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Ciclo XXV

Molecular Characterization of *Vibrio* spp. in Shellfish using Multilocus Sequence Analysis

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my parents

inspiration to go ahead

(A.K.M. Nurul Islam

Late Begum Shamsunnahar)

SUMMARY

Fish and shellfish are the second largest source of protein for man after meat products and in some countries, such as Japan, constitute the main source of protein. In recent years, indigenous marine bacteria were responsible for 20% of all diseases and 99% of fatalities associated with the consumption of fishery products (Cozzi and Ciccaglioni, 2005). Among these, the main causes of diseases are some species of Vibrionaceae, which can cause gastroenteritis, especially after the consumption of fish products, raw or undercooked, from temperate and warm Seas. *Vibrio* is a very diverse genus responsible of different human and animal diseases. The accurate identification of *Vibrio* spp. is very important to assess the risks in regard to public health and diseases of aquatic organisms. Thus, analyses of population structure for a reliable bacteria characterization in different ecological environments are necessary. In particular, sequence based identification methods are preferable over classical biochemical approaches. In this study, a Multilocus Sequence Analysis scheme was developed on the basis of four housekeeping genes (*gyr*B, *pyr*H, *rec*A and *atp*A) applied to 3 set of *Vibrio* strains (154 isolates from mollusks in 2007; 92 isolates from crustacean and 22 isolates form mollusks in 2011) and 29 reference strains. Concatenated sequences were used for phylogenetic and population analyses and the results were compared with biochemical identification tests (Alsina's scheme). The phylogeny provided a good clustering, showing 15 clusters and 6 single strains in the first set of strains; 10 clusters and 4 singletons in second set; and 4 clusters and 4 singletons in the third set of strains. The population analysis highlighted 17 subpopulations in first set and 12 subpopulations in second set of *Vibrio* strains that were well supported by phylogeny with few exceptions. Overestimations of risk due to biochemical identification have been found for *V. parahaemolyticus* and *V. vulnificus* and no *V. cholerae* strains were identified. The false negative results of Alsina's scheme need to be considered as it might represent a potential public health risk. These findings highlight the need of a rapid and robust identification of shellfish associated foodborne *Vibrio* spp. and, in addition, the connection of environmental information to genetic data could enhance the *Vibrio* spp. characterization.

Second part of the study gave special emphasis on the species *Vibrio parahaemolyticus*, a potential emerging pathogen in the North Adriatic Sea. Pathogenic strains of *V. parahaemolyticus* represent one of the main causes of foodborne gastroenteritis,

especially in Asia and USA (Su and Liu, 2007). The study examined 160 strains isolated from 43 edible mollusks sampled between January and October 2011, identified biochemically as *Vibrio parahaemolyticus* in the Food Microbiology laboratory of Istituto Zooprofilattico (IZSVe). The strains were characterized for the presence of genes typical for the species *Vibrio parahaemolyticus* (*tox*R and *tlh*) in order to confirm the biochemical identification and virulence genes (*tdh* and *trh*). Dubious or misidentified strains were subjected to MLSA (Multilocus Sequence Analysis) by evaluating the sequence of 4 housekeeping genes. Finally, 102 *Vibrio parahaemolyticus* strains were analyzed by the MLST protocol: portions of 7 genes (*dna*E, *gyr*B, *rec*A, *dtd*S, *pnt*A, *pyr*C and *tna*A) were sequenced and concatenated. With the obtained MLST information phylogenetic analyses were performed to determine the relationships between the different strains isolated in this study and secondly, any links with worldwide isolates. All strains of *V. parahaemolyticus* were found positive for *tox*R and *tlh*, no strain was *tdh* positive, while 6 strains had the positive reaction for *trh* gene. 72 non-redundant (63 new) STs were identified. A total of 54 clonal groups were highlighted, in which 17 are clonal complex. Two distinct populations of *V. parahaemolyticus* were marked by phylogenetic, structure and recombination analyses. The main result is that despite the high percentage of positive samples for *V. parahaemolyticus*, only a few strains were potentially pathogenic for humans. However, some possible genetic relationships with strains can emerge from a comparative study with the STs in the world database. The characterization could help to identify suspect genotypes and thus clarify the dynamics of the spread of potentially pathogenic strains.

RIASSUNTO

I prodotti ittici sono la seconda fonte di proteine per l'alimentazione dell'uomo e in alcuni Paesi, quali il Giappone, ne costituiscono la principale fonte. Negli ultimi anni, i batteri marini della flora indigena sono risultati responsabili del 20% delle malattie nell'uomo e del 99% dei decessi derivati dal consumo dei prodotti della pesca. Tra questi, le principali cause di malattie sono da ascrivere ad alcune specie di *Vibrionaceae* in particolare al genere *Vibrio*, che possono causare gastroenteriti, soprattutto a seguito di consumo di prodotti crudi o poco cotti, provenienti da mari temperati e caldi. L'identificazione accurata dei batteri appartenenti al genere *Vibrio* risulta quindi molto importante per valutare i rischi in materia di salute pubblica e per l'identificazione puntuale delle malattie degli organismi acquatici. Risulta quindi necessario sviluppare ed applicare metodi affidabili che possano caratterizzare le specie di vibrioni residenti nei prodotti commercializzati (es. molluschi bivalivi e crostacei). In particolare, i metodi di identificazione basati sull'analisi delle sequenze geniche sono preferibili rispetto ai classici approcci biochimici. In questo studio è stato sviluppato uno schema MLSA *Multilocus Sequence Analysis* impiegando quattro geni *housekeeping* (*gyr*B, *pyr*H, *rec*A e *atp*A), tale schema è stato valutato in 3 differenti data set di ceppi (154 isolati da molluschi nel 2007; 92 isolati di crostacei e 22 da molluschi isolati nel 2011) e 29 ceppi di riferimento e *Type strain*. I concatenameri sono stati utilizzati per le analisi filogenetiche e per gli studi di popolazione dei *Vibrio* isolati, confrontando al contempo i risultati dell'identificazione di specie con i test biochimici (schema di Alsina) applicati di routine all'identificazione dei Vibrioni. L'analisi della struttura di popolazione mediante il software STRUCTURE e l'analisi filogenetica risultano concordi nell'assegnazione dei principali taxa evidenziando una simile clusterizzazione dei gruppi in sottopopolazioni. Al contrario, il confronto tra la classificazione mediante MLSA e i test biochimici ha evidenziato varie discrepanze tra le quali una sovrastima di ceppi classificati come *V. parahaemolyticus* e *V. vulnificus.* Al contempo alcuni ceppi di *V. parahaemolyticus* sono risultati falsi negativi. Questi riscontri potrebbero indicare una limitazione dell'utilizzo delle prove biochimiche adottate di routine alla classificazione dei *Vibrio* potenzialmente patogeni per l'uomo e tale riscontro si riflette in un possibile rischio per la salute pubblica.

La seconda parte dello studio ha considerato nel dettaglio la caratterizzazione molecolare di *V. parahaemolyticus*. Questo batterio è oggi un patogeno emergente

derivato dal consumo di prodotti ittici, infatti ceppi patogeni di *V. parahaemolyticus* rappresentano una delle principali cause di gastroenterite di origine alimentare, in particolare in alcuni paesi dell'Asia e negli Stati Uniti. Questo batterio, a causa di mutamenti ambientali e delle abitudini dei consumatori (consumo di prodotti crudi provenienti da aree contaminate) potrebbe rappresentare una problematica igienico sanitaria anche nel Mare Adriatico settentrionale. In questa parte dello studio sono stati esaminati 160 ceppi isolati da 43 campioni di molluschi commestibili campionati tra gennaio e ottobre 2011 e identificati a livello biochimico dal laboratorio di microbiologia dell'Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). I ceppi sono stati caratterizzati per la presenza dei *marker* genici specie specifici (*toxR* e *tlh* - *Vibrio parahaemolyticus*) per confermare l'identificazione biochimica e quindi dei geni per i fattori di virulenza (*tdh* e *trh*). I ceppi risultati di dubbia o errata identificazione sono stati sottoposti a MLSA (*Multilocus Sequence Analysis)* valutando la sequenza dei 4 geni *housekeeping*. Infine tutti i ceppi risultati *Vibrio parahaemolyticus* (n° 102) sono stati analizzati mediante il protocollo MLST (http://pubmlst.org/vparahaemolyticus/.). Lo schema prevede l'analisi di sequenza di 7 porzioni geniche (*dna*E, *gyr*B, *rec*A, *dtd*S, *pnt*A, *pyr*C *and tnaA*). I concatenameri ottenuti sono stati utilizzati nelle analisi bioinformatiche di popolazione per determinare le relazioni tra i diversi ceppi isolati in questo studio e, in seconda battuta, per evidenziare eventuali collegamenti con ceppi isolati a livello mondiale. Per quanto concerne i fattori di virulenza tutti i ceppi di *V. parahaemolyticus* sono risultati *tdh* negativi, mentre 6 ceppi hanno presentato la positività per il gene *trh*. Nel complesso sono stati identificati 72 profili ST non ridondanti, 63 dei quali di nuova attribuzione rispetto al *database on-line*. L'analisi clonale dell'intero database ha evidenziato la presenza di 54 gruppi clonali dei quali 17 risultano essere ascritti entro un complesso clonale. Le analisi di popolazione nel loro complesso delineano la presenza di due gruppi principali di *V. parahaemolyticus*. Dallo studio emerge che, nonostante sia stata riscontrata un'alta percentuale di campioni positivi per *V. parahaemolyticus*, solo pochi ceppi risultano potenzialmente patogeni per l'uomo. Tuttavia, alcune possibili relazioni genetiche con ceppi isolati da casi di gastroenteriti in varie parti del mondo emergono dallo studio comparativo con il *database on-line*. La caratterizzazione molecolare potrebbe aiutare a individuare genotipi sospetti e quindi chiarire la dinamica della diffusione di ceppi potenzialmente patogeni.

CONTENTS

1. INTRODUCTION

1.1. General information about edible shellfish and its production in Venice lagoon

The mollusks are, in the majority of cases, sessile or sedentary burrowing animals that feed on small food particles present in the water or sediment, through an intense activity of filtration during which bacteria retain in their bodies that may be present in environment (Lee *et al*., 2008).

Among the products of fishing, edible shellfish bivalves are mostly exposed to possible contamination by chemical, microbiological and toxicological hazards. In Italy, shellfish aquaculture production is the main national product in 2006, 70.6% of the total aquaculture production came from shellfish farms and, in particular, the mussel farming accounted for 73% (ISMEA, 2008 http://www.ismea.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/4689, accessed on 07 January 2013). The production is based almost exclusively on mussels (*Mytilus galloprovincialis*) and Philippines clams (*Tapes philippinarum*), in addition to small amounts of clams (*Tapes decussatus*) and oysters (*Crassostrea gigas* and *Ostrea edulis*) (Prioli, 2008).

It is important to note that, in Italy; the mussel farming is practiced mainly in coastal areas that suffer the consequences of the high level of urbanization on inland waters (lagoon area of the Adriatic coast, the Po delta, Gulf of Taranto, Gulf of Liguria and Sicily). The current legislation does not ensure that the shellfish are free of some potentially pathogenic agents. In fact, the presence of bacteria indicative of fecal contamination is not correlated with the presence of viruses or bacteria such as Vibrionaceae, pathogenic micro-organisms normally present in the marine environment (Cozzi and Ciccaglioni, 2005).

The chains of bivalve mollusks start with the rearing or collection of different species in the production areas. These zones can be sea, estuary or lagoons which are the natural beds of bivalve mollusks or sites used for their cultivation. In both cases, their location and their boundaries must be defined and classified by the competent authority. The mussel production has a tradition settlement over time in different regions of Italy and in the last decades of the last century there has been a shift from cultivation in lagoons and coastal ponds to the open sea. This shift was mainly caused by the deterioration of the characteristics sanitary water basins that have restricted trade with the sea.

The sowing is practiced throughout the year, although they tend to avoid the hard days of winter, since at temperatures below 5-6°C the growth is practically zero. The

collection of the product of a commercial size takes place during the whole year with intensified sampling during the months of August and December.

The production and marketing of live bivalve mollusks, considered foods that are high risk, are governed by the Regulations (EC) 852/2004, 853/2004, 854/2004 and 2073/2005. The production areas intended for mussels are distinguished by the current legislation in classes A, B and C differ in the level of microbiological contamination.

Zone A: in these areas mollusks may be collected and used for direct human consumption. These mollusks must meet the following requirements:

- contain less than 230 *E. coli* per 100 g of pulp and liquid
- do not contain salmonella in 25 g of flesh;
- do not contain toxic or harmful substances of natural origin or released to the environment in a quantity that the assumption by food exceed the ADI (Acceptable Daily Intake) for humans;
- have a maximum level of radioactive nuclides not exceeding the limits to CEE;
- have a maximum of algal biotoxins PSP *(paralytic shellfish poison)* in the edible parts not exceeding 80 µg per 100 g (Measured by biological method);
- Do not give positive reaction with the biological testing methods, for presence of DSP toxins *(Diarrhetic shellfish poison);*
- have a maximum of ASP *(amnesic shellfish poison)*, not greater than 20 µg of domoic acid per gram (analysis method HPLC).

Zone B: mollusks from these areas may be allocated for direct human consumption only after treatment in a depuration center or after relaying in an area that meets the requirements microbiological, biological, chemical and physical prescribed for the area A. Mollusks collected from these areas must not exceed the levels of 4600 *E. coli* per 100 g of pulp and intravalvular liquid in 90% of samples. By purification or relaying, mollusks from these areas of production will get to meet the requirements for shellfish areas A.

Zone C: mollusks from these areas can be used for direct human consumption only after relaying over a period not less than two months, in a zone of the microbiological, biological, chemical and physical prescribed for Zone A, the housing can be with or without an intensive purification. Mollusks collected from these areas must not exceed 46000 *E. coli* per 100 g of pulp and intravalvular liquid in 90% of samples.

The shellfish harvested in the areas of class A may be destined for direct human

consumption provided they meet specific health requirements, while those from areas B and C have to be submitted after harvest to treatment in a purification centre.

In Italy, the consumption of shellfish attributes 7% of infection (Parisi, 2004), but it is believed that the available epidemiological data are underestimated and that the number of actual cases is about 20 times higher, particularly in the southern regions, where tradition consumption of raw shellfish continues (Normanno *et al*., 2006). In many cases, the consumption of shellfish causes only mild gastrointestinal symptoms that do not require any medical treatment.

1.2. Bacterial community of Shellfish and public health concern (Food safety issues)

In the context of food safety, it is extremely important to know the diffusion and the potential pathogenicity of some etiologic agents that may come into contact with the different types of food. Fishery products are an important source of protein supply for the people of the world, but often turn out to be responsible for food poisoning due to the presence of toxins or pathogens for humans. The microbial flora of fish and shellfish (mollusks and crustaceans) is closely related to the microbiological characteristics of the environment in which they live and their habits.

On the skin and gills prevails aerobic microbial flora consists of *Pseudomonas* spp., *Aeromonas* spp., *Acinetobacter* spp., *Moraxella* spp., *Cytophaga* spp., while aerobic or facultative anaerobes, such as *Vibrio* spp., *Alcaligenes* spp., *Flavobacterium* spp., *Xanthomonas* spp. can be found in the intestine (Croci and Suffredini, 2003).

During the filtration activity, mollusks retain in their bodies not only plankton necessary for their metabolism, but also bacteria and viruses that may be present in the environment.

