H and *rec*A). The mean G + C content of the DNA along with the d_N/d_S ratios (where d_N is the number of nonsynonymous substitutions per nonsynonymous site, and d_S is the number of synonymous substitutions per synonymous site), number of polymorphic sites, indexes of association (I^A and I^A_S) (Maynard et al., 1993) were calculated using START2 (http://pubmlst.org/software/analysis/start2/). Nucleotide diversity per site (π) was estimated using DnaSP v5 (Librado and Rozas, 2009). The clonal structure of the population was established using Phyloviz software by goeBURST 1.2.1 algorithm (Francisco et al., 2009). ClonalFrame v1.1 (Didelot and Falush, 2007) was used to construct a dot graph using a 50% majority rule consensus tree with ten runs of MCMC (Markov Chain Monte Carlo). For phylogenetic analysis, concatenated sequences were aligned and trees were performed for the whole collection with the program MEGA version X (Kumar et al., 2016) by Neighbor-joining (NJ)

Table 1
Origin and MLST data of *Vibrio* isolates employed in this study.

Bacteria code	Fish species	Isolation date	Isolated city	Isolated region	Fish lesion	MLSA result	Sequence type (ST)
V1	live marine animals	ND	ND	ND	ND	*V. alginolyticus*	130
V10	*Dicentrarchus labrax*	Jul-2016	Mugla	Aegean region	ND	*V. alginolyticus*	134
V13	*Dicentrarchus labrax*	Jan-2016	Mugla	Aegean region	ND	*V. alginolyticus*	136
V16	*Dicentrarchus labrax*	Apr-2017	Mugla	Aegean region	ND	*V. alginolyticus*	137
V19	*Dicentrarchus labrax*	Dec-2016	Kayseri	Central Anatolia	ND	*V. alginolyticus*	134
V20	*Sparus aurata*	Feb-2016	Kayseri	Central Anatolia	ND	*V. alginolyticus*	137
V22	*Dicentrarchus labrax*	Jun-2017	Elazıg	Eastern anatolia	ND	*V. alginolyticus*	136
V23	*Sparus aurata*	May-2017	Elazıg	Eastern anatolia	ND	*V. alginolyticus*	134
V26	*Dicentrarchus labrax*	Feb-2017	Mugla	Aegean region	ND	*V. alginolyticus*	134
V69	*Oncorhynchus mykiss*	May-2011	Rize	Black sea region	darkening in color, haemorhagia, fin erosion	*V. alginolyticus*	134
V72	*Dicentrarchus labrax*	Jan-2016	Mugla	Aegean region	No lesion	*V. alginolyticus*	134
V73	*Dicentrarchus labrax*	Jul-2017	İzmir	Aegean region	Anemia in liver	*V. alginolyticus*	140
V74	*Dicentrarchus labrax*	Jul-2017	İzmir	Aegean region	Anemia in liver	*V. alginolyticus*	140
V75	*Oncorhynchus mykiss*	Feb-2015	Mugla	Aegean region	No lesion	*V. alginolyticus*	141
V103	*Dicentrarchus labrax*	Jun-2015	Canakkale	Maramara region	High mortality	*V. alginolyticus*	142
V5	live marine animals	Jun-2008	Norway	ND	ND	*V. anguillarum*	131
V9	*Dicentrarchus labrax*	Jun-2016	Mugla	Aegean region	ND	*V. anguillarum*	133
V12	*Dicentrarchus labrax*	Jan-2016	Mugla	Aegean region	ND	*V. anguillarum*	135
V15	*Oncorhynchus mykiss*	May-2017	Mugla	Aegean region	ND	*V. anguillarum*	133
V18	*Oncorhynchus mykiss*	Dec-2016	Tunceli	Eastern anatolia	ND	*V. anguillarum*	133
V21	*Sparus aurata*	Jun-2017	Elazıg	Eastern anatolia	ND	*V. anguillarum*	133
V25	*Sparus aurata*	May-2017	Mugla	Aegean region	ND	*V. anguillarum*	135
V28	*Sparus aurata*	Jan-2017	Mugla	Aegean region	ND	*V. anguillarum*	133
V32	*Sparus aurata*	Jan-2017	Mugla	Aegean region	ND	*V. anguillarum*	133
V76	*Oncorhynchus mykiss*	Aug-2013	Mugla	Aegean region	Moribund fish	*V. anguillarum*	133
V80	*Oncorhynchus mykiss*	Feb-2015	Mugla	Aegean region	No lesion	*V. anguillarum*	133
V82	*Oncorhynchus mykiss*	Jan-2015	Mugla	Aegean region	No lesion	*V. anguillarum*	133
V85	*Oncorhynchus mykiss*	Feb-2011	Rize	Black sea region	darkening in color, haemorhagia, fin erosion	*V. anguillarum*	133
V88	*Oncorhynchus mykiss*	Feb-2014	Mugla	Aegean region	Darkening in color	*V. anguillarum*	133
V91	*Oncorhynchus mykiss*	Feb-2014	Mugla	Aegean region	Darkening in color	*V. anguillarum*	133
V92	*Oncorhynchus mykiss*	Feb-2014	Mugla	Aegean region	Darkening in color	*V. anguillarum*	133
V95	*Oncorhynchus mykiss*	Nov-2013	Mugla	Aegean region	Haemorhage in fin base	*V. anguillarum*	133
V96	*Oncorhynchus mykiss*	Nov-2013	Mugla	Aegean region	Haemorhage in fin base	*V. anguillarum*	133
V98	*Dicentrarchus labrax*	Jun-2015	Canakkale	Maramara region	High mortality	*V. anguillarum*	133
V99	*Oncorhynchus mykiss*	Apr-2015	Mugla	Aegean region	No lesion	*V. anguillarum*	133
V100	*Oncorhynchus mykiss*	Apr-2015	Mugla	Aegean region	No lesion	*V. anguillarum*	133
V101	*Dicentrarchus labrax*	Jun-2015	Canakkale	Maramara region	High mortality	*V. anguillarum*	133
V102	*Dicentrarchus labrax*	Jun-2015	Canakkale	Maramara region	High mortality	*V. anguillarum*	133
V104	*Dicentrarchus labrax*	Jun-2015	Canakkale	Maramara region	High mortality	*V. anguillarum*	133
V109		Feb-2017	Mugla	Aegean region	No lesion	*V. anguillarum*	133

(*continued on next page*)

Table 1 (*continued*)

Bacteria code	Fish species	Isolation date	Isolated city	Isolated region	Fish lesion	MLSA result	Sequence type (ST)
	Dicentrarchus labrax						
P143	*Dicentrarchus labrax*	Jan-2017	Antalya	Mediterranean region	Darkening in color, exophthalmos	*V. anguillarum*	133
V145	*Dicentrarchus labrax*	2014	Muğla	Aegean region	No lesion	*V. anguillarum*	133
V146	*Dicentrarchus labrax*	2014	Mugla	Aegean region	No lesion	*V. anguillarum*	133
V24	*Dicentrarchus labrax*	May-2016	Elazıg	Eastern anatolia	ND	*V. diabolicus*	139
V7	live marine animals	Apr-2007	Bangladesh	Decca	ND	*V. fluvialis*	132
V17	*Dicentrarchus labrax*	Aug-2017	Mugla	Aegean region	ND	*V. harveyi*	138
V110	*Sparus aurata*	Feb-2018	Mugla	Aegean region	No lesion	*V. harveyi*	143
V111	*Sparus aurata*	Feb-2017	Mugla	Aegean region	No lesion	*V. harveyi*	144
V147	*Sparus aurata*	2017	Mugla	Aegean region	No lesion	*V. harveyi*	145
V148	*Sparus aurata*	2017	Mugla	Aegean region	No lesion	*V. harveyi*	146
V6	live marine animals	ND	ND	ND	ND	*V. parahaemolyticus*	117

ND: No data, table order was construed according to the alphabetical order of species.

method using Jukes-Cantor method and by Maximum Likelihood (ML) algorithm using General Time Reverse model (GTR + G + I), with 1000 bootstrap pseudoreplicates in both cases. For the ML reconstruction, optimal evolution models were estimated from nucleotide data using MEGA X software considering 24 substitution types. The best model was selected using the Bayesian Information Criterion (BIC). A network constructed by the Median Joining method using the program NETWORK 4.1 (Fluxus-engineering) (Bandelt et al., 1999) was displayed to establish the relationship among haplotypes.

