Retrospective analysis of *Vibrio* spp. isolated from marketed crustaceans using multilocus sequence analysis

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Abstract

The genus *Vibrio* includes bacteria with different morphological and metabolic characteristics responsible for different human and animal diseases. An accurate identification is essential to assess the risks in regard to aquatic organisms and consequently to public health. The Multilocus Sequence Analysis (MLSA) scheme developed on the basis of 4 housekeeping genes (*gyr*B, *pyr*H, *rec*A and *atp*A) was applied to identify 92 *Vibrio* strains isolated from crustaceans in 2011. Concatenated sequences were used for the phylogenetic and population analyses and the results were compared with those from biochemical identification tests. From the phylogenetic analysis, 10 clusters and 4 singletons emerged, whereas the population analysis highlighted 12 subpopulations that were well supported by phylogeny with few exceptions. The retrospective analysis allowed correct re-attribution of isolated species, indicating how, for some pathogens, there may be an overestimation of phenotypic identification (*e.g. V. parahaemolyticus*). Use of the PubMLST *Vibrio* database highlighted a possible genetic link between Sequence Type (ST) 529 and ST195 (*V. alginolyticus*) isolated from a human case in Norway during 2018. In addition to the identification of major risk groups of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*, MLSA could be a valid support for species considered a minor risk, such as *V. alginolyticus*, *V. mimicus* and *V. fluvialis*. Due to the increased incidence of vibriosis in Europe, the application of different tools will also have to be considered to investigate the possible epidemiological links of the various species in the perspective of *Open Science* to protect the consumer.

Introduction

At present, few data are available about vibriosis incidence and the related outbreaks both, foodborne and extra-intestinal infections (Onohuean *et al.*, 2022). The underestimation of this foodborne illness is due to mild gastrointestinal symptoms that do not require any medical treatment; therefore, foodborne vibriosis is not a notifiable disease in most European countries (Amato *et al.*, 2022). However, due to climate change and extreme meteorological events (heatwave, flood, changes in water salinity), the number of cases related to *Vibrio* species causing vibriosis has dramatically increased in Europe in the last decade (Brehm *et al.*, 2021; ECDC, 2021; Amato *et al.*, 2022). Moreover, eating habits play an important role in the transmission of vibriosis. Italian consumers enjoy raw or slightly cooked seafood such as crustaceans or shellfish, that may be accidentally contaminated by foodborne pathogens, including *Vibrio* spp. This behaviour can increase safety concerns and the spread of outbreaks.

Vibrio is one of the most studied *genera* found in aquatic ecosystems and includes the major culturable bacteria in marine and estuarine environments; indeed, many species of *Vibrio* are part of the indigenous aquatic microbiota. According to recent species updates, there are around 147 species of *Vibrio* and 4 subspecies (Sampaio *et al.*, 2022), but the description of new species has led to a constantly changing taxonomy.

Austin (2010) suggested a classification of zoonotic *Vibrio* spp. in 2 groups named *higher risk* vibrios (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, the main species causing serious foodborne gastroenteritis in humans) and *lower risk* vibrios (*V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. metschnikovii* and *V. mimicus*).

Currently, the European legislation still lacks microbiological criteria on the punctual monitoring of *Vibrio* contamination in fishery products. However, the Italian guidelines related to EC Regulations 882/2004 (European Commission, 2004) and 854/2004 (European Commission, 2004) have indicated *Vibrio cholerae* (V. *cholerae O1, V. cholerae 0139, V. cholerae non-O1, V. cholerae non-O139*) and *V. parahaemolyticus* as hazards to detect in fishery products during the official controls. Moreover, the guidelines mentioned the methods suitable for the detection of the potentially enteropathogenic *Vibrio* species. Although the Regulation 625/2017 (European Commission, 2017) repeals the previous regulations (EC Reg. 882/2004 and 854/2004), the guidelines are still valid in Italy according to the note No. 0069887/2019 (Italian Health Ministry, 2019).