It has been widely demonstrated that the presence of bacteria of fecal contamination, is unrelated to that of Vibrionaceae, which are normally present in the marine environment, nor the presence of enteric viruses. The later, in fact, although coming from fecal contamination, are more resistant bacteria to common treatments for the reclamation of waste water and can therefore also be found in waters that are clear of fecal bacteria (Martinez-Urtaza *et al*., 2008; Su and Liu, 2007; Yeung and Boor, 2004).

Among the pathogens indigenous to marine environment, microorganisms belonging to the family Vibrionaceae play primary role in diseases due to the consumption of raw or undercooked seafood from warm temperate seas.

1.3. The genus *Vibrio* and its diversity

In 1854, the first *Vibrio* species i.e., *V. cholerae* was discovered by Italian physician Filippo Pacini in Florence (Thompson *et al*., 2004). The genus *Vibrio* includes Gram-negative bacilli with sizes between 0.5 to 0.8 μm in width and 2-3 μm in length, sometimes slightly curved and shaped furniture for the presence of a polar flagellum, enclosed in a continuous coating with the outer membrane of the cell wall. The vibrios show that aerobic metabolism is fermentative and do not produce spores. The growth of the majority of vibrios is stimulated by the presence of sodium and, for some species, this ion is essential.

It is one of the most studied and diverse genus of microorganisms found in the aquatic ecosystems and comprises the major culturable bacteria in marine and estuarine environments. Many species of vibrios are part of the indigenous aquatic bacterial flora and about half of them have been associated with infections in humans or aquatic animals. According to the Association of *Vibrio* Biologists (A*Vi*B) (http://www.vibriobiology.net/), there are 97 species of *Vibrio* and 2 subspecies (updated on January 2013*)*, but the description of new species has led to a constantly changing taxonomy.

Vibrio spp. are frequently isolated from edible shellfish and some species (such as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*) cause serious foodborne gastroenteritis in human (Thompson *et al*., 2004). In addition, some species, such as *V. anguillarum*, *V. salmonicida*, are pathogenic for fish, *V. splendidus*-related species for bivalves and *V. harveyi* and *V. campbellii* for shrimps (Austin and Austin, 2007; Le Roux *et al*., 2002). Moreover, several *Vibrio* species, for example *V. alginolyticus*, have been characterized as probionts (Gomez-Gil *et al*., 2000) and pathogens (Lee *et al*., 1996).

Some infections from *Vibrio* have importance, as included in those diseases requiring quarantine and compulsory notification to the World Health Organization (eg *V. cholerae*), as known to cause high mortality (eg *V. vulnificus*), or to cause a high number of poisoning in some countries (eg *V. parahaemolyticus* in Japan). In addition to these, other species are known pathogens in humans, but classified as less risky than the first three. Among these *V. mimicus*, so named for its resemblance to *V. cholerae* O1, *V. alginolyticus* and *V. damsela*, *V. fluvialis*, *V. harveyi*, *V. furnissii*, *V. hollisae*, *V. metschnikovii*, *V. anguillarum* and *V. tapetis* are remarkable as pathogens of vertebrates and aquatic invertebrates (Austin, 2010). The predominating *Vibrio* species associated with bivalves are *V. splendidus*, *V. alginolyticus*, *V. harveyi* and the combination of these

species (or some of them) is the most frequent cause of diseases affecting all life stages of bivalve mollusks (Beaz-Hidalgo *et al*., 2010). Originally, *V. anguillarum*, *V. alginolyticus*, *V. tubiashii* and/or *V. splendidus* were the recognized agents associated to larval vibriosis and bacillary necrosis of mollusks (Romalde and Barja, 2010).

Recently, Austin (2010) suggested a new classification of zoonotic *Vibrio* in two groups named Higher Risk Vibrios (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*) and Lower Risk Vibrios (*V. alginolyticus, V. fluvialis, V. furnissii V. harveyi, V. metschnikovii* and *V. mimicus*.

Brief descriptions of the major *Vibrio* species are given below:

Vibrio cholerae: it is the main cause of the human pandemics of cholera, which is caused by cholera-toxin producing strains that has been associated with toxigenic serogroup O1 (Morris, 2003). The source of some outbreaks has been linked with contaminated shellfish, including raw oysters and crabs, and involves non-O1 and non-O139 strains (Farama *et al*., 2008). An estimated 3-5 million cases and over 100,000 deaths occur each year around the world due to cholera (CDC, http://www.cdc.gov/cholera/general/ accessed on 20 January 2013).

Vibrio parahaemolyticus: described in the next section 1.4.

Vibrio vulnificus: it is an important etiologic agent of wound infections and septicemia in humans (CDC, 1996). In the USA, *V. vulnificus* has been regarded as being responsible for most of the seafood-related deaths since the first report in 1979 (Oliver, 2005). A capsular polysaccharide (CPS) is the primary virulence factor in *V. vulnificus* pathogenesis (Wright *et al*., 2001); type IV pili and various proteases, principally a serine protease also determined as pathogenicity factors (Wang *et al*., 2008). It was also recognized as a serious pathogen of eels in Japan, Spain and Denmark (Austin and Austin, 2007); cause disease in *P. monodon* in India (Jayasree *et al*., 2006).

Vibrio alginolyticus: it is a halophilic *Vibrio* implicated with ear, soft tissue and wound infections, of which antibiotic-resistance has been cited as a major issue (Horii *et al*., 2005). Gastroenteritis was thought to be a rare presentation of *V. alginolyticus* infection, but accounted for 12% of infections in one study (Hlady and Klontz, 1993). It is also pathogenic to finfish (sea bream, grouper, cobia etc.) and shellfish (shell disease and white spot in shrimp, mass mortalities in carpet shell clam larvae etc.) (Austin, 2010).

Vibrio harveyi: *V. harveyi* and related species represent major pathogens for aquatic animals, causing diseases responsible for severe economic losses in the aquaculture industry (Cano-Gomez *et al*., 2011). Among vibrios of the Harveyi clade, four species

(*V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*) known as the *V. harveyi* group, are well-known pathogen in marine reared fish, crustacean and shellfish (Gomez-Gil *et al*., 2004)

Vibrio fluvialis: it is a halophilic *Vibrio*, biochemically similar to *Aeromonas hydrophila*, first identified in 1975 in a patient with diarrhea in Bahrain (Furniss *et al*., 1977). *V. fluvialis* rarely causes wound infections or primary septicemia. It also causes disease of lobster, abalone etc.

Vibrio anguillarum: also known as *Listonella anguillarum*, is the causative agent of vibriosis, a deadly hemorrhagic septicemia disease affecting various marine and fresh/brackish water fish, bivalves and crustaceans (Frans *et al*., 2011)

1.4. *V. parahaemolyticus* and seafood safety

Identified for the first time by Japanese researchers in 1951 as an agent of food-borne gastroenteritis, *Vibrio parahaemolyticus* is now recognized as an important intestinal pathogen in many parts of the world especially in Japan where it is the main causative agent of intestinal poisoning, perhaps because of the widespread use of raw fish (Keusch *et al*., 2002).

It is a Gram-negative bacterium, rod-shaped curved, oxidase positive. From the genetic point of view, it has two circular chromosomes, one greater than about 3.2 Mb and a second of 1.9 Mb employed both in DNA replication. Under optimal conditions, the timing of replication are of 8-9 minutes and, likely, the division of the genome into two chromosomes this mechanism makes it faster and more efficient, in addition to improving the adaptability of *V. parahaemolyticus* to the external environment (Yamaichi *et al*., 1999; Han *et al*., 2008).

This bacterium is widely distributed in nature, native to the coastal marine environment (especially in tropical and temperate regions), but is also present in fish, crustaceans and mollusks. The *Vibrio* is responsible for outbreaks associated with the consumption of raw seafood (mostly shellfish) or undercooked. In Western countries, the main food vehicles of *Vibrio parahaemolyticus* consist of shellfish, especially mussels and oysters.

The bivalve organisms living in an environment naturally contaminated, through the filtration can accumulate within them a variety of bacterial species. The treatment to which the filter feeding bivalve mollusks undergo before being traded and during which assume uncontaminated water to remove bacteria such as *Salmonella* and *E. coli*, do not have important effects on the reduction of the microflora of *Vibrio* in the body

(Martinez-Urtaza *et al*., 2008)

The distribution and concentration of this microorganism is influenced by the action of the different environmental conditions of growth, among the most important factors are the temperature, salinity and turbidity. *V. parahaemolyticus* is mostly isolated in the hottest summer months and not in the winter when the water temperature drops below 20 ° C (Parveen *et al*., 2008; Yeung and Boor, 2004).

A quantitative evaluation of dose-response relationship between the levels of *V. parahaemolyticus* swallowed and the frequency and severity of the disease was conducted in Risk Assessment FDA in 2005. The dose-response relationship for *V. parahaemolyticus* estimated from studies on human nutrition surveillance and epidemiological data have shown a probability of disease of 50% at a dose of approximately 100 million cfu. This means that for every 100 portions at that dose level, about 50 people fall ill. At exposures of about 1000 cfu, the probability of disease is relatively low (<0.001). The certainty of disease occurs at exposure levels of approximately $1x10^9$ cfu (http://www.fda.gov/Food/ScienceResearch/ResearchAreas/RiskAssessmentSafetyAssessment/ucm185499.htm accessed on 06 January 2013).

The food infection by *V. parahaemolyticus* generally occurs after an incubation period of 4-96 hours. Clinical symptoms include diarrhea, abdominal pain, nausea, fever but in the case of immunocompromised individuals or liver problems may result in septicemia and death. In milder cases the disease is temporary and treatable without expert doctors and in severe cases can be administered antibiotics (Yeung and Boor, 2004).

Salinity is a prerequisite for the survival and multiplication of *V. parahaemolyticus*, with a range of tolerance of NaCl concentration between 0.5 and 10% and the optimum between first 3% (DePaola *et al*., 2000); in addition, this organism is susceptible to other physical and chemical factors, can survive for three weeks at 4 ° C with a following multiplication at 35 ° C for 48-72 hours, while the freezing to -18 ° C and -24 ° C for 15-28 weeks can permanently inactivate the organism. The heat treatment between 60 ° C and 100 ° C is lethal depending on the size of the population; also other treatments, such as hydrostatic pressure, irradiation, bactericides are effective, managing to reduce the presence of the bacterium (Su and Liu, 2007; Oliver and Kaper, 2007).

The primary basis of strains classification of *V. parahaemolyticus* is a serotyping scheme, which depends on the antigenic properties of the somatic (O) and capsular (K) antigens. The serotyping scheme is a combination of 11 O antigens and 71 K types.

O3:K6, known as pandemic serotype, was first identified in the US in 1998 and caused the largest outbreak associated with oyster consumption (Daniels et al., 2000). Later a pandemic spread of this clone to other continents has been reported. The isolation of the O3:K6 strain from US outbreaks raised concern about increased risks of *V. parahaemolyticus* infections from shellfish consumption. Usually the O3:K6 isolates had identical genotypes (*tdh* positive, *trh* and urease negative) and nearly identical arbitrarily primed PCR (AP-PCR) profiles and shared similar antibiotic sensitivity patterns (Okuda *et al*., 1997).

1.5. The virulence properties of *V. parahaemolyticus*

Using molecular biology techniques, fragments of genes coding for virulence factors are identified that are appropriate of this species represented by *tdh* and *trh*.

The pathogenicity of *Vibrio parahaemolyticus* seems to be related to the presence of two toxins: TDH, thermostable direct hemolysin and TRH, TDH-related hemolysin, whose genes are detectable by biomolecular techniques (PCR). The pathogenic strains are those generally associated with the Kanagawa phenomenon, given by the capacity to induce beta-hemolysis on a special blood agar plate containing fresh human or rabbit erythrocytes, induced by the toxin TDH (Oliver and Kaper, 2007).

The *trh* gene contains the information for the "factors related to the production of TDH" (nominated TRH), which were detected in strains of *V. parahaemolyticus* negative for hemolysis and isolated from patients suffering from gastroenteritis (Lynch *et al*., 2005).

Clinical strains of *V. parahaemolyticus* which showed a Kanagawa phenomenon-negative given the absence of the *tdh* gene produced a TDH-related hemolysin (TRH). The *trh* gene has 68% homology with the *tdh* gene demonstrated by epidemiological studies that have found a strong association between *trh* and *tdh* in clinical strains, and this has suggested that TRH is an important virulence factor with TDH (Oliver and Kaper, 2007).

The *tox*R gene was first discovered as the regulatory gene of the cholera toxin operon and was later found to be involved in the regulation of many other genes of *Vibrio cholerae*. The presence of the *tox*R gene in *V. parahaemolyticus* is a species identifier, but is not connected to toxigenicity, which is confirmed by the presence of toxins TDH or TRH (there are both or only one) (Zulkifli *et al*., 2009).

V. parahaemolyticus has many serotypes based on O and K antigens. Epidemics, increasing substantially in Japan and Thailand, are due to the increase in the incidence

of serotype O3:K6. Outbreaks O3:K6 have also taken place in the United States, after ingestion of raw fish. According to one study in Italy, O3:K6 strains showed the presence of *tdh* gene, but not of *trh* (Ottaviani *et al*., 2008).

In Italy, the work of Lleo *et al*. (2010) shows how *V. parahaemolyticus* isolated from water, sediment, fish and shellfish, have a great serological variability and more than 20% of the studied strains from all over the country and from different matrices are trh^+, two of which have the same serotype of a strain isolated from a clinical case; that may constitute a reservoir of bacterial infections and thereby a risk to human health.

Nowadays, for the lack of a European legislation concerning the control of species of *Vibrio* in the environment and in fish, it is difficult to correlate the clinical case in the presence of the agent. It is thus essential to determine the pathogenicity of the strains isolated through specific cultivation methods or biomolecular approach (Lleo *et al*., 2010).

1.6. Biochemical method of *Vibrio* spp. Identification (Alsina's scheme)

Classical biochemical tests are usually applied to characterize this diverse group, but the great phenotypic diversity of *Vibrio* spp. makes microbiological identification difficult (Alsina and Blanch, 1994a, 1994b).The Vibrios are generally isolated in pure culture using direct plating onto a selective agar medium for *Vibrio*, eg thiosulfate citrate bile salt sucrose agar (TCBS). Samples are usually incubated in selective enrichment medium, eg alkaline peptone water (APW), before plating onto a selective isolation medium. Moreover, these tests require several days and the results can vary with the experience of examiners and could not be always reliable. There are few official protocols specific for *V. cholerae* or *V. parahaemolyticus* isolation and identification, but they cannot be used to analyze other vibrios and may not always be accurate. The common biochemical commercial kits (BIOLOG-GN fingerprints and API 20E profiles) are not totally reliable to recognize *Vibrio* spp., and sometimes they are not able to distinguish *Vibrio* from other bacteria genera, such as *Listonella*, *Photobacterium*, *Aeromonas* (Austin *et al*., 1997, Ottaviani *et al*., 2003, Vandenberghe *et al*., 2003). In addition, when the samples come from environmental sources (seawater, sediments, seafood etc.), it will be more difficult to identify; various *Vibrio* species and related species may show similar biochemical characteristics. Nishibuchi (2006) commented that it has become impossible to establish a comprehensive scheme to differentiate *Vibrio* species using only biochemical characteristics. Identification based on

biochemical tests is not definitive, and the work is time-consuming and resource-intensive. For this reasons, researchers move to molecular genetic identification methods that are quicker and more definitive than biochemical tests.