2.4. Recombination analyses

The phi test for recombination, based on individual loci from the whole strain collection, was performed using SplitsTree v4 (Huson and Bryant, 2006). The *P*-value (a value of $P < 0.05$ was considered significant herein) indicated the DNA regions exhibiting the strongest evidence of mosaicism. By DnaSP v5 (Librado and Rozas, 2009), the minimal number of recombination events (R_{min}) were calculated.

Structure software (Falush et al., 2007; Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000) was used to identify groups with distinct allele frequencies and detect strains carrying foreign DNA among isolates. The number of clusters (K) was set from 2 to 39 and all runs were replicated five times. The best model of probability, estimated using a burn-in period of 100,000 interactions and an MCMC = 50,000, was at twenty-eight groups (K = 28). The best model of probability (K = 28) was estimated by webtool Structure Harvester (Earl and VonHoldt, 2012).

2.5. Demographic history and gene flow

DnaSP5 (Librado and Rozas, 2009) was employed to calculate the average number of nucleotides per site (π) and the number of segregating sites (θ). In addition, the Tajimás test (D) (Tajima, 1989) and Fu and Li statistics (F* and D*) (Fu and Li, 1993) were used to confirm if the mutant alleles are selectively neutral (Kimura, 1983). The haplotype structure based on haplotype frequency distribution was evaluated by Fús (F'S) (Fu, 1997) and Strobeck's (S) (Strobeck, 1987) statistics. Ramos-Onsis R2 test (Ramos-Onsins and Rozas, 2002) assessed the differences between the number of singleton mutations and the average number of nucleotide differences in the population and was used to detect a possible demographic expansion.

3. Results

3.1. Isolation, phenotypic characterization and identification

The diseased fish affected by the strains isolated in this study showed darkening in color, haemorrhagia in internal organs, skin and fin base, fin erosion, anemia in the liver and exophthalmia. Isolates were Gram-negative, motile, oxidase and catalase-positive, glucose fermentative, hydrolyzed to gelatin, tween 20 and tween 80 and sensitive to vibriostatic agent (Supplementary Table S3). All Turkish isolates included in this study were primarily identified as *Vibrio* sp. by 16S rRNA gene sequencing (data not shown), being presumptively assigned to *V. anguillarum*, *V. alginolyticus*, *V. harveyi*, *V. diabolicus, V. fluvialis* and *V. parahaemolyticus* (Supplementary Fig. S1).

3.2. Genetic diversity, clustering and phylogenetic analysis

We aligned the sequences for each strain at each MLST locus and confirmed that neither indels nor premature stop codons were present in any of the sequences. The DNA fragments used for the analysis are between 570 bp (*gyr*B) to 462 (*rec*A) (Supplementary Table S2), and the concatenated sequences produced a 2022-bp fragment. The locus with more polymorphic sites was *gyr*B (131 biallelic, 44 triallelic and 71 tetrallelic sites), in contrast with the *atp*A (108 biallelic, 45 triallelic and 5 tetrallelic sites). The most frequent allele detected in the four genes were allele 77 in *atp*A (59%), allele 107 in *gyr*B (66%), allele 84 in *pyr*H (63%) and allele 105 in *rec*A (65.6%). The G + C content means of these genes varied from 45.68% to 52.19% (Table 2).

The nucleotide diversity per site (π) and the number of segregation sites (θ) for the concatenated sequences were 0.12637 and 0.12509, respectively. Values of these estimators for each individual gene in the whole population are presented in Table 3. The d_N/d_S ratio is a common indicator of selection pressure, with $d_N/d_S > 1$ indicating positive selection and $d_N/d_S < 1$ indicating purifying selection for the gene sequence tested. For all loci, the ratio d_N/d_S was less than one (Table 3). This indicated that all loci were under strong purifying selection, which is optimal for MLST loci.

The concatenated sequences were constituted by 201 different alleles or STs (Table 3), 76 of which were sinapomorfic (shared and derived). Although we avoided using some strains from the original MLST, all old STs were checked with the aim to recognize new STs. Among the 51 Turkish *Vibrio* strains which were isolated in the present study, we determined 7 new STs (103, 105, 106, 107, 108, 109, 110) (Table 2).

Table 2
Sequence type and G + C content of Vibrio isolates.

Isolate	ST	atpA	gyrB	pyrH	recA	Mean %GC
Vi_2	1	48,47	50,35	49,3	48,05	**49,04**
Vi_9	2	48,67	47,89	47,9	48,05	**48,13**
Vi_10	3	47,24	49,3	48,9	45,67	**47,78**
Vi_11	4	47,03	49,3	49,5	45,89	**47,93**
Vi_12	3	47,24	49,3	48,9	45,67	**47,78**
Vi_13	5	47,03	49,12	49,7	46,32	**48,04**
Vi_14	6	47,03	48,77	49,5	45,89	**47,8**
Vi_16	7	47,03	48,6	46,51	50	**48,03**
Vi_18	8	47,85	45,09	46,71	48,27	**46,98**
Vi_21	9	48,26	51,93	52,69	50,65	**50,88**
Vi_22	10	46,63	48,77	49,5	45,45	**47,59**
Vi_23	11	47,44	49,3	49,3	45,89	**47,98**
Vi_24	12	47,44	49,12	49,5	45,89	**47,99**
Vi_25	13	47,03	49,12	49,5	46,1	**47,94**
Vi_26	14	47,44	46,32	47,7	46,54	**47**
Vi_27d	15	47,65	47,02	49,5	49,35	**48,38**
Vi_28	16	48,67	49,12	49,5	48,7	**49**
Vi_29	17	47,44	47,89	48,1	48,7	**48,04**
Vi_31	18	47,65	47,02	49,5	48,92	**48,27**
Vi_32	19	47,44	47,72	48,1	48,92	**48,05**
Vi_33	20	47,85	47,89	49,5	48,7	**48,49**
Vi_34	21	47,85	47,37	49,3	48,48	**48,25**
Vi_35	22	47,85	47,72	49,3	49,13	**48,5**
Vi_36	23	47,44	47,72	47,9	48,7	**47,94**
Vi_37	24	47,65	46,84	49,5	48,7	**48,17**
Vi_38	25	47,85	48,42	48,5	46,54	**47,83**
Vi_39	26	47,85	46,84	49,3	48,92	**48,23**
Vi_40d	27	47,85	48,77	49,5	48,48	**48,65**
Vi_41	28	47,85	47,19	48,3	47,62	**47,74**
Vi_42	29	49,69	46,32	48,7	48,27	**48,25**
Vi_43	30	48,06	47,37	49,3	48,7	**48,36**
Vi_44	31	48,06	47,37	49,3	48,92	**48,41**
Vi_45	32	47,85	46,32	48,7	48,05	**47,73**
Vi_46	33	47,85	46,32	48,7	48,05	**47,73**
Vi_47	5	47,03	49,12	49,7	46,32	**48,04**
Vi_48	32	47,85	46,32	48,7	48,05	**47,73**
Vi_49	32	47,85	46,32	48,7	48,05	**47,73**
Vi_50	34	47,85	46,67	49,1	48,27	**47,97**
Vi_52	35	46,42	48,07	48,3	47,4	**47,55**
Vi_53	36	46,42	47,02	47,5	47,4	**47,09**
Vi_55	37	47,24	48,6	49,3	46,32	**47,86**
Vi_56	38	48,47	47,19	48,5	47,84	**48**
Vi_57	39	47,24	49,3	49,5	46,1	**48,04**
Vi_58	40	47,65	48,95	49,3	46,32	**48,05**
Vi_59	3	47,24	49,3	48,9	45,67	**47,78**
Vi_61	41	46,83	47,19	47,9	47,4	**47,33**
Vi_63	42	47,65	47,72	47,9	48,92	**48,05**
Vi_64	43	47,65	48,95	49,5	48,7	**48,7**
Vi_66	43	47,65	48,95	49,5	48,7	**48,7**
Vi_67	44	47,44	48,42	47,5	48,48	**47,96**
Vi_68	45	47,65	49,12	48,1	47,84	**48,18**
Vi_69	46	47,85	46,32	48,7	48,27	**47,78**
Vi_70	47	47,44	47,89	49,3	48,27	**48,23**
Vi_71	48	47,85	49,12	48,5	46,54	**48**
Vi_72	49	47,03	49,12	49,5	45,89	**47,89**
Vi_74	32	47,85	46,32	48,7	48,05	**47,73**
Vi_79	50	47,03	46,84	47,31	47,19	**47,09**
Vi_80	51	48,06	47,37	49,3	48,7	**48,36**
Vi_81	52	47,65	49,3	48,3	46,32	**47,89**
Vi_1a	53	47,44	47,37	49,5	49,35	**48,42**
Vi_2a	54	45,6	45,44	47,9	47,4	**46,59**
Vi_3a	55	47,65	49,47	48,5	47,62	**48,31**
Vi_4a	56	47,85	49,12	48,1	47,4	**48,12**
Vi_5a	57	47,65	47,54	49,3	48,7	**48,3**
Vi_6a	58	48,26	48,77	48,1	44,59	**47,43**
Vi_7a	59	47,65	48,42	48,3	48,27	**48,16**
Vi_8a	60	48,06	47,72	49,3	48,92	**48,5**
Vi_10a	29	49,69	46,32	48,7	48,27	**48,25**
Vi_11a	61	48,26	47,19	48,9	47,84	**48,05**
Vi_12a	62	48,06	46,67	48,9	48,05	**47,92**
Vi_13a	63	47,44	47,02	49,3	48,48	**48,06**
Vi_14a	64	47,44	47,19	47,9	48,05	**47,65**
Vi_15a	65	47,03	48,6	46,71	50	**48,08**