Numerous phenotypic schemes and biomolecular methods have been developed to characterize and classify *Vibrio* species. Classical biochemical tests are usually applied to identify the *Vibrio* genus, but the

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great phenotypic diversity among strains makes their application difficult. Moreover, traditional analyses are time and resource-consuming. For this reason, researchers are focused on the simplification of the classical identification protocols to implement the specific ISO (ISO, 2017) and to test the efficacy of molecular methods for the detection of the most important *Vibrio* human pathogens and their putative virulence markers (Hartnell *et al.*, 2019). 16S rRNA gene sequencing can give an accurate identification of *vibrios* at the family and genus levels but identification at the species and strain levels requires the application of genomic analysis.

The Multilocus Sequence Analysis (MLSA) approach is a valid alternative to biochemical as well as fingerprint pattern-based methods for species identification. MLSA has proved to be a very practical and reliable method and one of the main advantages of it is the reproducibility among different laboratories. Several molecular markers, *e.g. rec*A, *pyr*H, *gyr*B and *atp*A in single or concatenated sequences, have been used to identify *Vibrionaceae* species and many different specific schemes are available for *Vibrio* spp. or *Vibrionaceae* (Rahman *et al.*, 2014).

The aim of this retrospective study was to apply the MLSA scheme previously developed by Rahman *et al.* (2014) to identify and characterize *Vibrio* spp. isolated from crustaceans of the northeast Italian market. The data were analysed using different approaches in order to define the *Vibrio* species associated with different commercialized crustaceans and the possible genetic relationships among the strains. The direct comparison of sequences and allelic profiles deposited on the public database *Vibrio* spp. PubMLST (https://pubmlst.org/organisms/vibrio-spp) allowed the definition of additional links between strains collected from shellfish and others implicated in human cases of vibriosis. A comparison between MLSA attribution and the biochemical identification highlighted some limits of the phenotypic methods. In the frame of open science, this study aimed to represent a first step of the hazard identification to characterize *Vibrio* spp. associated with crustacean marketing and consumption in northeast Italy.

Materials and Methods

The MLSA scheme was applied to identify *Vibrio* species isolated from fresh and defrosted samples of various crustacean species (*Palaemon* spp., *Crangon crangon*, *Squilla mantis*, *Hymenopenaeus muelleri*, *Carcinus aestuarii*). Samples were collected during a market survey in Venice from July to December 2011 (see the PubMLST *Vibrio* spp. website for sampling details: https://pubmlst.org/organisms/vibrio-spp; ID isolate collection from 1147 to 1244; Supplementary Table 1 and the paper of Caburlotto *et al.* (2016) for additional information. The shellfish originated mainly from the North Adriatic (Chioggia area, Venice Lagoon, Po Delta – Goro) and South Adriatic Sea.

Isolation of *Vibrio* strains and species identification by biochemical methods

In collaboration with the National Reference Laboratory for Fish, Crustacean and Mollusc Pathologies, IZSVe (Adria, Italy), the samples were prepared according to Caburlotto *et al.* (2016). In brief, for the first enrichment, 25 g of sample (crustacean pulp and a portion of the carapace) was homogenized in 225 mL of common alkaline peptone water (APW) and APW with 2% NaCl and incubated at 37°C for 18–24 hours. The *Vibrio* spp. were enumerated by the Most Probable Number (MPN) procedure and pure colonies were obtained from each enrichment medium streaked on thiosulphate citrate bile salt sucrose agar (TCBS) and on ChromAgar plates (37°C for 18–24 hours). The *Vibrio* presumptive colonies (6–8 per sample) were then subjected to Gram staining, resistance to vibriostatic O129, oxidase test, and O/F test and growth at different salt concentrations. Gram-negative, oxidase-positive and facultative anaerobic (+/+ for O/F test) isolates were identified with miniaturized biochemical tests