1.7. Multilocus Sequence Analysis (MLSA) scheme and *Vibrio* spp. Identification

DNA-based molecular methods have become more popular and widely acceptable due to their reproducibility, simplicity and high discriminatory power (Prakash *et al*., 2007).There are some multiplex PCR protocols for *Vibrio* identification, but they are directed only on clinically important species e.g. *V. cholerae*, *V. parahaemolyticus, V. vulnificus* (Bauer and Rorvik 2007, Neogi *et al*., 2010) and sometimes include *V. mimicus* and/or *V. alginolyticus* (Espineira *et al*., 2010, Tarr *et al*., 2007). 16S rRNA gene sequencing ("gold standard") can give an accurate identification of vibrios at the family and genus level but identification at the species and strain levels requires the application of genomic analyses (DNA-DNA hybridization, REP-PCR, AFLP etc.). These techniques are essential for species delineation but their use is restricted to few laboratories and inter-laboratory comparison of fingerprint patterns are very difficult (Thompson *et al*., 2005). MLSA (Multilocus Sequence Analysis) approach is a valid alternative to biochemical as well as fingerprint pattern based methods for species identification. It includes sequencing of several protein-coding housekeeping genes that display faster evolutionary rates than rRNA genes (Gevers *et al*., 2005). The choice of the protein coding genes is of great importance in this method because not all genes are really useful if the strains belong to tightly related species. The selected housekeeping genes should fulfill several criteria to work as alternative phylogenetic markers as suggested by Zeigler (2003): 1) the genes must be widely distributed among genomes, 2) the genes must be present as a single copy within a given genome, 3) the individual gene sequence must be long enough to contain sufficient information but short enough to allow sequencing in a convenient way and 4) the sequences must predict whole genome relationships with acceptable precision and accuracy to correlate with 16S rRNA and DNA-DNA hybridization data. MLSA has revealed phylogenetic clusters of closely related strains depending on the amount of recombination between clusters. *Ad hoc* Committee for the re-evaluation of species definition in bacteriology (Stackebrandt *et al*., 2002) recommended the use of MLSA as an alternative method for species delineation in bacteriology. Bishop and colleagues developed electronic taxonomy of viridians streptococci using MLSA approach, and proposed a generic open access

MLSA website for microbial electronic taxonomy (Bishop *et al.*, 2009). Based on MLSA approach, there is also online electronic taxonomy of Vibrios (http://www.taxvibrio.lncc.br/). MLSA is proved to be very practical and reliable and one of the most important advantages of this approach is the comparison of the obtained sequences between any laboratories, avoiding the problems of lack of comparability when using DNA-DNA data (Pascual *et al*., 2010). It has been widely demonstrated to be a good substitute for DNA-DNA hybridization in studies of the Vibrionaceae. Several molecular markers, e.g. *rec*A, *pyr*H, *rpo*A, *atp*A in single or in concatenated sequences, have been used to identify vibrionaceae species, but these analyses have been mainly applied on type strains (Thompson *et al*., 2004, 2005, 2007a). Recently, Preheim *et al*., (2011) applied MLSA approach for the study of population structure and ecology of Vibrionaceae.

1.8. Multilocus Sequence Typing (MLST) and *V. parahaemolyticus* strains characterization

The Multilocus Sequence Typing (MLST) is a method for the molecular typing proposed in 1998, able to discriminate micro-organisms until the level of strain in a universal way, by comparing the sequences of fragments of housekeeping genes (Maiden *et al*., 1998). MLST is an improved adaptation of MLEE (Multilocus Enzyme Electrophoresis) and has been advocated as the most reliable molecular tool for epidemiology. Both techniques index the variation in housekeeping genes; MLEE compares the electrophoretic mobility of enzymes, while MLST assigns alleles directly from the nucleotide sequences. The characterization by means of MLST turns out to be objective, reliable, transferable to a wide range of isolates and able to return information for the understanding of the epidemiology of outbreaks of contamination (Maiden, 2006).

The number of gene fragments varies based on the level of discrimination that someone wants to achieve relative to genera or species under examination. The classic schemes of MLST able to discriminate different strains belonging to the same species require the analysis of 6-8 fragments of housekeeping genes of length between 400 and 600 nucleotides. The housekeeping genes are the core components of the genome, which are necessary for the performance of the essential stages of cellular metabolism, coding for proteins essential for bacterial survival. The housekeeping genes are choice by the fact that they are found in every strains of a specific species or genus, with a limited level of

evolution.

Generally choosing very expressed genes, encoding a protein with a high degree of "codon-bias" (ie the probability that a given codon is used to encode an amino acid as compared to other codons that encode the same amino acid); equipped with a good power discriminating, of dimensions not excessively high in order to optimize the sequencing, nor limited, so as to contain a sufficient amount of information. The identification of genetic variations in different loci can be defined for each locus an allele, the combination of which generates for each strain its ST (Sequence Type). Analysis of this information allows you to determine the phylogenetic relationships among strains in examination, by creating a phylogenetic tree based on the concatenated sequences of all genes analyzed for each strain.

What makes this method applicable and available at the international level is the use of the web. Through a special database user can compare the data of his study with those in the database, so you can have a global overview of the distribution of pathogenic strains (Maiden, 2006).

The use of MLST is growing as a tool for routine typing, but its functionality also cover other purposes such as studies of antibiotic resistance, an association of particular genotypes to virulence, epidemiological, evolutionary analysis and population studies, estimates of the rate recombination and mutation spread also in diploid organisms (Urwin and Maiden, 2003).

To date, several MLST schemes are available for the typing of different microorganisms, including *Arcobacter* spp. (Miller *et al*., 2009), *Aeromonas* spp. (Martino *et al*., 2011) etc.; and most of the MLST schemes now published and available online at the website http://pubmlst.org. MLST analyses have also been successfully applied to *Vibrio* species like *V. parahaemolyticus* (Gonzalez-Escalona *et al*., 2008) and *V. vulnificus* (Bisharat *et al*., 2005, 2007) for epidemiological studies.

In this study we have chosen to follow the protocol proposed in the MLST database for *Vibrio parahaemolyticus* (http://pubmlst.org/vparahaemolyticus/, Gonzalez-Escalona *et al*., 2008) in order to compare our data with those already in the database.

The genetic profile used is 7 genes; 4 genes on chromosome I and 3 on chromosome II, in order to better represent the genetic distribution of organisms present.

This method is widely used by several authors for epidemiological studies or case reports of environmental sampling in order to have objective and clear guidance on membership of a population or of serotypes already typed in other parts of the world.

An example of application is represented from an article by Yu *et al*. (2011), in which 71 strains of *V. parahaemolyticus* isolates from clinical cases and shellfish were analyzed according to protocol along with 51 MLST profiles taken from the database of isolates from other continents. In this paper, we showed a correlation between the clinical samples isolated in different parts of China, but not connected to the food matrices studied, without relationships with strains obtained from the database.

Hart *et al*., (2009) have applied the MLST study in epidemiological studies. In this work they analyzed strains from clinical cases of disease outbreaks in Chile between 2006 and 2007, noticing a change in serotype pathogenic strains, and in 2006 all the samples belonged to the pandemic serotype O3:K6, while in 2007 it appeared form O3:K59 genomic regions with the same serotype of departure. This suggested that the pathogenic character can be moved laterally by a pandemic strain to another strains.

1.9. Objectives of the Thesis

The microorganisms of the genus *Vibrio* are common inhabitants of aquatic ecosystems for which their presence is more to be associated with fishery products. In Italy, the suitability of microbiological consumption of shellfish does not include the determination of microorganisms naturally present in the marine environment and potentially pathogenic as those belonging to the genus *Vibrio*, but may pose a danger to the consumer.

The aim of this thesis was to develop a MLSA approach to identify and characterize *Vibrio* spp. isolates from shellfish (Mollusks and Crustacea) in Venice Lagoon and Sea (Italy) and to compare molecular data with biochemical results. Four genes (*gyr*B, *rec*A, *pyr*H and *atp*A) have been analyzed. The data were analyzed using different approaches in order to evaluate the typology of the relationships among the strains. The population structure was evaluated to identify the presence of subpopulations.

The aim of the second part was to characterize strains of *Vibrio parahaemolyticus* isolated from edible mollusks by MLST to get a picture of the phylogenetic relationships and investigate existing virulence. In addition, to compare different allelic profiles found in the northern Adriatic with those isolated in the rest of the world to see what relations there may be global.

With this work we want to implement a first step in the Risk Analysis namely the Hazard Identification that characterize the hazard of *Vibrio* spp. associated to the marketing and consumption of shellfish in this area of Italy.

2. MATERIALS AND METHODS

2.1. Sampling

Three sampling were carried out in the northern Adriatic Sea and in Venice lagoon, one in 2007 (mollusks) and two in 2011 (mollusks and crustaceans).

1) A collection of 164 mollusks samples were analyzed from February 2007 to December 2007. Various bivalve species (*Ruditapes philippinarum*, *Ostreaedulis*, *Crassostrea gigas*, *Mytilus galloprovincialis*, *Ensis* spp., *Solen* spp., *Chamelea gallina*, *Callista chione*, *Cerastoderma* spp.), *Paracentrotus lividus* (sea urchin) were collected from Venice lagoon and sea, Italy (see map http://www.regione.veneto.it/NR/rdonlyres/C832ED55-B014-4E3B-8EE6-D90BAD9E541F/0/allegatoc_mappa_lowpdf.pdf). (Table S1 for details sampling information).

2) To verify the developed MLSA scheme to identify *Vibrio* species, a preliminary analysis was done using 15 fresh, frozen and unfrozen samples of various Crustacean species (*Palaemon* spp., *Crangon crangon*, *Squilla mantis*, *Hymenopenaeus muelleri*, *Carcinus aestuarii*) collected from fish market of Venice in 2011 (Table S2 for details sampling information). The shellfish were coming mainly from the North Adriatic, including the area of Chioggia, the Venice Lagoon, the Po Delta (Goro) and also included samples from Southern Adriatic which are abundantly sold in Veneto region.

3) A second sampling (from January 2011 to October 2011) of 133 Mollusks was done in different rearing areas and various depths (Table S3 for details sampling information) with the aim to isolate only the *Vibrio parahaemolyticus* strains. MLST scheme were then applied to characterize these isolated strains.

2.2. Isolation of *Vibrio* strains by Biochemical methods (Alsina's scheme)

In collaboration with Food Microbiology Laboratory, IZSVe (Legnaro and Adria, Italy), the samples were prepared following ISO/TS 21872-(1 and 2): 2007 (E) with some modifications. For the first enrichment, 25 g of sample (Mollusks pulp or crustacean pulp and a portion of the carapace) were homogenized in 225 ml of Alkaline Peptone water with 3% NaCl and incubated at 37°C for (18-24) hours. The second enrichment was done with Polymyxin B and incubated at the same temperature and time period of the first enrichment. The cultures obtained in the enrichment medium were streaked on thiosulphate citrate bile salt sucrose agar (TCBS) and on ChromAgar plates. The *Vibrio* presumptive colonies were then subjected to gram staining, oxidase test and O/F test.

Gram-negative, oxidase-positive and facultative anaerobes (+/+ for O/F test) isolates were identified with the dichotomous keys proposed by Alsina and Blanch (1994) through a series of 29 different biochemical tests. The scheme was designed for routine purposes to provide fast and presumptive identification of *Vibrio* spp., especially for environmental isolates. 47 different species were included in the scheme: 38 *Vibrio* spp., 3 *Photobacterium*, 1 *Plesiomonas* and 5 undetermined species. Several *Vibrio* species, for example *V. brasiliensis, V. chagasii, V. diabolicus, V. owensii, V. rotiferianus* and *V. shilonii* were not included in Alsina's scheme, but they were added in the subsequent analyses with MLSA approach.

2.3. MLSA approach

2.3.1. Design of Primers

Four housekeeping genes (*gyr*B, *pyr*H, *rec*A and *atp*A) were chosen for the MLSA analysis. Most of the available partial and full length sequences of the four *Vibrio* housekeeping genes were downloaded from the GenBank database and aligned by the ClustalW program (http://www.ebi.ac.uk). Primers were designed from the most conserved regions by using Primer3 software (http://frodo.wi.mit.edu/primer3/) and PriFi software for degenerated primers (Fredslund, 2005) with a length of 18 to 29 nucleotides. Primers for the amplification of the internal region of *atp*A were obtained from a previous study (Thompson *et al*., 2007b). The complete list of genes analyzed in this study and all primers used for PCR amplifications and sequencing is listed in Table1.

Table 1: Primers used for amplification and sequencing of *Vibrio* spp. isolates in MLSA analyses

Primers	Sequence (5′-3′)	Gene product	Trimmed amplicon length (bp)	Annealing temperature (°C)	Reference
Vi_gyrBdg2F	GARGTGGTRGATAACTCWATTGATGAAGC (29)	DNA gyrase, β subunit (*gyr*B)	570	55	This study
VigyrBR	CGGTCATGATGATGATGTTGT (21)				
VigyrBF	GAAGGTGGTATTCAAGCGTT (20)				
Vh_gyrB_F	CGTGAGCTTTCTTTCCTAAACTC (23)				
VipyrHdgF	CCCTAAACCAGCGTATCAACGTATTC (26)	Uridylate kinase (*pyr*H)	501	55	This study
VipyrHdgR	CGGATWGGCATTTTGTGGTCACGWGC (26)				

VirecAF	TGCGCTAGGTCAAATTGAAA (20)	Recombinase	462	55	This study
VirecAdgR	GTTTCWGGGTTACCRAACATYACACC (26)	A (*rec*A)			
Vi_atpAdg_F	ATCGGTGACCGTCARACWGGTAAAAC (26)	ATP	489	60	This study
Vi_atpAdg_R	ATACCTGGGTCAACCGCTGG (20)	synthase, α			
ViatpA-01-F	CTDAATTCHACNGAAATYAGYG (22)	subunit		57	Thompson
ViatpA-04-R	TTACCARGWYTGGGTTGC(18)	(*atp*A)			*et al*., 2007b

2.3.2. DNA extraction, PCR amplification and Sequencing

For DNA extraction, a single colony from a fresh culture was resuspended in 100µl nuclease-free water, vortexed at high speed for 5s, and incubated at 94°C for 10 min. The tube was vortexed again and centrifuged for 2 min at 14,000 rpm. The supernatant was transferred to a fresh tube and stored at -20°C.

The PCR amplification was performed in a Euroclone One Advanced thermal cycler (Celbio, Milan, Italy). The PCRs were performed in a final volume of 20 µl of amplification mix containing 1 U of GoTaq polymerase (Promega, Madison, WI), 1X GoTaq buffer, 2.5 mM MgCl2, 0.1mM each deoxynucleoside triphosphate (dNTP), 125nM each primer, and 5 ng of genomic DNA as the template.

For *atp*A, *pyr*H and *rec*A genes, amplification conditions comprised an initial 2 min denaturation step at 94°C followed by 35 cycles of 20 s at 94°C, 30 s at different annealing temperatures (55°C for *pyr*H and *rec*A; 60°C for *atp*A) depending on the amplified target, and 30 s at 72°C, with a final extension at 72°C for 7 min.