Table 2 (*continued*)

Isolate	ST	atpA	gyrB	pyrH	recA	Mean %GC
Vi_17a	66	47,65	47,02	49,7	49,35	**48,43**
Vi_18a	67	47,65	48,95	48,7	46,54	**47,96**
Vi_19a	68	47,44	47,54	48,7	48,05	**47,94**
Vi_20a	69	47,65	46,84	49,5	48,7	**48,17**
Vi_21a	70	47,65	48,77	48,5	48,05	**48,24**
Vi_22a	71	47,85	48,42	48,1	46,54	**47,73**
Vi_23a	65	47,03	48,6	46,71	50	**48,08**
Vi_24a	72	47,03	50,53	46,71	49,57	**48,46**
Vi_25a	70	47,65	48,77	48,5	48,05	**48,24**
Vi_26a	67	47,65	48,95	48,7	46,54	**47,96**
Vi_27a	73	48,06	48,42	48,7	46,32	**47,88**
Vi_28a	74	47,85	46,84	49,5	48,7	**48,22**
Vi_29a	75	48,26	48,77	48,1	46,32	**47,86**
Vi_30a	76	47,85	47,19	49,5	48,7	**48,31**
Vi_31a	77	47,03	48,6	46,51	49,78	**47,98**
Vi_32a	78	48,26	47,37	48,9	48,27	**48,2**
Vi_33a	79	47,44	47,89	48,1	48,7	**48,04**
Vi_34a	19	47,44	47,72	48,1	48,92	**48,05**
Vi_35a	80	47,44	47,54	48,3	48,48	**47,94**
Vi_36a	81	47,44	47,54	48,1	48,48	**47,89**
Vi_37a	82	47,85	48,95	48,1	46,97	**47,97**
Vi_38a	83	46,63	47,37	47,5	47,62	**47,28**
Vi_39a	84	48,26	47,19	48,7	47,84	**48**
Vi_40a	85	47,85	47,72	47,9	48,7	**48,04**
Vi_41a	86	48,26	47,54	48,3	48,7	**48,2**
Vi_42a	87	47,65	47,19	47,7	47,4	**47,49**
Vi_43a	88	47,44	47,72	48,1	48,48	**47,94**
Vi_44a	89	47,85	49,12	48,1	47,4	**48,12**
Vi_45a	90	47,85	48,77	49,5	48,05	**48,54**
Vi_46a	91	47,65	49,3	48,5	46,75	**48,05**
Vi_47a	92	47,44	48,95	48,1	46,97	**47,87**
Vi_48a	93	47,44	47,37	47,9	48,48	**47,8**
Vi_49a	94	47,65	49,3	46,51	46,97	**47,61**
Vi_50a	95	47,44	48,07	47,5	46,1	**47,28**
Vi_51a	96	46,83	47,72	47,5	45,02	**46,77**
Vi_52a	97	47,85	48,6	48,5	46,54	**47,87**
Vi_53a	98	47,85	50,35	47,11	50,87	**49,04**
Vi_54a	99	48,06	47,19	48,1	48,27	**47,91**
Vi_55a	100	48,06	48,77	49,3	48,27	**48,6**
Vi_56a	101	47,65	47,19	47,9	48,48	**47,81**
Vi_57a	102	47,03	48,6	46,71	50	**48,08**
Vi_58a	103	47,85	49,3	46,31	47,84	**47,82**
Vi_59a	104	47,24	48,95	46,11	47,19	**47,37**
Vi_60a	105	47,44	47,54	47,31	46,1	**47,1**
Vi_62a	106	48,47	47,19	49,5	47,62	**48,19**
Vi_63a	107	47,65	49,3	46,91	46,97	**47,71**
Vi_64a	108	47,03	48,77	45,91	46,97	**47,17**
Vi_65a	109	47,24	49,12	46,11	47,19	**47,41**
Vi_67a	110	47,24	49,12	46,11	50	**48,12**
Vi_68a	111	51,12	52,46	51,5	53,68	**52,19**
Vi_69a	112	47,85	48,42	48,3	48,27	**48,21**
Vi_70a	113	46,63	47,02	48,7	45,89	**47,06**
Vi_71a	114	47,24	49,12	45,91	47,19	**47,36**
Vi_72a	115	47,24	47,72	47,7	47,4	**47,52**
Vi_73a	115	47,24	47,72	47,7	47,4	**47,52**
Vi_74a	116	47,85	48,77	48,3	47,62	**48,14**
Vi_75a	117	47,85	48,95	48,3	46,97	**48,02**
Vi_76a	117	47,85	48,95	48,3	46,97	**48,02**
Vi_77a	117	47,85	48,95	48,3	46,97	**48,02**
Vi_78a	118	48,26	50,35	49,5	48,92	**49,26**
Vi_79a	119	46,63	47,19	48,5	45,89	**47,05**
Vi_80a	120	47,85	47,02	48,1	48,27	**47,81**
Vi_81a	121	47,24	49,3	46,31	47,19	**47,51**
Vi_1bd	117	47,85	48,95	48,3	46,97	**48,02**
Vi_2b	122	47,44	47,37	47,9	45,45	**47,04**
Vi_3b	123	47,44	47,37	47,11	45,89	**46,95**
Vi_4b	124	47,65	48,77	49,5	48,27	**48,55**
Vi_5b	125	47,85	49,47	47,5	47,84	**48,17**
Vi_6b	126	47,44	47,54	47,11	46,1	**47,05**
Vi_7b	127	47,44	47,54	47,31	45,89	**47,05**
Vi_8b	87	47,65	47,19	47,7	47,4	**47,49**
Vi_9b	128	47,24	48,95	45,71	47,4	**47,32**
Vi_10b	129	47,65	47,89	49,1	48,48	**48,28**
V1	130	47,44	47,54	47,9	48,7	**47,9**
V5	131	47,24	48,77	49,5	45,67	**47,8**

(*continued on next page*)

Table 2 (*continued*)