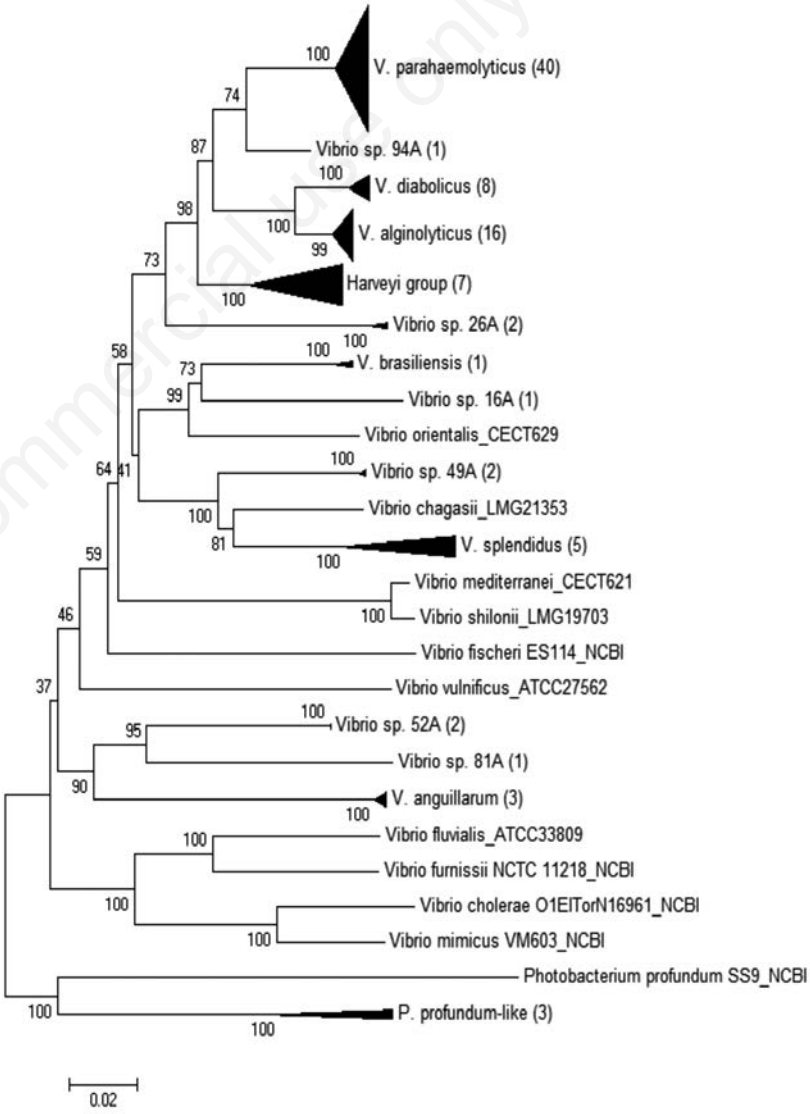

Figure 1. Neighbour-joining phylogenetic tree (compressed) with concatenated sequences of 4 housekeeping genes for the *Vibrio* strains isolated from crustacean samples in 2011. Numbers in brackets describe the number of strains included in the reference species group (black triangle).

(API20NE, bioMérieux, Florence, Italy).

MLSA approach - DNA extraction, polymerase chain reaction amplification and sequencing

The MLSA scheme followed in this study was from Rahman *et al.* (2014). In brief, four housekeeping genes (*gyr*B, *pyr*H, *rec*A and *atp*A) were chosen for the MLSA.

DNA were extracted by boiling from 107 pure colonies classified as *Vibrionaceae* by API20NE (102 *Vibrio* species and 5 other *genera*). The Polymerase chain reaction (PCR) amplification was performed in a Euroclone One Advanced thermal cycler (Celbio, Milan, Italy) following different amplification conditions described in detail in Rahman *et al.* (2014) and on *Vibrio* spp. PubMLST (https://pubmlst.org/static/organisms/vibrio-spp/Vibrio_primers.pdf). The amplicon of each gene was verified by BLAST search for an initial species attribution.