The reaction mixture was subjected to a touchdown PCR for *gyr*B gene as follows: an initial step at 95°C for 2 min, followed by 40 cycles each of denaturation at 95°C for 10 s, annealing at changing temperatures (i.e., the temperature changed from 65°C to 55°C in 0.5°C decrements during the first 20 cycles) for 30 s, extension at 72°C for 50s and with a final extension at 72°C for 7 min. Amplified products were analyzed by electrophoresis on 1.8% agarose–Tris-acetate- EDTA (TAE) gels, stained with SYBR Safe (Invitrogen, Carlsbad, CA), and visualized on a UV transilluminator.

Conditions for direct sequencing without any additional purification of templates were used, except for a few cases when standard PCR conditions (0.2 mM dNTPs, 250 nM both primers) were used, followed by Illustra^{TM} ExoStar purification using manufacturer's standard operating protocol (GE Healthcare Life Sciences UK Limited, UK).

Bidirectional sequencing of the four target genes was performed using the respective primer pairs used for PCR amplifications as sense and antisense sequencing primers,

except for *gyr*B gene where sequencing reactions were carried out using VigyrBF as sense primer and the same reverse primer used for the amplification as antisense sequencing primer. In addition, Vh_gyrB_F was used for the amplification and sequencing of some strains that were not amplified byVi_gyrBdg2F primer.

The nucleotide sequences were determined using the BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and the electrophoresis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) automated sequencer, according to the manufacturer's instructions. Finally, the sequences of the amplicons were verified by BLAST search (Altschul *et al*., 1997) to indicate whether they had homology to the respective genes for which the primers were designed.

2.3.3. Phylogenetic analyses of MLSA data

Analysis, editing, and comparison of the chromatograms and sequences obtained for the four genes were performed using FinchTV software (Geospiza). The consensus sequence for each gene fragment was determined by the alignment of the forward and reverse sequences by ClustalW program (http://www.ebi.ac.uk). The coding sequences used for the housekeeping genes were read in frame. Allele sequences that differed from each other by one or more polymorphisms were attributed to a unique allele number in the order of discovery. Each unique allelic profile, as defined by the allele numbers of the four loci, was assigned a sequence type (ST). The same ST was used for the strains that shared the same allelic profile. Multiple alignments containing the concatenated sequences were straightforward and were performed according to the genomic gene order: *gyr*B, *pyr*H, *rec*A and *atp*A. All the analyzed MLSA sequences had the same length (2022 nucleotides).

Diversity indices, such as the G+C content of each locus, number of polymorphic sites, average numbers of synonymous and non-synonymous sites, Tajima's *D*, nucleotide diversity per site (π), and the average number of nucleotide differences per site (θ), were calculated using DnaSP version 5.10 (Librado and Rozas, 2009).

For phylogenetic analysis, concatenated sequences were aligned and analyzed by using MEGA v5.04 (Tamura *et al*., 2011). Genetic distances were computed by Kimura two-parameter model and the phylogenetic tree was constructed using the neighbor-joining method. At the same time, a phylogenetic tree was also constructed for each gene to create a comparison between the four single gene trees and the concatenated one.

In order to better describe the phylogenetic relatedness among isolates, we also sequenced 16 *Vibrio* reference strains and included the sequences of 13 *Vibrio* spp. and 1 *Photobacterium profundum* strains downloaded from NCBI database (Table 2). The taxon names of each cluster were attributed according to the available representative reference/NCBI strains clustered in the same group. When the isolates were considered related but clearly distinct, the species name was used with the addition of '-like' (e.g. *V. mediterranei*-like) but if the isolates were considered not closely related to the reference strains, the strain name representative for the cluster was used (e.g. *Vibrio* sp. Vi20).

Table 2: Reference/Type strains included in the MLSA analyses

Serial number	Species name	We sequenced	Sequences downloaded from NCBI				
			Strain code	Accession numbers			
				gyrB	pyrH	recA	atpA
1	*Vibrio alginolyticus*	ATCC 17749^{T}	Strain 40B	ACZB01000013	ACZB01000030	ACZB01000012	ACZB01000013
2	*Vibrio anguillarum*	ATCC 43305	Strain 775	NC_015633	NC_015633	NC_015633	NC_015633
3	*Vibrio brasiliensis*	-	LMG 20546^{T}	AEVS01000075	AEVS01000115	AEVS01000055	AEVS01000057
4	*Vibrio campbellii*	CECT 523^{T}	-				
5	*Vibrio chagasii*	LMG 21353^{T}	-				
6	*Vibrio cholerae*	-	O1 biovar El Tor str. N 16961	NC_002505	NC_002505	NC_002505	NC_002505
7	*Vibrio diabolicus*	LMG 23867	-				
8	*Vibrio fischeri*	-	Strain ES 114	NC_006840	NC_006840	NC_006840	NC_006840
9	*Vibrio fluvialis*	ATCC 33809^{T}	-				
10	*Vibrio furnissii*	-	NCTC 11218	NC_016602	NC_016602	NC_016602	NC_016602
			BAA-1116^{T}	NC_009783	NC_009783	NC_009783	NC_009783
11	*Vibrio harveyi* *	ATCC 14126^{T}	Strain HY01	AAWP01000066	AAWP01000277	AAWP01000115	AAWP01000035
			Strain 1DA3	ACZC01000040	ACZC01000013	ACZC01000012	ACZC01000017
12	*Vibrio mediterranei*	CECT 621^{T}	-				
13	*Vibrio mimicus*	-	Strain VM 603	ACYU01000116	ACYU01000183	ACYU01000010	ACYU01000044
14	*Vibrio orientalis*	CECT 629^{T}	-				
15	*Vibrio owensii*	LMG 25443^{T}	-				
16	*Vibrio parahaemolyticus*	ATCC 17802^{T}, ATCC 43996	RIMD 2210633	NC_004603	NC_004603	NC_004603	NC_004603
17	*Vibrio rotiferianus*	LMG 21460^{T}	-				
18	*Vibrio shilonii*	LMG 19703^{T}	-				
19	*Vibrio splendidus*	LMG 19031^{T}	LGP 32	FM954972	FM954972	FM954972	FM954972
20	*Vibrio vulnificus*	ATCC 27562^{T}	-				
21	*Photobacterium profundum*	-	Strain SS9	CR378663	CR378672	CR378673	CR378674

Total 29 reference strains (21 species) included in the analyses with one *P. profundum* as outgroup.
* Lin *et al*. (2010) identified strains BAA-1116 and HY01 as *Vibrio campbellii* by Comparative genomic analyses.

2.3.4. Recombination analyses

Evidence of recombination was investigated using SplitsTree 4.10 software (Huson and Bryant, 2006). Split networks were constructed with EqualAngle algorithm both for individual loci and for the concatenated sequences, and then analyzed using the Pairwise Homoplasy Index (PHI) test (Bruen *et al*., 2006) implemented in SplitsTree4.10 to identify alleles with significant evidence of recombination.

2.3.5. STRUCTURE analyses

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. The following parameters were used: 5 iterations, following a burn-in period of 100,000 iterations; Markov chain Monte Carlo [MCMC] = 50,000 with a *K* between 1 and 20. Finally, the evaluation of K was performed as suggested by Evanno *et al*., 2005.

2.3.6. Statistical Methods for Rater and Diagnostic Agreement

The McNemar test (McNemar, 1947) is a way to test marginal homogeneity in K×K tables. McNemar test was done using MH (Marginal Homogeneity) Program (v. 1.2) (http://www.john-uebersax.com/stat/mh.htm). This program is used for the analysis of agreement among raters, diagnostic tests etc. We use dichotomous categorical ratings ie Yes/No, Present/Absent. Very often agreement studies are an indirect attempt to *validate* a new rating system or instrument. That is, lacking a definitive criterion variable or "gold standard," the accuracy of a scale or method is assessed by comparing its results when used by different raters. We used this program to compare our developed MLSA approach with Classical biochemical methods (Alsina's scheme) to identify the several clusters of *Vibrio* spp. Both the Bhapkar test (Bhapkar, 1966) and the Stuart-Maxwell test (Stuart, 1955; Maxwell, 1970) were done to test overall marginal homogeneity for all categories simultaneously.

2.4. MLST characterization

2.4.1. Isolation of *V. parahaemolyticus* strains by biochemical and MLSA

V. parahaemolyticus strains isolated in 2011 (Table S3 for details information) were used for MLST characterization. As reported in the previous section (2.2), they were isolated and identified to species level by classical biochemical techniques (Alsina's scheme) in the Food Microbiology Laboratory of IZSVe, Legnaro (Italy). Species specific *tox*R and *tlh* genes were also checked for confirmation. The suspected isolates were then subjected to MLSA identification (described in previous sections). Finally, all strains recognized as *V. parahaemolyticus* were characterized using MLST scheme developed by Gonzalez-Escalona *et al*., 2008.

2.4.2. Primer specific for *V. parahaemolyticus* MLST, PCR amplification and sequencing

PCR amplification was carried out using primers described on the *V. parahaemolyticus* MLST website (http://pubmlst.org/vparahaemolyticus). The seven housekeeping genes loci analyzed by MLST were dispersed on both chromosomes (Table 3).

Table 3: List of genes used and their position in the chromosomes

Genes	**Chromosomes**
*dna*E (DNA polymerase III, alpha subunit) *gyr*B (DNA gyrase, subunit B) *rec*A (Recombinant A, protein)	Chromosome I
*dtd*S (Threonine dehyrogenase) *pnt*A (Transhydrogenase alpha subunit) *pyr*C (Dihydroorotase) *tna*A (Tryptophanase)	Chromosome II

Table 4: Primers used in the MLST study, with the sequence and length of the amplicons

Loci	**Sequences**	**Amplicon size (bp)**
*rec*A	GAAACCATTTCAACGGGTTC CCATTGTAGCTGTACCAAGCACCC	773
*gyr*B	GAAGGBGGTATTCAAGC GAGTCACCCTCCACWATGTA	629
*dna*E	CGRATMACCGCTTTCGCCG	596

*dtd*S	GAKATGTGTGAGCTGTTTGC TGGCCATAACGACATTCTGA GAGCACCAACGTGTTTAGC	497
*pnt*A	ACGGCTACGCAAAAGAAATG TTGAGGCTGAGCCGATACTT	470
*pyr*C	AGCAACCGGTAAAATTGTCG CAGTGTAAGAACCGGCACAA	533
*tna*A	TGTACGAAATTGCCACCAAA AATATTTTCGCCGCATCAAC	463

The PCR amplification was performed in a Euroclone One Advanced thermal cycler (Celbio, Milan, Italy). Conditions for direct sequencing without any additional purification of templates were used. The PCRs were performed in a final volume of 20 µl of amplification mix with the following composition. Concentration of MgCl2 can vary in the reaction mixture to obtain better amplification.

Table 5: The concentration of reagents for MLST PCR

Reagents	**Concentration**
Reaction Buffer 5X	1X
$MgCl_2$ 25 µM	2.5 mM
dNTPs each 25 µM	0.125mM
Forward Primer 10 µM	125nM
Reverse Primer 10 µM	125nM
Taq polymerase 5 U/µl	1U
Distilled H_2O	Add until 20µl
Template DNA of 2 ng/µl	1 ng

Table 6: The reaction mixture was subjected to the following PCR conditions.

Steps	**Temperature (°C)**	**Time**	**Cycles**
Activation of Taq polymerase	94 °C	2 min.	1 cycle
Denaturation	96 °C	1min.	30 cycles
Annealing	58 °C	1 min.	
Extension	72 °C	1 min.	
Final extension	72 °C	10 min.	1 cycle

For the *rec*A gene, the best results were obtained by increasing the annealing temperature to 60°C.

Amplified products were analyzed by electrophoresis on 1.8% agarose–Tris-acetate-EDTA (TAE) gels, stained with SYBR Safe (Invitrogen, Carlsbad, CA), and visualized on a UV transilluminator.

PCR products were sequenced in both directions with primers M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGACC-3′). The nucleotide sequences were determined using the BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), and the electrophoresis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) automated sequencer, according to the manufacturer's instructions. Finally, the sequences of the amplicons were verified by BLAST search (Altschul *et al*., 1997) to indicate whether they had homology to the respective genes for which the primers were designed.

2.4.3. MLST data treatment and phylogenetic analyses

Chromatograms and sequences obtained for the seven genes from the 102 strains of *V. parahaemolyticus* were treated as described in the section **2.3.3.** Variety of information were collected from the database http://pubmlst.org/vparahaemolyticus/ on individual loci and on isolates examined.

The database was used to derive the ID numbers of alleles present in our dataset by entering the nucleotide sequence, ST derived from different allelic profiles and information on isolates already present in the database. Multiple alignments containing the concatenated sequences were straightforward and were performed according to the genomic gene order. All analyzed MLST sequences had the same length (3669 nucleotides).

Diversity indices were calculated using DnaSP version 5.10 (Librado and Rozas, 2009).

For phylogenetic analysis, concatenated sequences were aligned and analyzed by using MEGA v5.04 (Tamura *et al*., 2011). Genetic distances were computed by the Kimura two-parameter model, and the phylogenetic tree was constructed using the neighbor-joining method.

2.4.4. Recombination analyses

Evidence for recombination between STs of each allele was investigated by using different approaches. Split-decomposition trees were constructed with 1,000 bootstrap replicates based on parsimony splits as implemented in SplitsTree 4.0 (Huson and

Bryant, 2006). The resulting trees, for individual loci and for the concatenated sequences, were analyzed using the Pairwise Homoplasy Index (PHI) test (Bruen *et al*., 2006) to identify alleles with significant evidence of recombination.

Recombination was also investigated by analyzing all STs with 7 algorithms implemented in the RDP3 program (RDP, Chimaera, GENCONV, MaxChi, Bootscan, Siscan and 3Seq) (Martin *et al*., 2010). Evidence for recombination was accepted if significant ($P < 0.001$) and obtained with at least three tests implemented in the RDP3 software.

2.4.5. Structure analysis

Structure allows analyzing data derived from the MLST to identify the different genomic cluster and providing, in addition, a display of mixed genomic profiles. The analysis was done as described in the section **2.3.5.**

2.4.6. eBURST, PHYLOViZ and ConalFrame analyses

Strain relationships were analyzed using the eBURST program (http://eburst.mlst.net/default.asp) to identify potential clonal complexes and founders (Feil *et al*., 2004). This software uses a model of bacterial evolution simple but effective, in which an ancestral genotype increases in frequency in the population and begins to diversify to produce a cluster of closely related genotypes that are all descended from the founding genotype. This cluster of genotypes is called "clonal complex". The output is a radial diagram, which shows the center of the founder genotype. The input given by the operator in eBURST is the allelic profile and the STs calculated for each isolates. The identification of clonal groups can be made by considering how closely isolates are related that share 6 of the 7 alleles and in such case, the group is called Single Clonal Complex, while a less stringent approach assumes to reunite isolates into a single group that share the same allele at least five loci. The primary founder is identified as the Sequence Type (ST) that differs from other STs in a single locus (Single Locus Variants, SLV). The analysis with eBURST was performed using the default parameters, in which STs are attributed to the same group only with Single Locus Variant.