Isolate	ST	atpA	gyrB	pyrH	recA	Mean %GC
V6	117	47,85	48,95	48,3	46,97	**48,02**
V7	132	48,88	51,4	51,3	52,81	**51,1**
V9	133	47,24	49,47	49,1	46,1	**47,98**
V10	134	47,65	47,54	48,1	48,7	**48**
V12	135	47,24	49,12	49,5	46,1	**47,99**
V13	136	47,44	47,37	48,3	48,48	**47,9**
V15	133	47,24	49,47	49,1	46,1	**47,98**
V16	137	47,44	47,54	48,1	48,48	**47,89**
V17	138	47,85	48,6	49,5	48,27	**48,55**
V18	133	47,24	49,47	49,1	46,1	**47,98**
V19	134	47,65	47,54	48,1	48,7	**48**
V20	137	47,44	47,54	48,1	48,48	**47,89**
V21	133	47,24	49,47	49,1	46,1	**47,98**
V22	136	47,44	47,37	48,3	48,48	**47,9**
V23	134	47,65	47,54	48,1	48,7	**48**
V24	139	47,85	47,02	49,5	49,13	**48,38**
V25	135	47,24	49,12	49,5	46,1	**47,99**
V26	134	47,65	47,54	48,1	48,7	**48**
V28	133	47,24	49,47	49,1	46,1	**47,98**
V32	133	47,24	49,47	49,1	46,1	**47,98**
V69	134	47,65	47,54	48,1	48,7	**48**
V72	134	47,65	47,54	48,1	48,7	**48**
V73	140	47,44	47,54	47,9	48,05	**47,74**
V74	140	47,44	47,54	47,9	48,05	**47,74**
V75	141	58,49	47,54	47,9	48,05	**50,5**
V76	133	47,24	49,47	49,1	46,1	**47,98**
V80	133	47,24	49,47	49,1	46,1	**47,98**
V82	133	47,24	49,47	49,1	46,1	**47,98**
V85	133	47,24	49,47	49,1	46,1	**47,98**
V88	133	47,24	49,47	49,1	46,1	**47,98**
V91	133	47,24	49,47	49,1	46,1	**47,98**
V92	133	47,24	49,47	49,1	46,1	**47,98**
V95	133	47,24	49,47	49,1	46,1	**47,98**
V96	133	47,24	49,47	49,1	46,1	**47,98**
V98	133	47,24	49,47	49,1	46,1	**47,98**
V99	133	47,24	49,47	49,1	46,1	**47,98**
V100	133	47,24	49,47	49,1	46,1	**47,98**
V101	133	47,24	49,47	49,1	46,1	**47,98**
V102	133	47,24	49,47	49,1	46,1	**47,98**
V103	142	47,24	47,19	48,1	46,1	**47,16**
V104	133	47,24	49,47	49,1	46,1	**47,98**
V109	133	47,24	49,47	49,1	46,1	**47,98**
V110	143	47,85	48,6	49,5	48,27	**48,55**
V111	144	47,65	48,6	49,5	48,27	**48,5**
P143	133	47,24	49,47	49,1	46,1	**47,98**
V145	133	47,24	49,47	49,1	46,1	**47,98**
V146	133	47,24	49,47	49,1	46,1	**47,98**
V147	145	47,24	48,6	49,5	48,27	**48,4**
V148	146	47,24	48,6	49,5	48,05	**48,35**
V alfacsensis CAIM 1831T	147	48,47	47,37	47,7	48,48	**48,01**
V alginolyticus ATCC 17749T	130	47,44	47,54	47,9	48,7	**47,9**
V anguillarum NCIMB 6T	131	47,24	48,77	49,5	45,67	**47,8**
V artabrorum Vb 118T	148	47,24	47,37	49,3	48,05	**47,99**
V atlanticus Vb 1111T	149	47,44	46,84	46,11	46,97	**46,84**
V azureus LC2 − 005T	150	47,44	47,37	47,31	46,75	**47,22**
V barjaei 3062T	151	47,44	48,42	46,71	50	**48,14**
V bivalvicida 605T	152	47,03	49,47	48,7	45,67	**47,72**
V brasiliensis LMG 20546T	153	46,63	48,07	48,3	46,75	**47,44**
V campbellii CECT 519T	154	48,06	49,12	48,5	48,92	**48,65**
V celticus CECT 7224T	155	46,83	46,84	47,9	47,62	**47,3**
V chagasii LMG 21353T	156	50,31	47,54	47,5	46,1	**47,86**
V cholerae CECT 514T	157	50,92	51,58	50,1	52,16	**51,19**
V cidicii 2756-81T	158	48,26	51,58	52,89	51,52	**51,06**
V cincinnatiensis NCTC 12012T	159	46,22	48,77	50,9	49,35	**48,81**
V crassostreae LMG 22240T	160	48,67	46,67	48,3	48,27	**47,98**
V cyclitrophicus LMG 21359T	161	47,24	46,49	47,9	46,32	**46,99**
	162	47,85	47,02	49,3	49,13	**48,33**

Table 2 (*continued*)

Isolate	ST	atpA	gyrB	pyrH	recA	Mean %GC
V diabolicus CNCM I-1629T						
V fluvialis NCTC 11327T	163	48,67	51,4	51,3	52,81	**51,05**
V fortis LMG 21557T	164	47,65	49,47	47,5	47,62	**48,06**
V furnissii NCTC 13120T	165	51,12	52,11	51,5	53,46	**52,05**
V gallaecicus CECT 7244T	166	46,01	46,32	47,9	43,94	**46,04**
V gigantis LGP 13T	167	48,67	46,67	48,5	47,19	**47,76**
V harveyi ATCC 14126T	168	47,65	46,84	48,7	48,05	**47,81**
V harveyi 1DA3	169	48,26	48,77	49,1	48,05	**48,55**
V hepatarius LMG 20362T	170	47,44	50,88	47,31	48,27	**48,47**
V hyugaensis 090810aT	171	48,06	47,02	49,3	48,27	**48,16**
V ichthyoenteri ATCC 700023T	172	48,06	47,19	46,91	46,32	**47,12**
V jasicida CECT 7692T	173	48,06	46,67	49,1	48,05	**47,97**
V kanaloae LMG 20539T	174	47,03	46,84	46,71	47,4	**47**
V lentus CECT 5110T	175	47,44	46,49	47,5	47,62	**47,26**
V maritimus CAIM 1455T	176	47,65	50,88	44,71	50,87	**48,53**
V mediterranei CECT 621T	177	47,24	49,3	45,91	47,19	**47,41**
V metoecus OP3HT	178	50,51	49,47	49,9	52,81	**50,67**
V metschnikovii NCTC 8443T	179	46,42	48,6	49,3	49,13	**48,36**
V mimicus LMG7896T	180	49,08	51,05	50,1	50,87	**50,27**
V mytili CAIM 528T	181	47,44	47,72	49,3	48,7	**48,29**
V natriegens LMG 10935T	182	47,44	47,72	47,9	50,22	**48,32**
V navarrensis CIP 103381T	183	48,26	52,46	54,49	52,81	**52,01**
V ordalii ATCC 33509T	184	48,06	48,42	49,7	45,45	**47,91**
V orientalis CECT 629T	185	49,49	48,42	48,3	49,13	**48,84**
V owensii LMG 25443T	186	49,28	47,02	48,5	47,84	**48,16**
V panuliri CAIM 703T	187	46,83	47,02	48,5	46,1	**47,11**
V parahaemolyticus ATCC 17802T	117	47,85	48,95	48,3	46,97	**48,02**
V pelagius LMG 3897T	188	47,85	49,3	45,91	47,62	**47,67**
V pomeroyi LMG 20537T	189	47,03	47,02	47,9	46,54	**47,12**
V ponticus CAIM 1751T	190	48,26	49,12	47,9	47,4	**48,17**
V rotiferianus LMG 21460T	191	47,85	48,77	47,9	47,84	**48,09**
V sagamiensis NBRC 104589T	192	47,24	44,39	43,91	47,19	**45,68**
V salilacus DSG-S6T	193	45,6	48,6	49,9	48,48	**48,15**
V scophthalmi LMG 19158T	194	48,47	48,25	48,1	46,97	**47,95**
V sinaloensis LMG 25238T	195	47,44	50	49,3	46,75	**48,37**
V splendidus LMG 19031T	196	47,24	45,61	47,9	46,75	**46,88**
V tasmaniensis LMG 20012T	197	47,03	46,32	46,31	47,62	**46,82**
V thalassae CECT 8203T	198	47,03	48,6	46,31	48,27	**47,55**
V toranzoniae CECT 7225T	199	47,65	45,26	46,91	46,1	**46,48**
V tubiashii ATCC 19109T	200	47,24	48,25	49,1	47,84	**48,11**
V variabilis CAIM 1454T	201	48,47	50,18	46,11	50,22	**48,74**
V vulnificus ATCC 27562T	118	48,26	50,35	49,5	48,92	**49,26**
Mean	–	**47,68**	**48,3**	**48,49**	**47,75**	**48,05**

Moreover, other new 43 STs were described in this work, composed of some strains employed in the analysis. The most heterogeneous species cluster in *V. alginolyticus/diabolicus,* including 32 different ST and commonly collected from live marine animals.

goeBURST analysis (Fig. 1) revealed 22 clonal complexes (CC, STs related by SLV or/and DLV) and 1202 singletons (STs). Some of these CC

Table 3
Genetic characteristics, evolutionary variation and demographic history statistics of *Vibrio* population.