Phylogenetic analysis of MLSA data

The concatenated sequences were aligned for phylogenetic analysis by using MEGA v5.04 (Tamura *et al.*, 2011) according to the Kimura two-parameter model and the phylogenetic tree was constructed using the neighbour-joining method.

In order to better describe the phylogenetic relatedness among isolates, we also sequenced 16 *Vibrio* reference strains and included the sequences downloaded from the NCBI database (Supplementary Table 2). The taxon name of each cluster was attributed according to the available reference/NCBI strains clustered in the same group. When the isolates were considered related but clearly distinct, the strain name representative for the cluster was used (*e.g. Vibrio* sp. Vi20). All strains were also screened for virulence genes markers by specific PCR protocols (genes: *Tox*R, *tlh*, *tdh* and *trh*; (Bej *et al*., 1999; Kim *et al*., 1999).

Structure analysis and genetic relationship

The linkage model was used to identify groups with distinct allele frequencies in STRUCTURE software (Falush *et al.*, 2003). This procedure assigns a probability of ancestry for each polymorphic nucleotide for a given number of groups, *K*, and it estimates *q*, the combined probability of ancestry from each of the *K* groups for each individual isolate (Rahman *et al.*, 2017). This analysis was conducted in order to verify the phylogenetic species attribution and to compare MLSA and API20NE classifications.

All the new sequences, allelic profiles and new sequence types were submitted to the public database *Vibrio* spp. (https://pubmlst.org/organisms/vibrio-spp). The PHYLOViZ (www.phyloviz.net/) program was applied to verify the possible relationships between the epidemiological information (geographic area of isolation, *Vibrio* species, year of isolation and source of isolation such as clinical/environment) provided by the public database *Vibrio* spp. and the genotypic profiles of Italian strains. On the date of analysis (2022-07-05), the database included 969 allele sequences, 1184 isolates and 272 genomes of vibrios.

Results and Discussion

Among 107 putative *Vibrio* strains isolated from crustacean samples (Supplementary Table 1), 7 strains amplified only with the *atp*A gene and were identified as *Shewanella* spp. by BLAST search. Another 8 strains did not amplify with one or more genes of the MLSA scheme and were excluded by the subsequent analyses (Supplementary Table 1). However, it was possible to define the genus of these strains, where 7 are *Vibrio* and 1 *Photobacterium* (Supplementary Table 1).

Phylogenetic analysis with a neighbour-joining tree showed 10 clusters and 4 singletons (Figure 1). In particular, the database was mainly formed by *V.*