PHYLOViZ (http://www.phyloviz.net/wiki/, Francisco et al. 2012) is a platform that allows the integration of typing analysis based on gene sequences with a series of data, such as epidemiological, environmental, geographic etc. It has two expansions

(goeBURST and Minimum Spanning Tree) which allow visualization of the phylogenetic relationships among isolates. The results are represented by a tree without roots. The input is represented by a table showing the different allelic profiles, which is reworked by an algorithm that builds a tree by simply connecting Single Locus Variant, or by evaluating the Euclidean distance between the characters considered for each isolates. The distance is calculated as the sum of the differences between all loci. PHYLOViZ was used not only for the determination of clonal complexes, but also to verify the possible relations among environmental information (Origin, presence of virulence genes etc.) of the individual strain with the data of genotypic characterization.

ClonalFrame is a software for the inference of bacterial microevolution that allows estimation of the clonal relationship between the members of a dataset; at the same time, the presence of recombination. The analysis allows viewing the position of homologous recombination events that changed the clonal inheritance.

The evaluation of r / m (ratio of recombination to mutation) was performed as suggested by Vos and Didelot (2009) for the entire population analyzed.

2.5. PCR identification and Virulence genes PCR of *V. parahaemolyticus*

In this study, two genes were investigated to confirm the identification of *V. parahaemolyticus* and another two to verify the virulence properties of the isolates (Table 7). For the assessment of the virulence genes, every reaction was performed three times so as to have a confirmation of the test. Two positive controls of *V. parahaemolyticus* which contained these genes (ATCC 43996, ATCC 17802) were used.

Table 7: Primers used in this study with sequence, relative length of the amplicons, the authors and the objective of the analysis.

Genes	Sequence	Length (bp)	Authors	Scope
*tox*R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	Kim *et al*., 1999	Identification
tlh	AAAGCGGATTATGCAGAAGCACTG GCTACTTTCTAGCATTTTCTCTGC	450	Bej *et al*., 1999	
tdh	GTAAAGGTCTCTGACTTTTGGAC TGGAATAGAACCTTCATCTTCACC	270		Virulence
trh	TTGGCTTCGATATTTTCAGTATCT CATAACAAACATATGCCCATTTCCG	500		

The reaction mixture was prepared to have a volume of 20 µl of the final product. While for *tlh* and *tdh* has been possible to operate a PCR-duplex in which the reactions occur simultaneously for the two genes, *tox*R and trh require separate reaction, because the amplicons would not be distinguishable in the agarose gel.

Table 8: Composition of the reaction mixture for single reaction with the relative amount expressed in µl to a final volume of 20 µl.

Reagents	**Quantity (µl)**		
	***tox*R**	***tlh+tdh***	***trh***
Green Buffer 1X	4.0 µl	4.0 µl	4.0 µl
$MgCl_2$ 25 µM	2.0 µl	3.4 µl	2.4 µl
dNTPs 25 µM	0.1 µl	0.2 µl	0.2 µl
Primer foward10µM	0.5µl	1.0 µl +1.0 µl	1.0 µl
Primer reverse10µM	0.5µl	1.0 µl +1.0 µl	1.0 µl
Taq polymerase 5 U/µl	0.16 µl	0.2 µl	0.2 µl
H_2O BDH	7.64 µl	4.2 µl	6.2 µl
DNA	5 µl	5 µl	5 µl

The amplification of the different genes requires a different thermal cycling reported in Table 9.

Table 9**:** Thermal cycles used for the amplification of species-specific and virulence genes PCR of *V. parahaemolyticus* in this study.

tlh-tdh-trh			
Steps	**Temperature (°C)**	**Time**	**Cycles**
Activation of Taq polymerase	94 °C	3 min.	1 cycle
Denaturation	94 °C	1 min.	25 cycles
Annealing	55 °C	1 min.	
Extension	72 °C	2 min.	
Final extension	72 °C	5 min.	1 cycle

***tox*R**			
Steps	**Temperature (°C)**	**Time**	**Cycles**
Activation of Taq polymerase	94 °C	3 min.	1 cycle
Denaturation	94 °C	1 min.	20 cycles
Annealing	63 °C	1 min.	
Extension	72 °C	1.5 min.	
Final extension	72 °C	3 min.	1 cycle

Amplified products were analyzed by electrophoresis on 1.8% agarose–Tris-acetate-EDTA (TAE) gels, stained with SYBR Safe (Invitrogen, Carlsbad, CA), and visualized on a UV transilluminator.

3. RESULTS

3.1. *Vibrio* spp. from mollusks 2007: Biochemical identification

In 2007, 123 mollusks samples out of 164 (75%) were positive for *Vibrio* spp. 168 strains were isolated and identified as various species of *Vibrio* according to Alsina's scheme (Table 10). The most common species found (for a total of 33 isolates) was *V. parahaemolyticus* followed by *V. alginolyticus* (29 isolates) and *V. vulnificus* (21 isolates). 3 strains were identified as *Vibrio* spp. and no *V. cholerae* were isolated in this study.

Table 10: List of biochemically identified (with Alsina's scheme) 168 *Vibrio* strains isolated from Mollusks in 2007

Sl. No.	Species name	Number as Alsina's scheme
1	*V. alginolyticus*	29
2	*V. anguillarum*	10
3	*V. campbellii*	3
4	*V. fischeri*	3
5	*V. fluvialis*	17
6	*V. harveyi*	6
7	*V. logei*	4
8	*V. marinus*	1
9	*V. mediterranei*	15
10	*V. mimicus*	3
11	*V. nereis*	4
12	*V. parahaemolyticus*	33
13	*V. pelagius*	6
14	*V. splendidus*	10
15	*V. vulnificus*	21
16	*Vibrio* spp.	3
	Total	**168**

3.2. *Vibrio* spp. from mollusks 2007: Identification by MLSA approach

3.2.1. Genetic diversity and phylogeny based on multilocus data

154 out of 168 isolates were analyzed with the MLSA approach: 14 isolates were not included in the analysis (7 strains biochemically identified as *Vibrio* spp. were not able to be revitalized and 7 isolates were not amplified using the four MLSA housekeeping genes). 8 strains (Vi_20, Vi_51, Vi_54, Vi_60, Vi_62, Vi_73, Vi_9a, and Vi_16a) were not amplified with *gyr*B primers. An alternative forward primer (Vh_gyrB_F) was

designed within 60 bp upstream of the Vi_gyrB_F primer, in order to maintain the same final *gyr*B fragment length. All the 8 strains were amplified with this primer and they showed a 3 bases insertion.

The examination of the nucleotide variability revealed 18 times more synonymous substitutions than non-synonymous substitutions. The mean G+C content of the four genes was very similar and varied from 47.6% (*atp*A) to 48.2% (*pyr*H). The genetic equilibrium of alleles was analyzed by using the Tajima's *D* neutrality test (Tajima, 1989). All of the obtained *D* values were comprised between -2 and 2, supporting a neutral selection of the considered genes (Table 12). The nucleotide diversity (the average number of nucleotide differences per site from two randomly selected sequences) was high in all genes (ranging from 0.083 for *atpA* to 0.139 for *pyrH*). The sequence variability among all *Vibrio* strains was 38.8%, which corresponded to 784 polymorphic sites (nucleotide diversity of 0.118) in the concatenated sequence. The genotypic diversity was high, and 137 distinct STs were identified. This high number of different alleles was expected because distinct species/taxa were processed. Only 12 STs include more than one strain; ST 33 and ST 125 included 4 isolates, ST 3 had 3 isolates and ST 5, 20, 30, 48, 72, 75, 78, 95 and 123 included 2 isolates.

Table 11: STs with more than one strain among isolates of 2007

ST	**Strain ID**
3	Vi_10, Vi_12, Vi_59
5	Vi_13, Vi_47
20	Vi_32, Vi_34a
30	Vi_42, Vi_10a
33	Vi_45, Vi_48, Vi_49, Vi_74
48	Vi_64, Vi_66
72	Vi_15a, Vi_23a
75	Vi_18a, Vi_26a
78	Vi_21a, Vi_25a
95	Vi_42a, Vi_8b
123	Vi_72a, Vi_73a
125	Vi_75a, Vi_76a, Vi_77a, Vi_1b

*out of 137 STs, 12 different ST have more than one strains and 125 STs have one strain

Table 12: Nucleotide diversity observed within the *Vibrio* spp. (154 strains of 2007) characterized in this study

Locus	Fragment size (bp)	No. of alleles	G+C content	No. (%) of polymorphic sites	No. of parsimony informative sites	Synonymous changes	Non-synonymous changes	Tajima's *D* test	θ	π
*gyr*B	570	111	0.481	232 (40.7)	214	223	15	0.15625	0.162	0.133
*pyr*H	501	87	0.482	196 (39.1)	175	211	8	0.23421	0.170	0.139
*rec*A	462	106	0.478	190 (41.1)	174	198	6	0.12117	0.170	0.138
*atp*A	489	79	0.476	167 (34.1)	132	183	16	-0.65010	0.093	0.083
Concatenate	2022	137	0.479	785 (38.8)	697	818	44	0.09115	0.140	0.118

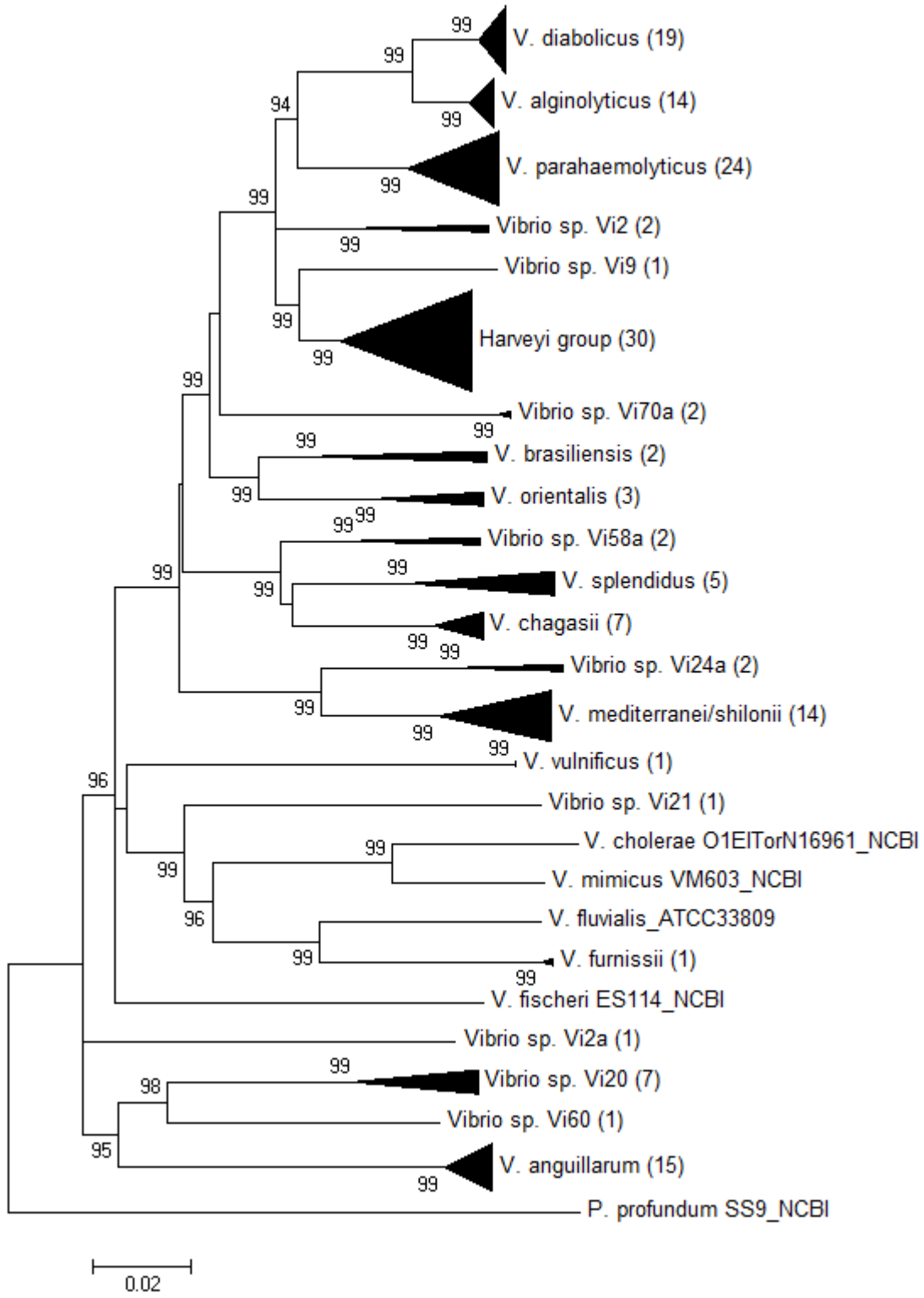

Figure 1: Neighbor-joining phylogenetic tree (compressed) with concatenated sequences of four housekeeping genes for the *Vibrio* strains isolated from mollusks in 2007. The numbers in brackets describe the number of strains included in the reference species group which is represented by a black triangle.

The phylogenetic tree obtained with the concatenated sequences of the 4 genes showed 15 clusters and 6 single strains (Figure 1). All bootstrap values were highly supported, demonstrating a high reliability of the phylogenetic relationships that were described. Some isolates did not cluster in specific groups and as a consequence they have not defined with a species name. They separately clustered in 9 different groups; 4 (*Vibrio* sp. Vi9, Vi21, Vi60 and Vi2a) with only one isolate, 4 (*Vibrio* sp. Vi2, Vi24a, Vi58a and Vi70a) with two isolates and one (*Vibrio* sp. Vi20) with 7 isolates. The phylogenetic analysis conducted on each gene mostly supported the distribution of the concatenated sequences, although little variations on some species clustering (such as *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. diabolicus*) are visible (supplementary figure 1).

3.2.2. Evidence of recombination

Evidence for recombination in the MLST loci was also investigated with the SplitsTree program, which used the split decomposition method separately on each locus and on the concatenated sequences of all STs (Fig 2). Individual genes were not significantly affected by intragenic recombination, but in all cases, parallelogram formation was evident that is indicative of some recombination events. Significant recombination ($P=1.8 \times 10^{-14}$) was found with concatenated sequence of all STs. The concatenated sequences of the most represented species (*V. alginolyticus*-*V. diabolicus* group, *V. anguillarum*-like, *V. harveyi*-group, *V. mediterranei* and *V. parahaemolyticus*-like) were analyzed separately and showed significant presence of recombination ($P=1.2 \times 10^{-6}$) within the *V. alginolyticus*-*V. diabolicus* group (Fig 3).