Locus	Number of alleles	Number of polymorphic site (PIM)	R_{min}	π	θ	d_N/d_S	D	D*	F*	F_S	S	R_2
*atp*A	130	208 (186)	57	0,090	0,121	0,033	−0,801	−0,632	-0,851	−22,35	1	0,105
*gyr*B	160	246 (228)	77	0,137	0,124	0,0329	0,333	0,650	0,577	−21,83	1	0,158
*pyr*H	133	212 (198)	59	0,133	0,123	0,027	0,263	1051	0,767	−9,14	1	0,156
*rec*A	160	207 (184)	59	0,144	0,133	0,0242	0,262	−0,009	0,154	−29,73	1	0,160
Concatenated	201	873 (796)	253	0,126	0,125	NA	0,033	0,301	0,189	−7,79	1	0,145

PIM, parsimony informative sites; R_{min}, minimal number of recombination events; π, average number of nucleotides differences per site; θ, number of segregating sites; d_N/d_S,ratio of mean non-synonymous substitutions per non-synonymous site/mean synonymous substitution per synonymous site; D, Tajima's D statistic; D* and F*, Fu and Li statistics; F_S, Fu's statistic; S, Strobecks's statistic; R_2, Onsis R2 statistic; NA, not available.

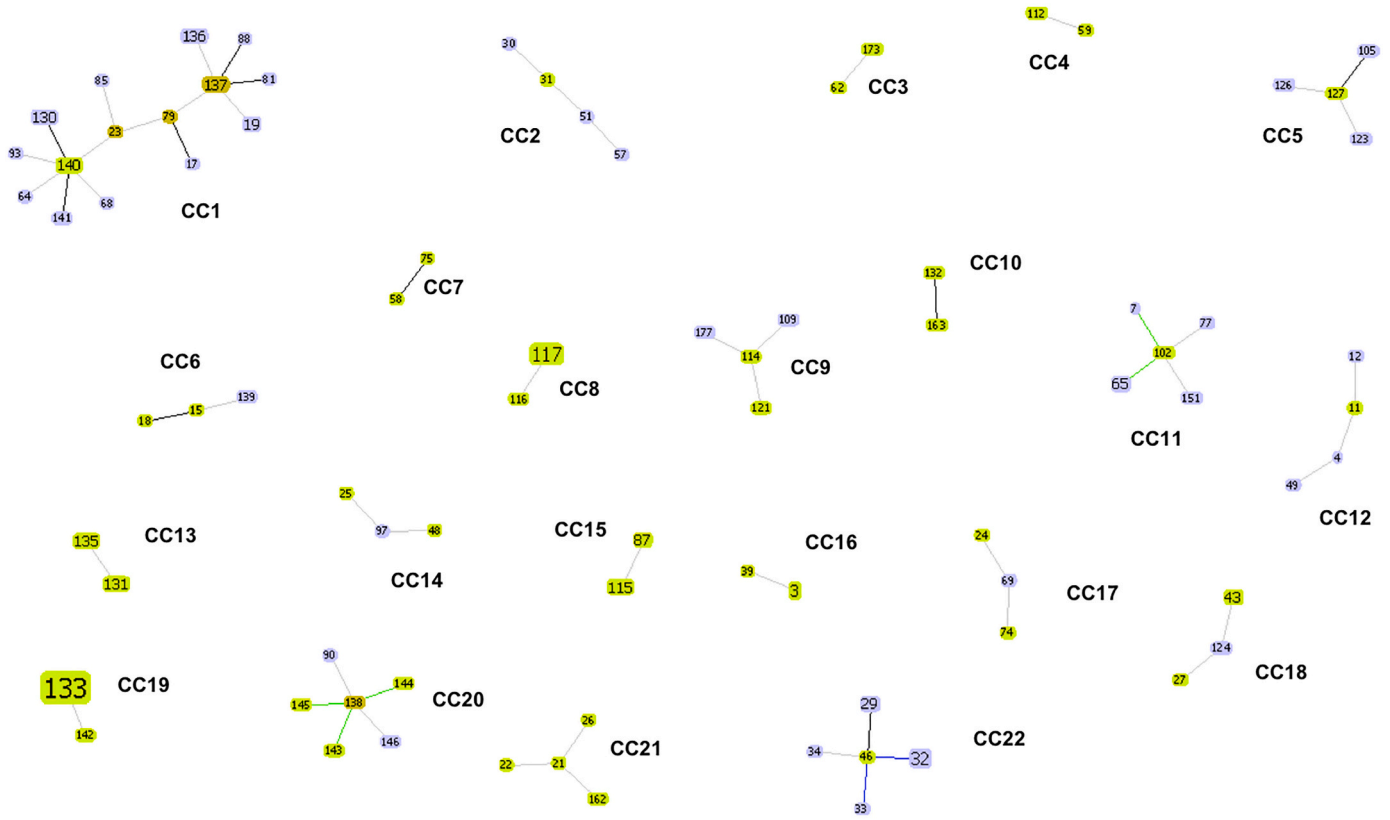

Fig. 1. Population snapshot obtained by goeBURST analysis. Each number corresponds to the ST. The size of each circle reflects the number of strains belonging to the ST. Single-locus variants (SLVs) are in black (link drawn without recourse to tiebreak rules) and blue (Link drawn using tiebreak rule 1). Double-locus variants (DLVs) are in grey and green (link drawn using tiebreak rule 2). CC, Clonal Complex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are composed by the isolates belonging to the same species which a low genetic variability as CC1 *V. alginolyticus*, *V. orientalis* CC4 CC5 *V. chagasii*,CC9 i*V. mediterranei*,*V. fluvialis* CC10; *V. barjaei* CC11and the CC15 composed by Vi_42a, Vi_8b, Vi_72a and Vi_73a probably a new species belonging to Harveyi clade. On the other hand, the species with more genetic diversity are divided in many CC as *V. diabolicus* CC2, CC6, CC18 and CC21, *V. parahaemolyticus* CC7, CC8 and CC15, *V. anguillarum* CC12, CC13, CC19, and CC16 and *V. harveyi* CC18 and CC20. Other species more close genetically are mixed in the CC3 and CC22, *V. owensii*, V. jasicida and V. hyugaensis isolates. Most of the Turkish isolates belong to ST133 identified as *V. anguillarum*.

The phylogenetic relationship of all aquatic species within the genus *Vibrio* was constructed by performing a NJ and ML tree (Supplementary Fig. S1). Before starting with the MLST studies, phylogenetic trees were performed with all *Vibrio* spp. (data not shown) with the aim of a correct identification of the isolates then, all *Vibrio* spp. not related with the strains used were deleted. The phylogenomic tree showed a clear identification of the different *Vibrio* spp. except in the case of *V. jasicida/V. hyugaensis* and *V. ordalii/V. anguillarum*. The isolates were classified in 28 *Vibrio* spp. but most of them were identified as *V. alginolyticus*, *V. diabolicus*, *V. parahaemolyticus* and *V. anguillarum/V. ordalii*. Many strains deposited in the pubmlst database were reclassified (Supplementary Table S1). The isolates V103, Vi_2, Vi_28, Vi_41, Vi_67, Vi_2a, Vi_24a, Vi_27a, Vi_38a, Vi_42a, Vi_54a, Vi_56a, Vi_67a, Vi_72a, Vi_73a, Vi_80a and Vi_8b are not clearly identified. Turkish strains (51 of 256) were grouped into six different species *V. anguillarum* (28 strains), *V. alginolyticus* (15 strains), *V. harveyi* (5 strains), *V. diabolicus, V. fluvialis* and *V. parahaemolyticus* (one of each).