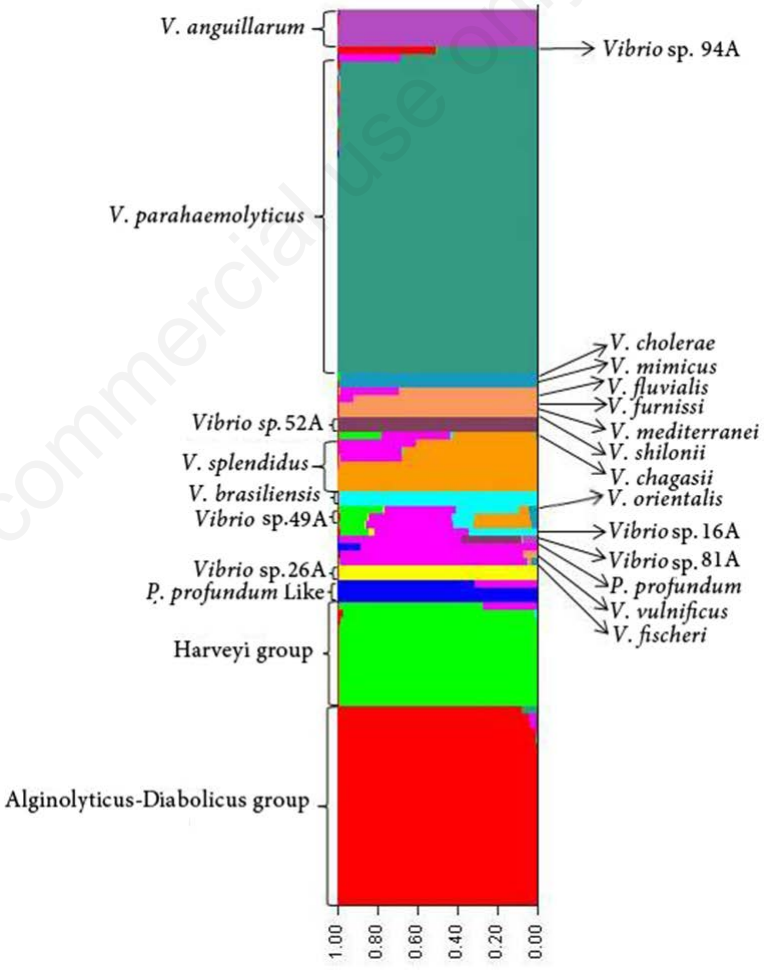

Figure 2. Population clustering (strains isolated from crustacean samples in 2011) identified by STRUCTURE software on the concatenated sequences of 4 genes. A single colour corresponds to a single population, while columns with mixed colours include strains carrying DNA from different populations. The analysis showed 12 ancestral groups. Groups with more than one isolate are indicated on the left side and single strains are showed on the right side (*V. cholerae, V. mimicus, V. fluvialis, V. furnissii, V. mediterranei, V. shilonii, V. chagasii, V. orientalis, V. vulnificus, V. fischeri* and *P. profundum* only represent the reference strains, not isolates in our study).

parahaemolyticus (43% of total *Vibrio* strains), *V. diabolicus* (9%), *V. alginolyticus* (17%), *V. harveyi* group (7.6%) and *V. splendidus* (5.4%). The only *higher risk Vibrio* species identified in crustacean samples was *V. parahaemolyticus*, while there were no *V. cholerae* or *V. vulnificus*. The most represented *lower risk* vibrios were *V. alginolyticus* followed by *V. diabolicus* and *V. splendidus*. In one study by Traoré *et al.* (2012) to assess the risk of *Vibrio* spp. transmission from crustaceans to humans, they identified 40% of the isolates as *V. alginolyticus*, 36% as *V. parahaemolyticus* and 24% as the nontoxigenic *V. cholerae*. Koralage *et al.* (2012) in their investigation on the prevalence of *Vibrio* spp. in shrimp farms, found *V. parahaemolyticus* was the most common (91.2%) followed by *V. alginolyticus* (18.8%), *V. cholerae* non-O1/non-O139 (4.1%) and *V. vulnificus* (2.4%). At the market level, the prevalence of many *Vibrio* species (such as *V. mimicus*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus*) was found in around 20% of crustacean samples (Álvarez-Contreras *et al.*, 2021).

The Harveyi clade contains 4 species (*V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*) that are pathogens for marine animals (Pretto, 2020; Harrison *et al.*, 2022). The BLAST analysis highlighted the putative attribution of only two species of this group, *V. harveyi* and *V. rotiferianus*.

Structure analysis recognized 12 subpopulations with the highest delta *K* value of 31.136 (Figure 2). The population structure confirmed the phylogenetic analysis, while *V. alginolyticus-diabolicus* were included in the same genetic population. Both analyses agreed with the previous findings on the MLSA application, with a similar *Vibrio* species definition as described by Rahman *et al.* (2014).