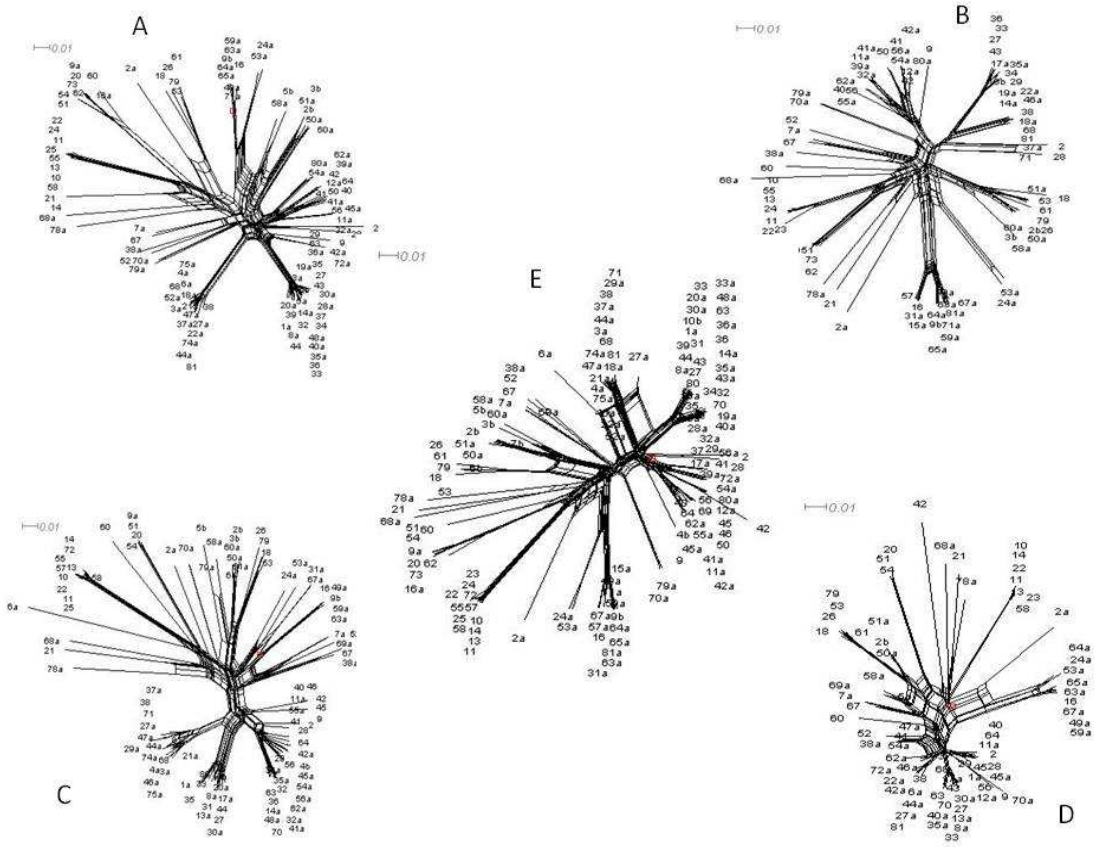

Figure 2: Isolates of mollusks in 2007: SplitsTree graphs of the four single loci and concatenated sequences of all STs constructed in SplitsTree v4.0: *gyr*B (A), *pyr*H (B), *rec*A (C), *atp*A (D) and concatenated (E). Significant evidence of recombination was obtained in concatenated tree by using the PHI test.

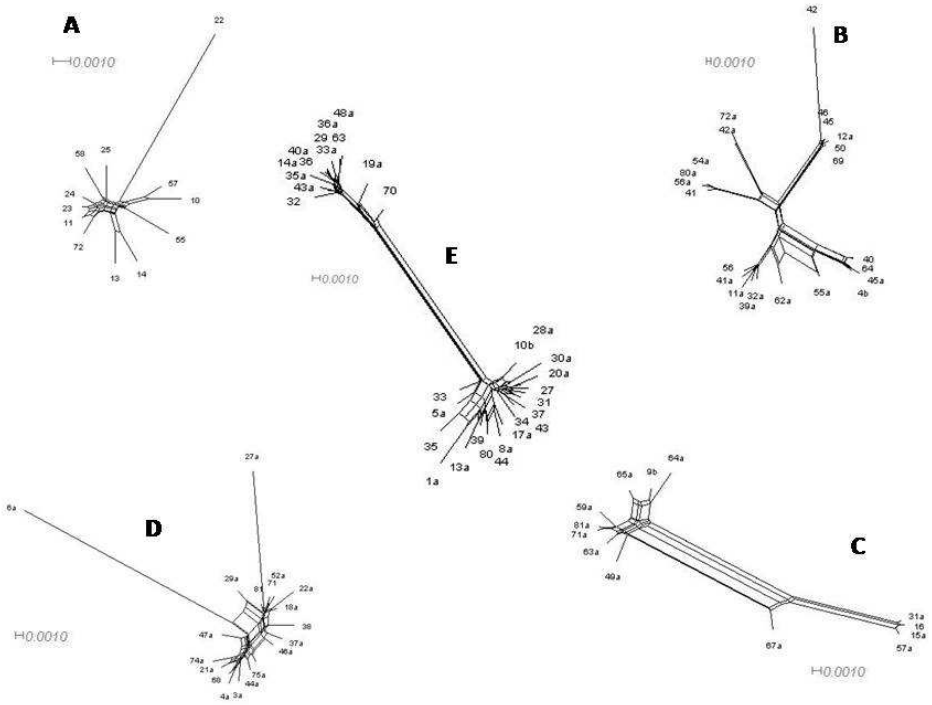

Figure 3: Isolates of mollusks in 2007: SplitsTree graphs of the most representative *Vibrio* groups using concatenated sequences of all 4 loci constructed in SplitsTree v4.0: *V. anguillarum*-like (A), *V. harveyi*-group (B), *V. mediterranei* (C), *V. parahaemolyticus*-like (D) and *V. alginolyticus*-*V. diabolicus* group (E). Significant evidence of recombination was obtained in *V. alginolyticus*-*V. diabolicus* group by using the PHI test.

3.2.3. STRUCTURE analyses

Structure software was used to identify the main groups (which differed in terms of their allele frequencies) and more subtle recombination events to detect strains carrying foreign DNA. 17 sub-populations were identified for this analysis (corresponding to seventeen colors in Fig. 4) because repeated analyses showed that the model probability was best supported at a *K* value of 17 according to Evanno *et al*., 2005. Among the same species, most strains were homogeneous. Some strains presented mixed colors in the corresponding column, demonstrating the import of gene sequences from other species.

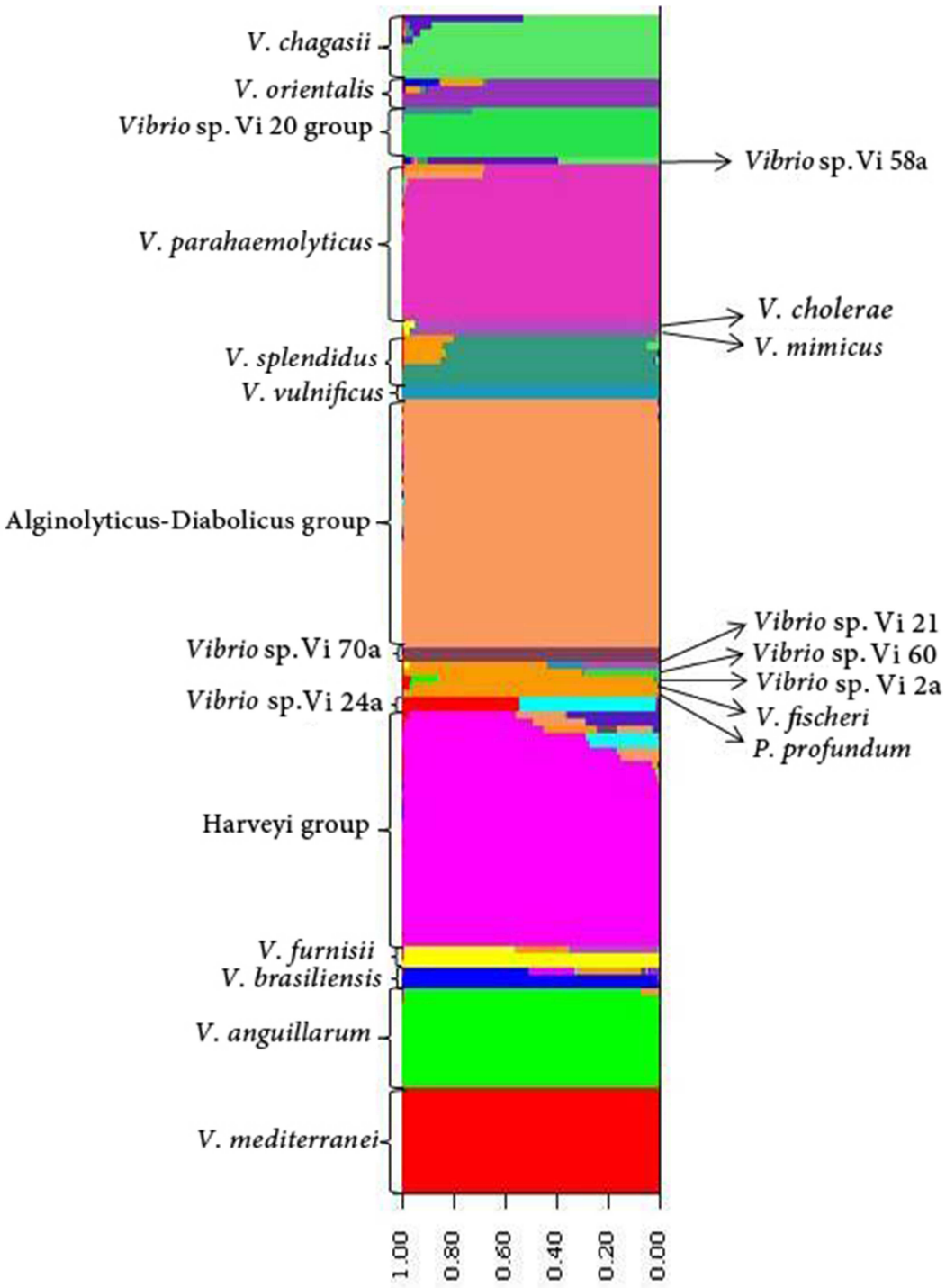

Figure 4: Populations clustering (strains of 2007) identified by STRUCTURE software on the concatenated sequences of four genes. Every single color corresponds to a single population, while columns with mixed colors include strains carrying DNA from different populations. The analyses showed seventeen ancestral groups. Groups with more than one isolates indicate in left side and single strains showed in right side.

3.2.4. Comparison between biochemical and MLSA identification

The agreement between the Alsina's and MLSA identifications was evaluated. MLSA identified 33 strains as *V. alginolyticus – V. diabolicus* group while 26 strains were found according to Alsina's scheme; in case of *V. parahaemolyticus*, 23 strains were identified with MLSA and 33 by Alsina's scheme. The McNemar test was used for testing the difference between paired proportions (Biochemical vs. MLSA). The results are reported in table 13. The p value highlighted no differences for some species such as *V. alginolyticus* and *V. splendidus*. But for the most part, especially for the highly represented species (*V. chagasii*, *V. fluvialis*, *V. harveyi*- group and *V. vulnificus*) the test highlighted discrepancy among MLSA and Alsina's scheme.

Table 13: Comparison between two approaches with McNemar Test (154 strains of 2007)

McNemar Tests for Each Category						
Category-k	Frequency		Proportion (Base Rate)		Chi-squared (a)	p
	Alsina	MLSA	Alsina	MLSA		
V. alginolyticus- *V. diabolicus* group	26[a]	33[b]	0.169	0.214	4.455	0.0348
V. anguillarum (like)	10	15	0.065	0.097	1.190	0.2752
V. brasiliensis (like)	0	2	0.000	0.013	exact test	0.5000
V. harveyi group	9[c]	30[d]	0.058	0.195	14.226	0.0002*
V. chagasii	0	12	0.000	0.078	12.000	0.0005*
V. fischeri	3	0	0.019	0.000	exact test	0.2500
V. fluvialis	17	0	0.110	0.000	17.000	0.0000*
V. furnissii	0	1	0.000	0.006	exact test	10.000
V. logei	4	0	0.026	0.000	exact test	0.1250
V. marinus	1	0	0.006	0.000	exact test	10.000
V. mediterranei (like)/ *V. shilonii*	14	14	0.091	0.091	exact test	10.000
V. mimicus	3	0	0.019	0.000	exact test	0.2500
V. nereis	4	0	0.026	0.000	exact test	0.1250
V. orientalis	0	3	0.000	0.019	exact test	0.2500
V. parahaemolyticus (like)	33	23	0.214	0.149	8.333	0.0039
V. pelagius	4	0	0.026	0.000	exact test	0.1250
V. splendidus (ll)	9	5	0.058	0.032	1.333	0.2482
V. vulnificus (B2)	14	1	0.091	0.006	13.000	0.0003*
Vibrio spp.	3	15	0.019	0.097	8.000	0.0047

(a) or exact test

* p < Bonferroni- adjusted significance criterion of 0.003.

Tests of Overall Marginal Homogeneity			
Bhapkar chi-squared	= 124.716	df = 18	P=0.0000
Stuart-Maxwell chi-squared	= 68.910	df = 18	P=0.0000

Bowker Symmetry Test			
Chi-squared =	78.867	df = 171	p = 1.0000

[a] all are *V. alginolyticus*, no *V. diabolicus*; [b] 14 *V. alginolyticus* and 19 *V. diabolicus*; [c] 3 *V. campbellii* and 6 *V. harveyi*; no *V. owensii*, *V. rotiferianus*; [d] includes all four species of Harveyi group (*V. campbellii*, *V. harveyi*, *V. owensii*, *V. rotiferianus*).

3.2.5. Preliminary analyses of *Vibrio* spp. by MLSA approach isolated from Crustacean samples in 2011

The *Vibrio* isolates from various Crustacean species (Table S2) were also verified using developed 4 genes MLSA scheme in collaboration with IZSVe, Adria (Italy). Fresh, frozen and unfrozen samples of crustacea were used for analyses. The *Vibrio* strains were isolated using biochemical methods and confirmed by MLSA scheme. The *V. parahaemolyticus* strains were also checked using species specific *tox*R and *tlh* genes; and *tdh*, *trh* for virulence properties. Among 107 *Vibrio* strains isolated from crustacean samples, seven strains amplified only with *atp*A gene and identified as *Shewanella* spp. by BLAST search. Another 8 strains didn't amplify with one or another gene. Finally, 92 strains were analyzed using MLSA approach, of which 52 (56.5%) strains had the same identification as biochemical method (Table 14 and S2). In case of *V. parahaemolyticus*, 11 false positive and 4 false negative strains were identified as compared to Biochemical approach. In total, 40 *V. parahaemolyticus* were identified by MLSA, whereas 47 as biochemical. Phylogenetic analysis with neighbor joining tree showed 10 clusters and 4 singletons (Fig. 5). Structure analysis identified 12 subpopulations with highest delta K value of 31.136 (Fig. 6). All 40 *V. parahaemolyticus* were positive with *tox*R and *tlh* genes but negative with *tdh* and *trh* genes.