The structure of the Median Joining (MJ) algorithm network (Fig. 2) is similar to the phylogenetic tree, with some exceptions. *V. ordalli* (h_184) and Vi_22 (h_10) were together in the same branch in *V. anguillarum/V. ordalli* group, but in MJ network, these strains were separated but in the same group. *V. mytili* (h_181) and *V. natriegens* (h_182) were shared branch in the ML tree; however, in the network, *V. mytili* was close to *V. diabolicus* (h_162) and *V. natriegens* to *V. parahaemolyticus* (h_117). The isolate Vi_2a (h_54) was included in the

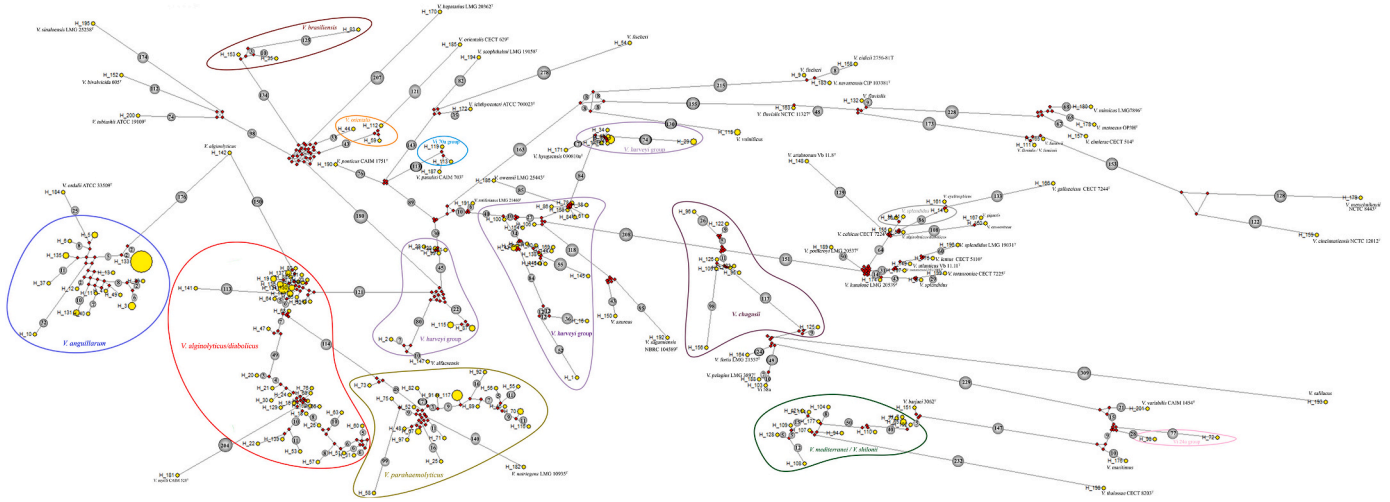

Fig. 2. Median joining network of *Vibrio* sp. isolates employed in the study based on the concatenated sequence (*atp*A, *gyr*B, *pyr*H and *rec*A). The size of the circles is related to the number of strains within each ST. Red circles represent putative intermediate sequences. Number in grey circles indicate the nucleotide substitutions. Lines without numbers correspond with one nucleotide substitution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Mediterranei clade in the phylogenetic tree while in the network belonged to *Scophtalmi* clade. Vi_2 (h_1) and Vi_28 (h_16) were identified as *Vibrio* sp. closely to *V. parahaemolyticus* and *V. alfacsensis* but in the network were affiliated to *V. harveyi* 1DA3 (h_169). The isolates grouped into the two branches of *V. harveyi* (h_168 and h_169) swiped the positions in the MJ network.

3.3. Recombination and mutation

The phi test (Pairwise Homoplasy Test) indicates non-significant statistical evidence for recombination in any gene. However, putative recombination events were detected with the minimal number of recombination events (R_{min}), 57, 77, 59 and 59 in *atp*A, *gyr*B, *pyr*H, *rec*A, respectively. Moreover, the hypothesis that some recombination could also occur is supporting by the linkage disequilibrium estimated from allelic profiles for the whole isolates collection, I_A parameter (2.0039 ($P = 0.000$)) and standardized $I_A^S = 0.0721$ ($P = 0.000$)), significantly different from zero. Moreover, the presence of homoplasies was shown by the interconnection network structures in the split graphs, based on each gene (Supplementary Fig. S2) and on the concatenated sequences (Fig. 3).

To investigate the population structure, we performed population analysis using Structure software. This software classifies individuals for a given number of clusters (K) represented by colors using one color for each ancestor lineage (Fig. 4). We performed Structure analysis by setting K from 2 to 39, executing the simulations five times for each K. The best model estimated by Structure Harvester (Earl and VonHoldt, 2012) was K = 28, therefore, 28 genetic ancestors. The admixture of these lineages resulted in 37 profiles (Fig. 4). The Turkish strains showed a predominance of profiles with ≥80% of ancestor lineages (profiles 5, 16, 21, 25, 30 and 36). The isolates V7 (profile 7, *V. fluvialis*), V24 (profile 8, *V. diabolicus*), V103 (profile 37, *V. alginolyticus*) and V147 (profile 22, *V. harveyi*) were the isolates with more admixture. On the contrary, Italian isolates showed a high degree of admixture. It's interesting to note that the Italian isolates Vi_42a, Vi_8b Vi_72a and Vi_73a (identified as a possible new *Vibrio* spp.) belonged to a different ancestry lineage (profile 2, yellow bars). All species encompass many different profiles and/or share the profile with other species except *V. vulnificus* (profile 4), *V. barjaei* (profiles 18 and 19, green bars) and *V. harveyi* ATCC 14126^T (profiles 21, 22 and 23, red bars). The patterns did not show any relation with host, geographical point or isolation year.

3.4. Gene flow and demographic trends

The neutral theory of molecular evolution holds that most variation at the molecular level evolutionary changes occurs at the molecular level does not affect fitness; therefore, the genetic variation is explained by random processes (stochastic processes) as genetic drift. The statistics Tajima's D, Fu and Lis (F* and D*), and the Ramos-Onsis R_2 were different to cero rejecting the neutral theory. Tajima's D and F* and D* were positive for all genes (except *atp*A and *rec*A) and concatenated sequences reflecting a minimal presence of rare polymorphisms, typical of a population in contraction and under balancing selection. On the contrary, the negative values of Fu's F_S, high values of Strobeck's statistic (S = 1) and low values of Ramos-Oasis R_2 test are consistent with deviation from neutrality due to either selection or population expansion (Table 3).

4. Discussion

Molecular identification of *Vibrio* genus by PCR and sequence analysis of one single gene has some incongruences in the species identification, a reliable and conceivable alternative is is identify and typing species by MultiLocus Sequence Analysis (MLSA) and by MultiLocus Sequence Typing (MLST) (Choopun et al., 2002). Similar to the reports, we found that *V. alginolyticus* and *V. anguillarum* are the most prominent species isolated from marine fish.

The population structure and evolution of bacteria are primarily based on the MLST analysis which is one of the most useful methods. Despite other authors employed several genes to MLSA studies, the MLST scheme for Vibrio spp. (Rahman et al., 2014) was established in four genes (atpA, gyrB, pyrH and recA) on the pubmlst database. The population presented only 77 nonsynonymous changes of the 873 polymorphic sites, indicating a strong selection against amino acid changes, a typical feature of housekeeping genes (Pérez-Losada et al., 2006). Determination of high number singletons in analyzed strains, about 89.5%, which resulted by the goeBurst scheme, also reflects some genetic discontinuity among the strains. More elaborated analysis by creating the median-joining network showed a tight relationship between some species of the *Vibrio* genus, despite the presence of missing intermediates in the population structure in this study.