Finally, 92 strains were analysed using the MLSA approach, of which 52 (56.5%) strains had the same identification as for the biochemical method. The Sankey diagram (Figure 3) showed the cases of misclassification between methods. In *V. parahaemolyticus*, 11 false positive and 4 false negative strains were identified as compared to the biochemical approach. In total, 40 *V. parahaemolyticus* were identified by MLSA, whereas 51 were identified using the biochemical method. 10% of *V. parahaemolyticus* were finally assigned to the genus *Shewanella* according to the *atp*A sequence. The *V. parahaemolyticus* strains were also checked using species-specific *tox*R and *tlh* genes; moreover, the *tdh* and *trh* genes were tested to assess the virulence properties. No virulence factors were detected; moreover, the identification of strains by MLSA totally agreed with the *tox*R results. The application of these genetic markers is strongly recommended to identify *V. parahaemolyticus* and to detect putative enteropathogenic isolates (CSR 212/2016, 2016). Moreover, for the three major *Vibrio* species (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*), the application of standardized protocols for biochemical identification is considered as an important prerequisite for diagnostic laboratories, especially for environmental strains as in the case of shellfish and crustacean samples (Hartnell *et al.*, 2019). No probable enteropathogenic strains were detected in the present *Vibrio* database; however, the number of samples/strains should be enlarged. The *V. parahaemolyticus* strains included in this retrospective analysis are only a part of those described by Caburlotto *et al.* (2016). However, the main focus of this retrospective work was to elucidate the feasibility of the MLSA approach on strains collected from this matrix and to define a first detailed evaluation of all the *Vibrio* species from crustaceans. In particular, the API20NE identification tended to underestimate several minor species such as *L. anguillarum*, *V. harveyi* group and *V. splendidus*. Moreover, the MLSA allowed the classification of undefined *Vibrio* sp. strains such as *V. alginolyticus-diabolicus* genetic clusters.

MLSA/Multi-locus Sequence Typing (MLST) is also a suitable tool to highlight links between isolates from different sources. In this regard, the comparison of sequence types (STs) available on public databases allowed fast and easy detection of epidemiological relatedness. Moreover, the possibility of comparing data from whole genome analysis could increase the resolution and detail of these comparisons.

The analysis of allelic profiles showed the presence of 72 STs with the definition of 67 new STs that were deposited in the *Vibrio* spp. PubMLST website. Interestingly, from the same crustacean sample, not only the co-