Table 14: List of 92 strains with their designated species name in comparison between biochemical and MLSA identification (Crustacean samples of 2011)

Sl. no.	Species name	Biochemical	MLSA Phylogeny identification
1	*Vibrio alginolyticus*	17	16
2	*Vibrio anguillarum*	1	3
3	*Vibrio brasiliensis*	-	1
4	*Vibrio campbellii/harveyi/owensii*	2	5
5	*Vibrio chagasii*	-	-
6	*Vibrio cholerae*	-	-
7	*Vibrio diabolicus*	-	8
8	*Vibrio fischeri*	-	-
9	*Vibrio fluvialis*	-	-
10	*Vibrio furnissii*	-	-
12	*Vibrio mediterranei*	-	-
13	*Vibrio mimicus*	-	-
14	*Vibrio orientalis*	-	-
16	*Vibrio parahaemolyticus*	47	40
17	*Vibrio rotiferianus*	-	2
18	*Vibrio shilonii*	-	-
19	*Vibrio splendidus*	1	5
	Vibrio tubiashii	1	-
20	*Vibrio vulnificus*	-	-
	Listonella pelagius	1	-
21	*Photobacterium profundum* (Like)	1	3
22	*Vibrio* spp.	21	-
23	*Vibrio* sp. 16A	-	1
24	*Vibrio* sp. 26A	-	2
25	*Vibrio* sp. 49A	-	2
26	*Vibrio* sp. 52A	-	2
27	*Vibrio* sp. 81A	-	1
28	*Vibrio* sp. 94A	-	1

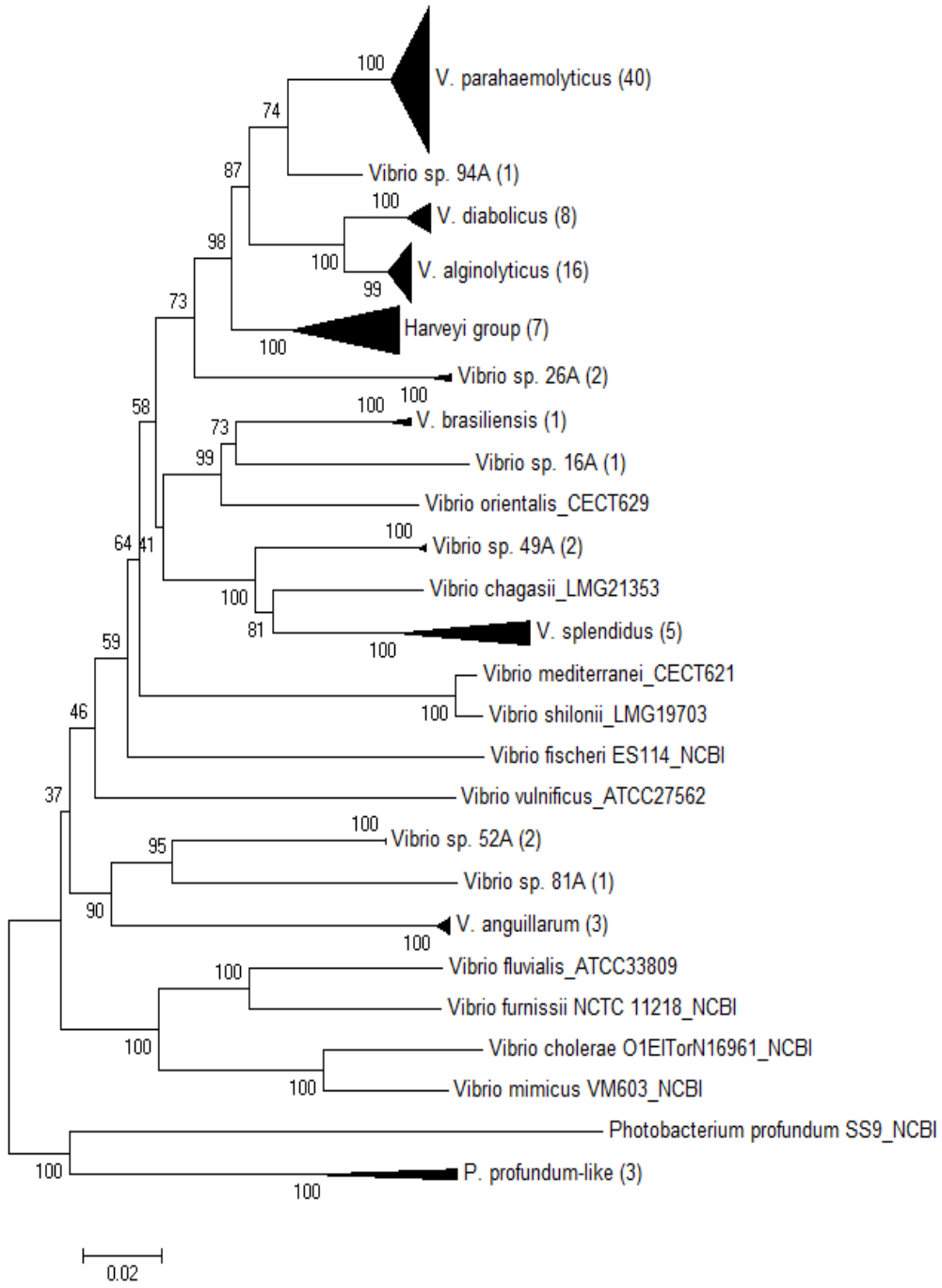

Figure 5: Neighbor-joining phylogenetic tree (compressed) with concatenated sequences of four housekeeping genes for the *Vibrio* strains isolated from Crustacean samples in 2011. The numbers in brackets describe the number of strains included in the reference species group which is represented by a black triangle.

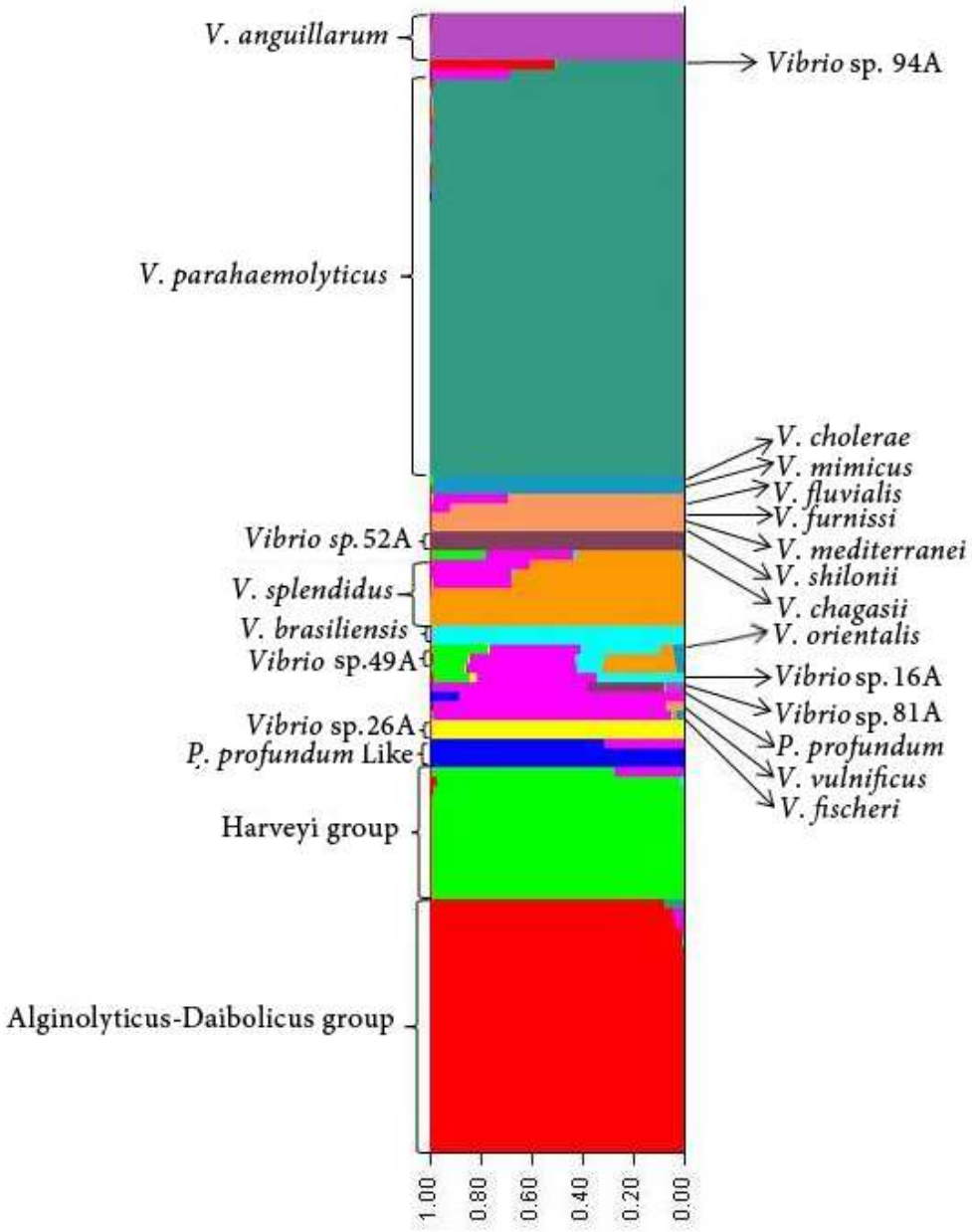

Figure 6: Populations clustering (strains isolated from Crustacean samples in 2011) identified by STRUCTURE software on the concatenated sequences of four genes. Every single color corresponds to a single population, while columns with mixed colors include strains carrying DNA from different populations. The analyses showed twelve ancestral groups. Groups with more than one isolates indicate in left side and single strains showed in 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 represents the reference strains, no isolates in our study.)

3.3. MLST characterization of *V. parahaemolyticus* strains (Mollusks samples of 2011)

3.3.1. Isolation and identification of *V. parahaemolyticus* strains by biochemical methods (Alsina's scheme), species specific genes PCR and MLSA approach

133 mollusks samples in 2011 were analyzed and 44 samples were found positive (33.08%) for *Vibrio* spp. from which 160 strains were isolated using biochemical methods (Alsina's scheme). The strains were evaluated for the presence of *V. parahaemolyticus* specific *tox*R and *tlh* genes. 102 strains identified as *V. parahaemolyticus* were also searched for *tdh* and *trh* virulence genes (Table 16). No strains were found positive for *tdh* and only 6 strains were found positive for *trh* gene. 26 doubtful isolates were then analyzed with 4 genes MLSA approach developed in this study (Table 15 and Fig. 7). At the end of multiple analyses, 102 strains (Table 17) were then characterized using MLST.

Table 15: MLSA identification of doubtful strains of *Vibrio* isolated from Mollusks in 2011

Strains number	Biochemical Identification	MLSA Identification
1	*V. parahaemolyticus*	*V. diabolicus*
2	*V. alginolyticus*	*V. diabolicus*
3	*V. parahaemolyticus*	*V. parahaemolyticus*
4	*V. parahaemolyticus*	*V. parahaemolyticus*
5	*V. cholerae*	*V. cholerae*
6	*V. cholerae*	*V. cholerae*
7	*Vibrio spp.*	*V. fluvialis*
19	*V. parahaemolyticus*	Harveyi group as *gyr*B, didn't amplify with *rec*A
20	*V. parahaemolyticus*	*P. profundum* - like
24	*V. vulnificus*	*V. anguillarum* - like
30	*V. vulnificus*	*V. diabolicus*
43	*V. vulnificus*	*V. parahaemolyticus*
44	*V. vulnificus*	*V. parahaemolyticus*
45	*V. vulnificus*	*V. parahaemolyticus*
68	*V. parahaemolyticus*	*V. parahaemolyticus*
69	*V. parahaemolyticus*	*V. parahaemolyticus*
71	*V. vulnificus*	*V. alginolyticus* as *gyr*B, didn't amplify with *rec*A
95	*V. parahaemolyticus*	*Vibrio* spp. 95L
96	*V. vulnificus*	*V. parahaemolyticus*
105	*V. vulnificus*	*V. parahaemolyticus*
115	*V. vulnificus*	*Harveyi group*
116	*V. vulnificus*	*Harveyi group*
117	*V. vulnificus*	Harveyi group as *gyr*B, didn't amplify with *rec*A
133	*V. vulnificus*	Harveyi group
135	Blue colony	*Enterobacter cloacae* as *atp*A, didn't amplify with other 3 genes
151	*V. vulnificus*	*V. diabolicus*

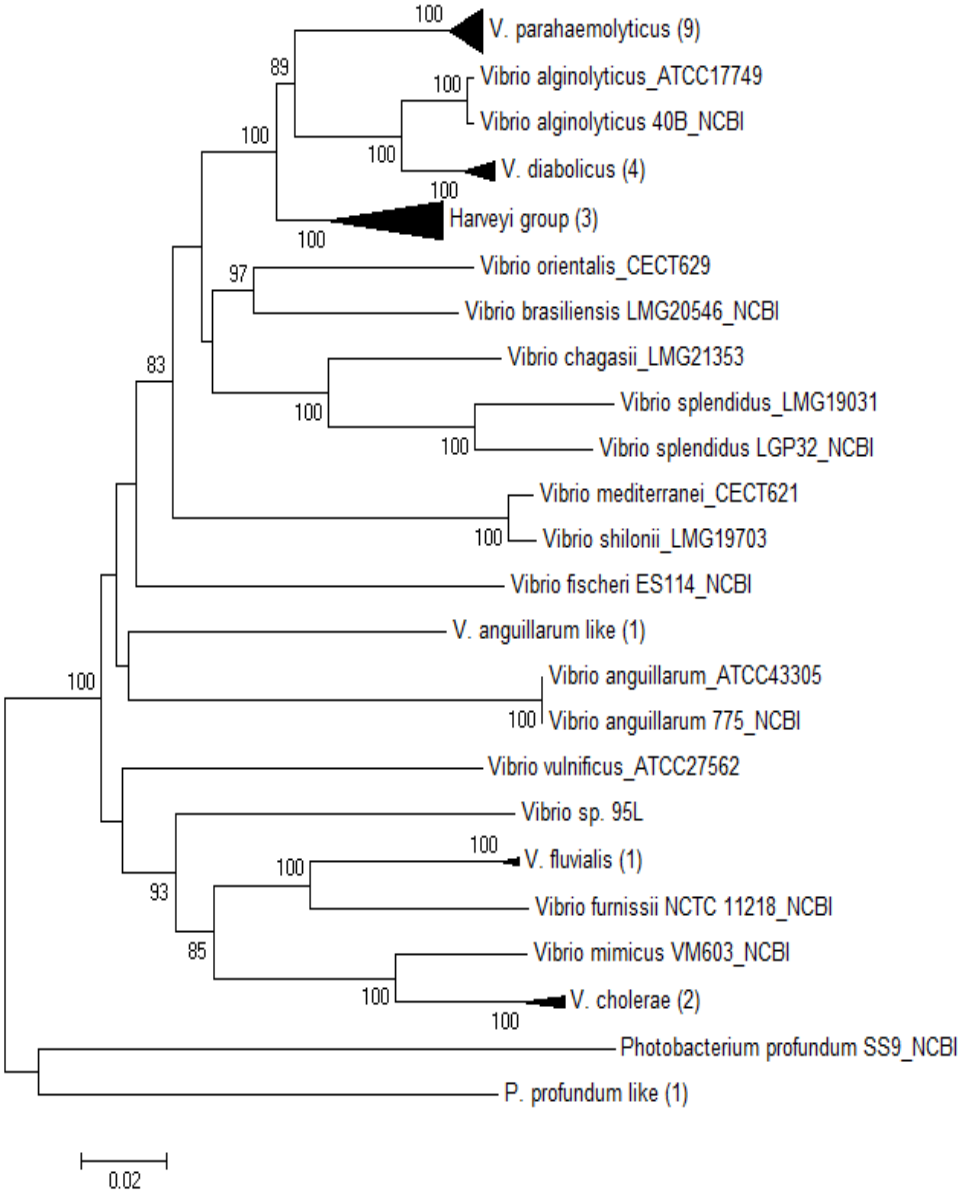

Figure 7: Neighbor-joining phylogenetic tree (compressed) with concatenated sequences of four housekeeping genes for the doubtful *Vibrio* strains isolated from Mollusks samples in 2011. The numbers in brackets describe the number of strains included in the reference species group which is represented by a black triangle.