On the other hand, it is important to highlight the limited number of isolates available in the GenBank for the recently identified fish pathogenic *Vibrio* spp.(Sawabe et al., 2013) such as *V. anguillarum, V.*

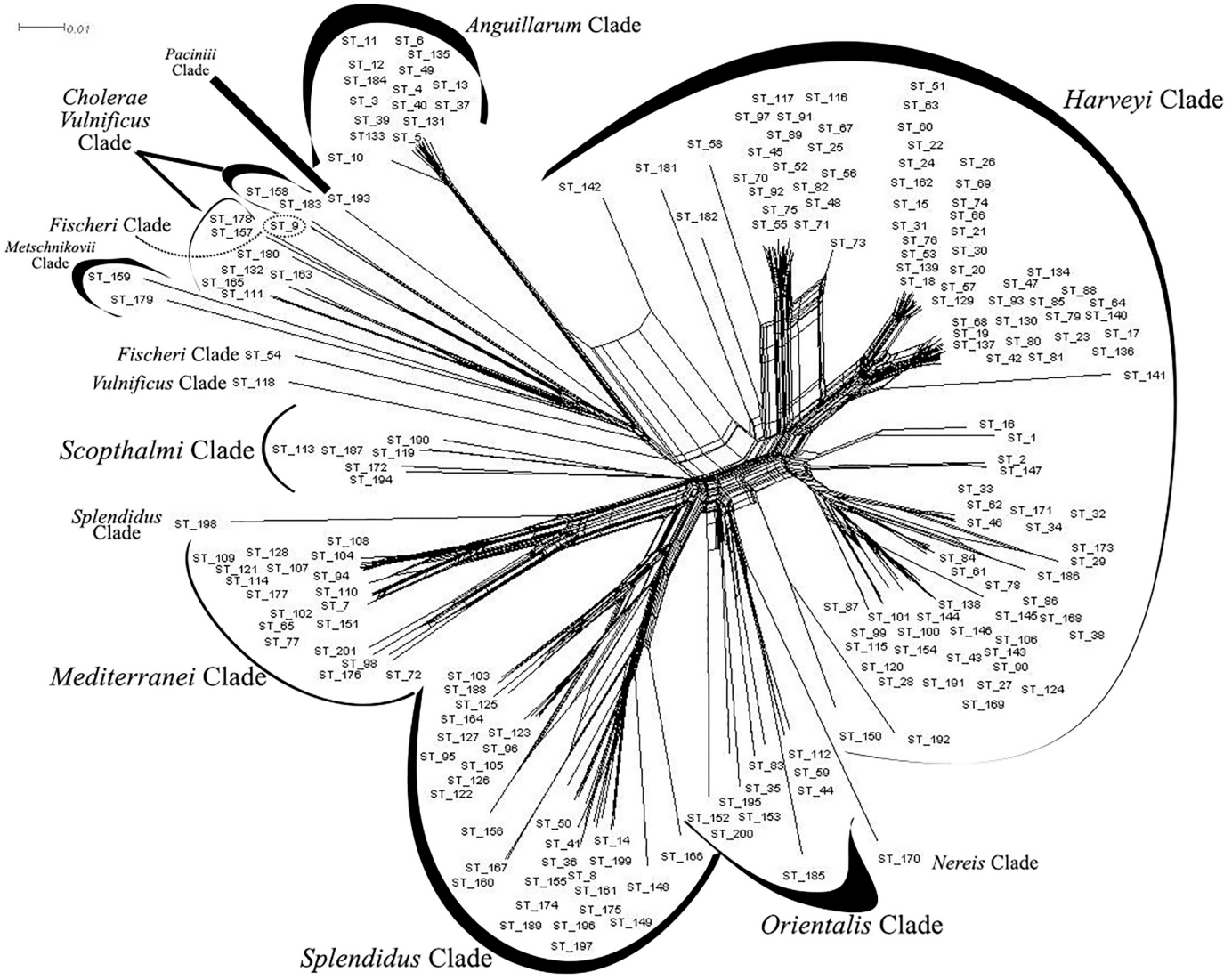

Fig. 3. Neighbor-Net graph based on the concatenated sequences of the four housekeeping genes. The formation of an interconnecting network structure was suggestive of recombination. Scale represents the genetic distance (Clades were determined according to Sawabe et al., 2013 and Jiang et al., 2022).

alginolyticus, V. harveyi, and this could cause a perturbation in the genetic structure (Balboa and Romalde, 2013; Bisharat et al., 2007; Bruto et al., 2017; Kim and Cho, 2015; Sawabe et al., 2013; Steinum et al., 2016; Tao et al., 2012; Warner and Oliver, 2008; Wollenberg and Ruby, 2009). *V. parahaemolyticus* and *V. cholera* are the most common strains into the *Vibrio* genus in the world because they cause pandemic disease in humans, and they have been found to have a high degree of genetic diversity within the species (Makino et al., 2003). On the contrast of these species, we found that *V. vulnificus, V. crassostreae, V. fischeri, V. anguillarum, V. ordalii, V. tapetis* and other pathogenic species reported that have a light linkage disequilibrium and commonly two lineages revealed by MLST analysis.

The phylogenetic analysis, on the basis of the concatenated sequences, showed some species in a separated monophyletic branch, which was consistent with a recent taxonomic study on the genus *Vibrio* (Sawabe et al., 2013). From all studied aquatic *Vibrio* isolates here, phylogenetic tree showed four big groups differentiating the species *V. alginolyticus, V. diabolicus, V. parahaemolyticus* and *V. anguillarum,* each of them grouping isolates from live marine animals including rainbow trout, sea bream and sea bass isolates, and the rest of isolates suggesting that the changes in the sequences can be caused by geographical isolation. Abayneh et al. (2012) reported that the MLSA approach is capable of resolving isolates according to their geographical origin or host. In a recent report, Vibrionaceae has been reported as a very complex group with determining 51 distinct clades including 21 newly defined ones by Jiang et al. (2022) and MLSA was reported that it is still an effective and reliable tool for delineating new species and monophyletic groups/clades. However, our results by MLST analysis did not establish any association between geographical location, isolation source or date, and further research needs to be clarified if any relation between the isolates.

The bacteria belonging to the family Vibrionaceae present a high level of genotypic diversity supported by a high recombination/mutation rate, especially because vibrios are natural constituents of marine environments and spend much of their life cycle outside the host and have adapted to fluctuating conditions, including temperature shifts, nutrient limitation, osmotic stress, and predator (Ceccarelli et al., 2019). In this study, the high recombination (R_{min}), 253 events for concatenated sequence, supports genotypic diversity of aquatic *Vibrio* spp..

The construction of the Median-Joining network revealed a high number of mutations to separate some species (*V. anguillarum, V. alginolyticus, V. parahaemolyticus, V. mediterranei/V. shilonii*); therefore, the high number of mutations together with the I_S^A value showed that, although the impact of recombination is an important evolution factor,