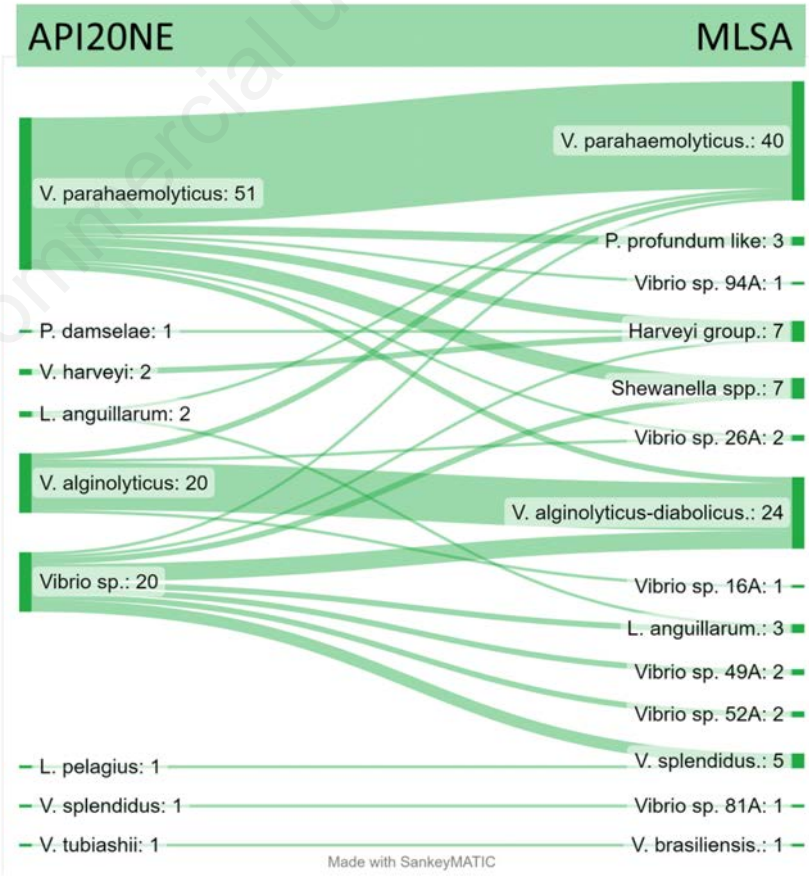

Figure 3. Sankey diagram of the whole *Vibrio* dataset (99 strains). The two blocks of nodes are related to the different identification methods (phenotypic API20NE *vs* Multilocus Sequence Analysis). Each node is the taxonomic attribution according to each classification method; the stream fields between the blocks represent the different attribution of these clusters in relation to each *Vibrio* species.

presence of different *Vibrio* species detected, but also different STs for each species (see isolate details) were found. The sequence analysis of a large number of *Vibrio* isolates per sample allowed the definition of a complex picture of the *Vibrio* strains associated with crustaceans, as in the case of *Crangon crangon* (sample 269/ITT) where four different *V. parahaemolyticus* STs were detected, or *Palaemon elegans* (sample 234/TT) with the presence of many different *Vibrio* species and different STs. These observations suggest screening many isolates per sample to better define the genetic variability of each *Vibrio* species.

Strain relationships were analysed using the PHYLOViZ program to identify potential clonal complexes (CCs) and founders. First, a recognition of the most represented species in the *Vibrio* spp. PubMLST database was performed by full MLST analysis (Figure 4A). The Minimum Spanning Tree-like structure formed with all available isolates showed two major branches represented by *V. alginolyticus-diabolicus* and *V. parahaemolyticus*. Only four species are displayed in Figure 4A, while others were not included in the analysis; moreover, the species attribution is not reported for all isolates of the database.

Clonal relationships among the STs collected from Italian samples and worldwide isolates at the triple-locus-variant level are reported in Figure 4B. The analysis evidenced 65 CCs, the biggest of which included 480 STs (a core cluster formed by *V. alginolyticus-diabolicus*). This CC also included many STs derived from strains collected from different cases of vibriosis. Several STs from crustaceans and molluscs are inside this CC; moreover, the few additional clonal complexes are formed mainly by *Vibrio* isolates from shellfish.

A clear relation was highlighted from ST529 and ST195 originating from human vibriosis (Figure 4B). ST195 was defined for the strain NO_VA_18_16, a *V. alginolyticus* isolated in Norway during 2018 (Amato *et al.*, 2022). In many countries of the Nordic-Baltic region, a dramatic increase of vibriosis cases associated with heatwaves was reported (ECDC, 2021; Amato *et al.*, 2022). *V. alginolyticus* was the cause of 34% of vibriosis infections during the period 2014–2017; 90% of cases were ear- or wound-related while only a few strains were isolated from faeces (2%). Despite the lesser importance of *V. alginolyticus* species as a foodborne pathogen, the manipulation, preparation and processing of crustaceans could be a risk for fishers and operators due to the probability of skin and soft tissue lesions (Neill *et al.*, 2020). The application of MLSA/MLST schemes and public

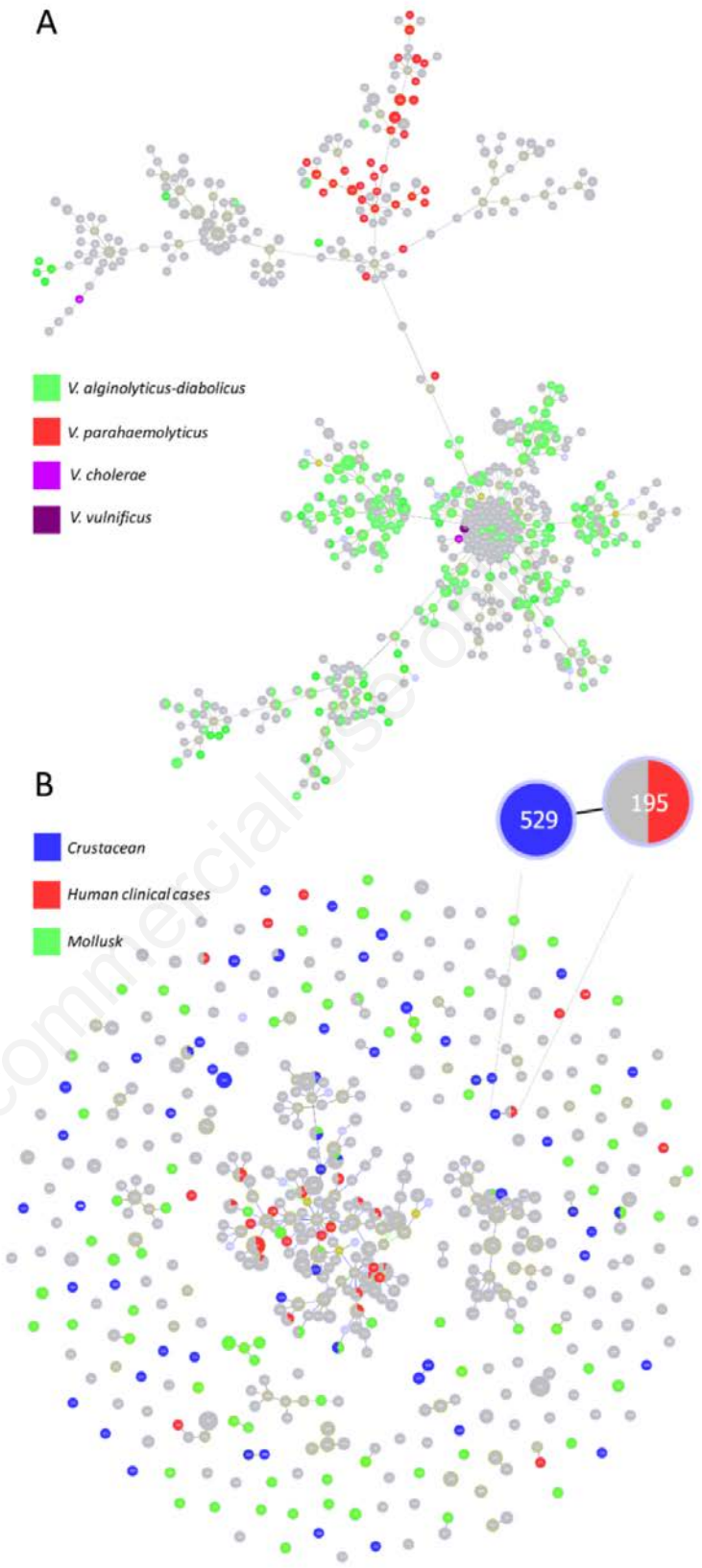

Figure 4. A) Full MST of all PubMLST database isolates; the sequence types are coloured according to the species attribution (4 species were considered); B) goeBURST analysis at the triple-locus-variant level to define clonal complexes. The proportion of each node is related to the frequency of each sequence type. The close-up shows a specific link between sequence types from crustaceans and human cases.

websites can help researchers to postulate new epidemiological links and sources of infections also for low-risk species and to discover new threats.

Conclusions

This retrospective analysis demonstrated that MLSA is a very fast and accurate analytic method to discriminate *Vibrio* species. The distribution and clustering of the analysed species achieved a high supported degree of discrimination that confirmed the results of previous analyses conducted on *Vibrio* spp. The 4 genes used in this study are sufficient to give suitable results and represent, of course, a faster way to analyse the genus *Vibrio*.

Sharing the data on public databases can deepen the understanding of seafood, such as crustaceans, as a vehicle of *Vibrio* spread and their distribution in the final product and can provide detailed information on their potential pathogenicity.

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