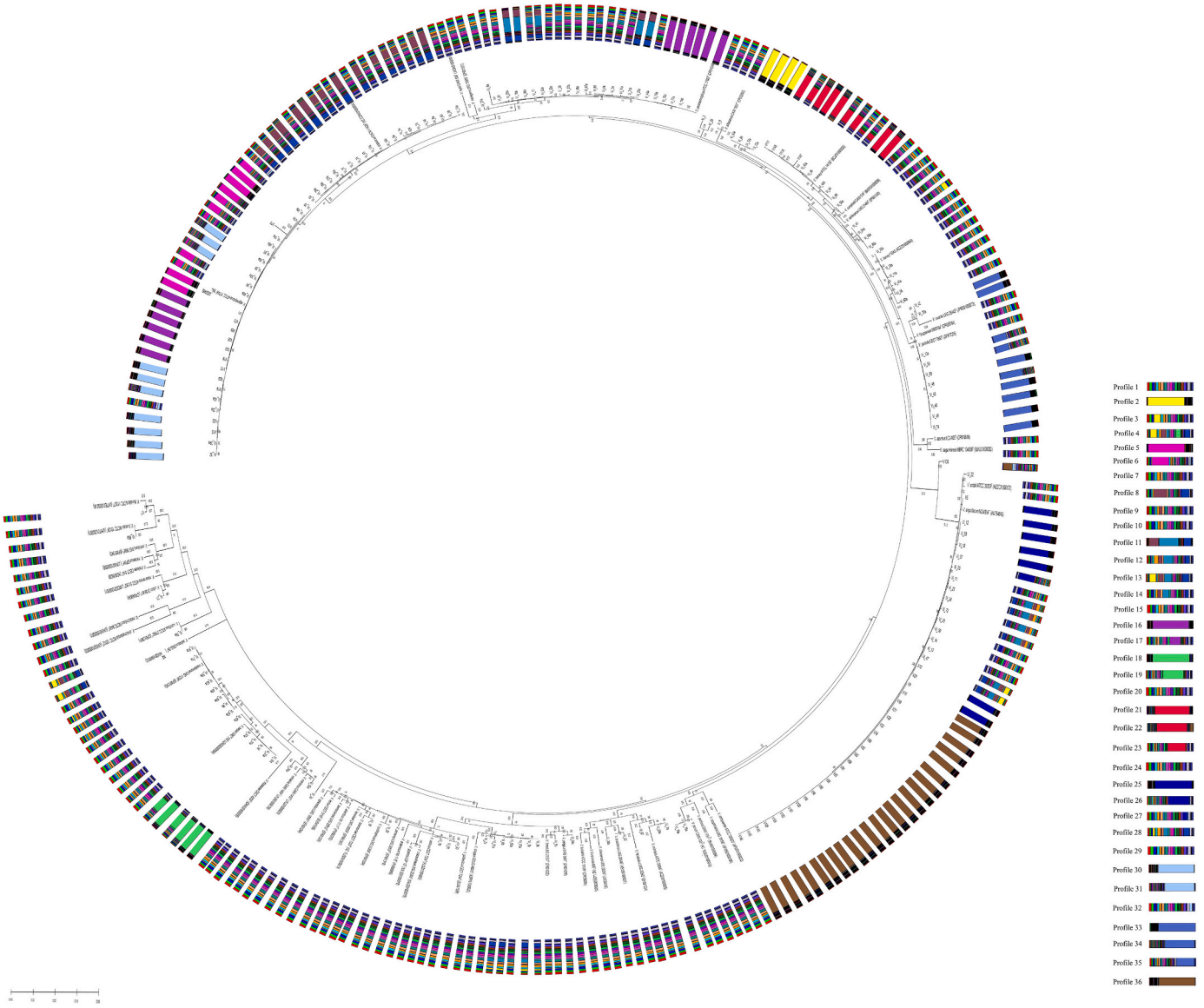

Fig. 4. Phylogenetic reconstruction and gene flow based on the concatenated sequences (*atp*A, *gyr*B, *pyr*H and *rec*A) using 256 isolates of genus *Vibrio* by Neighbor-Joining algorithm. Only bootstrap values above 70% are shown. Coloured bars represent the genetic ancestors identified with Structure analysis. The length of the coloured bars indicates the proportion of the genetic ancestor.

it is not enough for random allele association (de Las Rivas et al., 2006).

Structure analysis was performed to examine any possible influence of recombination among the species of the population. MLST studies on *Pseudomonas* (Bennasar et al., 2010; Lalucat et al., 2020; Martino et al., 2011; Pirnay et al., 2009) indicated that the different ancestral lineages were associated with the different species, but in this study, many isolates presented the existence of a high degree of admixture among the ancestor lineages. This non-association between genetic ancestors and *Vibrio* spp. can be explained by a strong presence of recombination in the evolution of most of the *Vibrio* spp. included in this study. Despite these results, the presence of clones within the population or the disequilibrium among the alleles of some genes showed an intermediate panmictic population (Hoffmann et al., 2010; Yan et al., 2011). Our results demonstrated that the clonal and epidemiologic relation among isolates belonging to different species is a complex work and the real impact of the recombination is not easy to evaluate (Didelot and Maiden, 2010). *V. anguillarum* serotype O1 associated with fish disease displays a high degree of sequence conservation (Steinum et al., 2016; Hansen et al., 2020), unfortunately the serotype identification of our strains is not available. Turkish isolates showed two different ancestral lineages but with an admixture degree very low. With this data we can hypothesize that *V. anguillarum* has a homogenetic structure in Turkey as previously reported (Steinum et al., 2016), contrasting with theEuropean isolates. This could be explained by limited fry imported by Turkey and the physical barrier of the Aegean sea. Moreover, a high genetic variation was found among *V. anguillarum* serotype O2A strain in the study performed by Hansen et al. (2020) but we can relate the high admixture degree in European isolates with serotype. The different information reflected by the indexes used for the study of the demographic history of the population showed a controversial model for evolution (Bastardo et al., 2015). The negative *Fs*, positive Strobeck's values (1), and R_2 close to zero (between 0,1052 to 0,1602) allow the acceptance of the neutral theory in the *Vibrio* population in the recent expansion. On the other hand, Tajima's D and Fu and Li's F* and D* the neutrality test for the overall population resulted in positive values rather than negative Fu's statistics (concatenated values), which determined the population existence of a bottleneck event. Moreover, Ramos-Onsis R_2 statistic also suggested a possible population expansion (Ramos-Onsins and Rozas,

2002) of *Vibrio* with values close to zero. *Fs* is a more sensitive indicator for population expansion than D^*, but R_2 statistic is particularly suited for small-size samples with recombination (Balboa and Romalde, 2013). There are a total of 457 isolates in the pubmlst database for *Vibrio* spp. (https://pubmlst.org/organisms/vibrio-spp) when we write this article, thus determination of population expansion in a high number of aquatic strains (256 strains) in the present study could be commented on using Fs and S additional R_2 statistics. This finding could be supported if some groups were in epidemiological expansion, inducing the spreading of the more virulent members in the population. Such hypothesis could be connected with the last outbreaks of pathogenic *Vibrio* spp. in the world focusing on *Anguillarum, Splendidus, Coralliilyticus, Pectenicida, Orientalis* and *Harveyi* clades (Amaro et al., 2020; Deng et al., 2020; Dubert et al., 2017; Ina-Salwany et al., 2019; Jiang et al., 2022). These data are reflected in the independent evolution of the lineages of *V. alginolyticus, V. anguillarum, V. parahaemolyticus* and *V. harveyi* group composed by reference or type isolates from Europe and Asia besides America.

Some genes showed a deviation from the general trend of the population, indicating unique pressures, spurious results, or a mix of factors. These contradictory results made it impossible to distinguish with confidence between expansion and balancing selection in our population, probably due to the different demographic histories among the species and a possible deviation of the data caused by the high number of isolates of *V. anguillarum, V. parahaemolyticus* and *V. harveyi* group.

5. Conclusions

We conclude that *V. anguillarum* and *V. alginolyticus* are predominant species in Turkish fish farms and they have biochemically heterogenetic characteristics. While 201 identified sequence types have been published when we write this article, seven new STs were found in Turkish isolates. The findings clearly showed that *Vibrio* have dissimilar genotypes based on isolation source or country. This study clearly demonstrated the complications of evolution conclusions due to the high diversity present within *Vibrio* populations and the fact that genetic interactions within the genus modify population expression at different levels. Newly identified STs indicated that the genes underwent recombination frequently. The basic knowledge of the population indicates that the structure is panmictic with traces of epidemiological expansion. The data reported in this study indicate that a high level of population genetic diversity exists in aquatic *Vibrio* spp.. The tools employed provide new information on the genetic mechanisms behind the emergence of the aquatic *Vibrio* spp.. The genetic heterogeneity highlighted the importance of better understanding the short-term and long-term impacts generated by the therapeutic treatments used in aquaculture for Vibriosis. The genetic variations published in this study shed light on determining virulence factors, genetic relationship of virulence strains/species and on the development of intervention strategies for monovalent/polyvalent vaccine production for different *Vibrio* spp. In order to produce an effective fish vaccine, the following aspects should be described for each of the main *Vibrio* spp. in which vaccination is employed: both the biochemical, antigenic and genetic heterogeneity of the etiological agents; and their geographical distribution and host range. Thus, our findings clearly demonstrated that population genetic, genetic heterogeneity, geographical distribution and host range of *Vibrio* spp. will be useful to achieve progress in fish vaccinology, development of polyvalent vaccines and genetic interactions between inter/intra-species.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738728.

Data availability statement

The data of sequences has been uploaded to the pubmlst database (https://pubmlst.org/organisms/*Vibrio*-spp), which is online accessible.

CRediT authorship contribution statement

Muhammed Duman: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Noemí Buján:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Soner Altun:** Funding acquisition, Investigation, Project administration, Resources. **Jesús L. Romalde:** Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Izzet Burcin Saticioglu:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing or financial interests.

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