Table 16: Analysis on the presence (+) or absence (-) of the genes *toxR, tlh, tdh, trh* in *V. parahaemolyticus* strains (Mollusks samples 2011)

Id	*tox*R	*tlh*	*tdh*	*trh*
1	-	-		
2	-	-		
3	+	+	-	-
5	-	-		
6	-	-		
7	-	-		
8	+	+	-	-
9	+	+	-	-
11	+	+	-	-
14	+	+	-	-
17	+	+	-	-
18	+	+	-	-
19	-	-		
20	-	-		
21	+	+	-	-
24	-	-		
25	+	+	-	-
30	-	-		
31	+	+	-	+
34	+	+	-	-
36	+	+	-	-
37	+	+	-	-
39	+	+	-	-
40	+	+	-	+
41	+	+	-	+
42	+	+	-	-
43	+	+	-	-
44	+	+	-	-
45	+	+	-	-
46	+	+	-	-
47	-	-		
48	+	+	-	-
49	-	-		
50	+	+	-	-
51	+	+	-	-
52	+	+	-	-
53	+	+	-	-
54	+	+	-	-
55	+	+	-	-
56	+	+	-	-
57	+	+	-	-
58	+	+	-	-
62	+	+	-	-
63	+	+	-	-
64	+	+	-	-
65	+	+	-	-
66	+	+	-	-
67	+	+	-	-
68	+	+	-	-
69	+	+	-	-
70	+	+	-	-
71	-	-		
72	+	+	-	-
73	+	+	-	-
74	+	+	-	-
75	+	+	-	-
76	-	+	-	-
77	+	+	-	-
78	+	+	-	-
79	-	+	-	-
80	+	+	-	-
81	+	+	-	-
85	+	+	-	-
86	+	+	-	-
87	-	+	-	-
88	+	+	-	-
89	+	+	-	-
90	+	+	-	-
92	+	+	-	-
93	+	+	-	-
94	-	+	-	-
95	-	-		
96	+	+	-	-
97	+	+	-	-
99	+	+	-	-
100	+	+	-	-
101	+	+	-	-
102	+	+	-	-
103	+	+	-	-
104	+	+	-	-
105	+	+	-	-
106	+	+	-	-
107	+	+	-	-
108	+	+	-	-
109	+	+	-	-
110	+	+	-	-
111	+	+	-	-
112	+	+	-	-
113	+	+	-	-
115	-	-		
116	-	-		
117	-	-		
118	+	+	-	-
120	+	+	-	-
121	+	+	-	+
122	+	+	-	-
124	+	+	-	+
128	+	+	-	-
129	+	+	-	-
132	+	+	-	-
133	-	-		
134	+	+	-	-
135	-	-		
136	+	+	-	-
137	+	+	-	-
140	+	+	-	-
142	+	+	-	-
143	+	+	-	-
145	+	+	-	-
146	+	+	-	-
147	+	+	-	-
148	+	+	-	-
149	+	+	-	-
150	+	+	-	+
151	-	-		
152	+	+	-	-
153	+	+	-	-
154	+	+	-	-
155	+	+	-	-
156	+	+	-	-
158	+	+	-	-
159	+	+	-	-
160	+	+	-	-

3.3.2. MLST scheme and genetic diversity

Using the BLAST program, it has been possible to query the pubMLST database and identify alleles for each locus analyzed. Table 17 shows the allelic profiles highlighted by the analysis of isolates and Sequence Type (ST) resulting from the combination of alleles of single loci.

Table 17: Allelic profiles of the 102 strains analyzed and the resulting Sequence Type (STs). * Indicates alleles or ST that is not in the database during analyses (October 2012).

Strains number	*dna*E	*gyr*B	*rec*A	*dtd*S	*pnt*A	*pyr*C	*tna*A	ST
3	134	104	131	223*	91	69	2	**535***
8	31	115	22	12	3	91	68	**141**
9	197*	268*	146	224*	45	11	144*	**536**
11	197*	268*	146	224*	45	11	144*	**536***
14	197*	268*	146	224*	45	11	144*	**536***
17	29	269*	75	225*	54	18	24	**537***
18	47	270*	168	13	2	204*	23	**538***
21	197*	268*	146	224*	45	11	94	**539***
25	60	197	31	18	106	150	26	**540***
31	42	248	19	76	129	46	69	**470**
34	12	180	195*	19	21	11	17	**541***
36	26	271*	196*	225*	28	49	23	**542***
39	5	272*	68	19	136*	205*	87	**543***
40	114	46	39	47	26	47	34	**544***
41	33	272*	197*	226*	18	206*	145*	**545***
42	28	273*	-	227*	28	207*	2	**-**
43	3	159	-	19	100	11	110	**-**
44	3	159	-	19	100	11	110	**-**
46	198*	274*	198*	120	23	208*	26	**546***
48	42	275*	199*	75	137*	46	24	**547***
50	2	113	72	94	26	83	23	**411**
51	51	4	77	67	60	8	24	**423**
52	199*	276*	199*	228*	61	14	23	**548***
53	137	277*	70	229*	4	209*	14	**549***
54	93	118	6	19	26	93	81	**550***
55	95	119	93	230*	74	89	70	**551***
56	17	278*	200*	57	94	210*	7	**552***
57	4	13	11	91	18	9	23	**481**
58	80	88	111	231*	37	95	61	**553***
62	3	2	82	50	4	78	66	**121**
63	119	101	31	232*	46	11	38	**554***
64	28	164	201*	233*	23	82	57	**555***
65	11	279*	102	234*	41	211*	26	**556***
66	0	280*	202*	167	21	11	146*	**-**

67	42	281*	199*	75	137*	46	24	**557***
68	9	213	165	185	2	46	1	**396**
69	132	136	203*	19	46	212*	147*	**558***
70	200*	84	204*	76	138*	213*	26	**559***
72	4	13	11	91	18	9	23	**481**
73	93	13	205*	235*	117	214*	80	**560***
74	201*	139	24	5	139*	27	94	**561***
75	139	116	0	19	60	134	24	**-**
76	4	13	11	74	60	9	23	**562***
77	51	4	77	67	60	8	24	**423**
78	202*	282*	31	19	140*	45	148*	**563***
79	42	13	11	91	18	9	23	**564***
80	83	49	-	70	141*	215*	57	**-**
81	4	13	11	38	18	9	23	**6**
85	17	16	-	98	24	16	149*	**-**
86	95	119	-	230*	74	89	70	**-**
87	4	13	11	91	18	9	23	**481**
88	28	144	116	138	142*	177	61	**565***
89	188	261	206*	236*	69	3	1	**566***
90	49	13	31	237*	50	11	150*	**567***
92	31	283*	75	55	4	216*	23	**568***
93	104	284*	207*	238*	69	217*	26	**569***
94	4	13	11	38	18	9	23	**6**
96	41	285*	70	89	23	218*	147*	**570***
97	4	13	11	91	18	9	23	**481**
99	3	286*	-	239*	143*	219*	148*	**-**
100	184	104	-	29	144*	96	26	**-**
101	14	30	-	78	27	7	13	**-**
102	153	13	134	240*	83	3	9	**571***
103	17	64	137	60	94	11	51	**572***
104	80	88	111	231*	37	95	61	**553***
105	203*	16	208*	241*	26	220*	51	**573***
106	49	13	31	237*	50	11	150*	**567***
107	204*	104	209*	33	26	221*	17	**574***
108	205*	106	39	106	50	54	33	**575***
109	206*	25	210*	151	31	222*	73	**576***
110	137	57	22	242*	45	171	24	**577***
111	2	287*	211*	19	129	82	42	**578***
112	137	288*	212*	123	128	138	2	**579***
113	207*	289*	213*	243*	145*	223*	151*	**580***
118	152	57	17	14	99	54	14	**581***
120	80	106	-	244*	23	101	145*	**-**
121	158	290*	-	153	139*	46	26	**-**
122	4	13	11	91	18	9	23	**481**
124	42	248	19	76	129	46	69	**470**
128	28	291*	61	245*	114	50	23	**582***
129	86	292*	17	246*	12	54	86	**583***

132	131	-	-	-	61	224*	152*	-
134	25	293*	-	185	117	3	87	-
136	4	13	11	91	18	9	23	**481**
137	44	13	214*	50	26	225*	23	**584***
140	4	13	11	247*	18	9	23	**585***
142	207*	294*	215*	248*	146*	226*	131	**586***
143	95	34	216*	230*	74	89	70	**587***
145	3	25	187	75	26	166	57	**588***
146	208*	292*	217*	249*	147*	227*	24	**589***
147	209*	111	167	188	116	228*	61	**590***
148	2	198	72	94	26	11	94	**591***
149	31	295*	-	250	4	45	153*	-
150	3	111	167	188	116	18	33	**592***
152	31	283*	75	55	4	216*	23	**568***
153	165	22	70	177	39	11	115	**398**
154	34	4	218*	251*	4	229*	33	**593***
155	210*	88	81	252*	4	230*	51	**594***
156	20	245	31	253*	61	157	23	**595***
158	42	281*	199*	75	137*	46	24	**557***
159	31	104	219*	254*	61	37	1	**596***
160	144	92	69	114	54	71	154*	**597***

The gene sequences for each strain were aligned using the ClustalW software available online to verify the correspondence of the amplified regions, as well as the absence of gaps in the alignment. As regards the concatenated sequences, 86 STs (72 non-redundant) were obtained of which 63 STs appear to be different (new) from those available in the online pubMLST database, or because they present a new allele or because the allelic profile manifested in the complex is not present in the database.
Table 18 shows the general information of the loci analyzed

Table 18: Information per locus; new allele is a sequence not present in the database.

Locus	**Number of strains analyzed**	**Number of strains not amplified**	**Number of new Alleles**
*dna*E	141	1	14
*gyr*B	102	1	14
*rec*A	102	15	25
*dtd*S	141	1	32
*pnt*A	102	0	12
*pyr*C	102	0	27
*tna*A	102	0	11

Table 19: Information obtained by the software DnaSP, such as GC content, the number of polymorphic sites, the number of parsimony informative sites, the number of synonymous and non-synonymous mutations, Tajima's D test, the values of Θ and π for each locus.

Locus	Dimension (bp)	No. of Allele	G+C content	No. of polymorphic sites	No. of parsimony informative sites	Synonymous changes	Non-Synonymous changes	Tajima's *D* test	Θ	π
*dna*E	555	58	0.487	49	31	46	5	-1.08376	0.016987	0.011173
*gyr*B	591	64	0.476	52	32	54	1	-0.70250	0.016929	0.013189
*rec*A	726	54	0.450	70	49	66	5	0.48362	0.019097	0.021909
*dtd*S	456	67	0.501	70	42	69	4	-0.48491	0.029481	0.025018
*pnt*A	429	46	0.438	40	22	34	8	-1.41817	0.017906	0.009753
*pyr*C	489	61	0.481	49	30	40	8	-1.26560	0.019244	0.011544
*tna*A	423	41	0.488	37	24	37	5	-1.24350	0.016798	0.010047

Table 20: Comparison of information obtained by the software DnaSP, such as GC content, the number of polymorphic sites, the number of parsimony informative sites, the number of synonymous and non-synonymous mutations, the Tajima's D value, the values of Θ and π for concatenated database and our dataset.

Concatenated	Dimension (bp)	No. of Allele	G+C content	No. of polymorphic sites	No. of parsimony informative sites	Synonymous changes	Non-Synonymous changes	Tajima's D test	Θ	π
Dataset	3669	72	0.473	346	209	325	32	-0.72902	0.019456	0.015393
Database	3669	597	0.473	884	643	799	146	-1.60326	0.034576	0.016148

Using the software DnaSP some parameters were highlighted relating to each of the genetic loci and to concatenated sequences under investigation, such as the percentage of G-C, the number of polymorphic sites, the relative abundance of synonymous and non-synonymous mutations, values of Tajima's D test, Θ and π. The results are summarized in Table 19 and 20 above.

The G+C content oscillates between the values of 0.438 (*pnt*A) and 0.501 (*dtd*S) with an average value of 0.474. The lowest number of polymorphic sites was found in tnaA (37), while the highest in the recA and dtdS (70); as regards to parsimony informative sites, they oscillate between the 22 of pntA and 49 of recA. The values of Tajima's D test assume between -1.41817 (*pnt*A) and 0.48362 (*rec*A). The nucleotide difference (π) calculated for the different loci were comprised within values of 0.009753 (*pnt*A) and 0.025018 (*dtd*S), while Θ calculated per π oscillates between 0.16798 (*tna*A) and 0.29481 (*dtd*S).

3.3.3. Phylogeny based on MLST data

Phylogenetic tree was obtained from alignment of the 72 concatenated sequences through the software MEGA shown in Figure 8. The bootstrap values highlighted phylogenetic analyses were generally low.

A global tree was constructed with 597 STs where the layout of the strains analyzed in this study had shown within the complex profiles of the database (Fig. 9). Red are highlighted the strains isolated from edible mollusks in this study. It should be noted that two main branches were formed and 8 ST (all obtained from the database) were detached markedly from the basic structure.

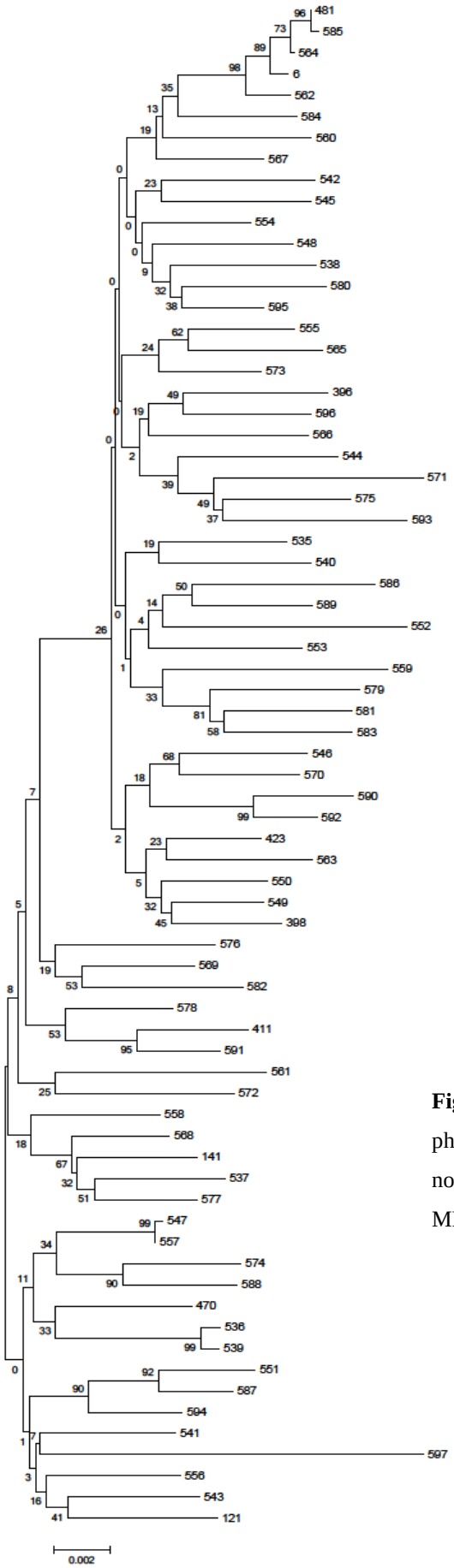

Figure 8: Topology of the phylogenetic reconstruction of the 72 non-redundant ST obtained using the MEGA software.

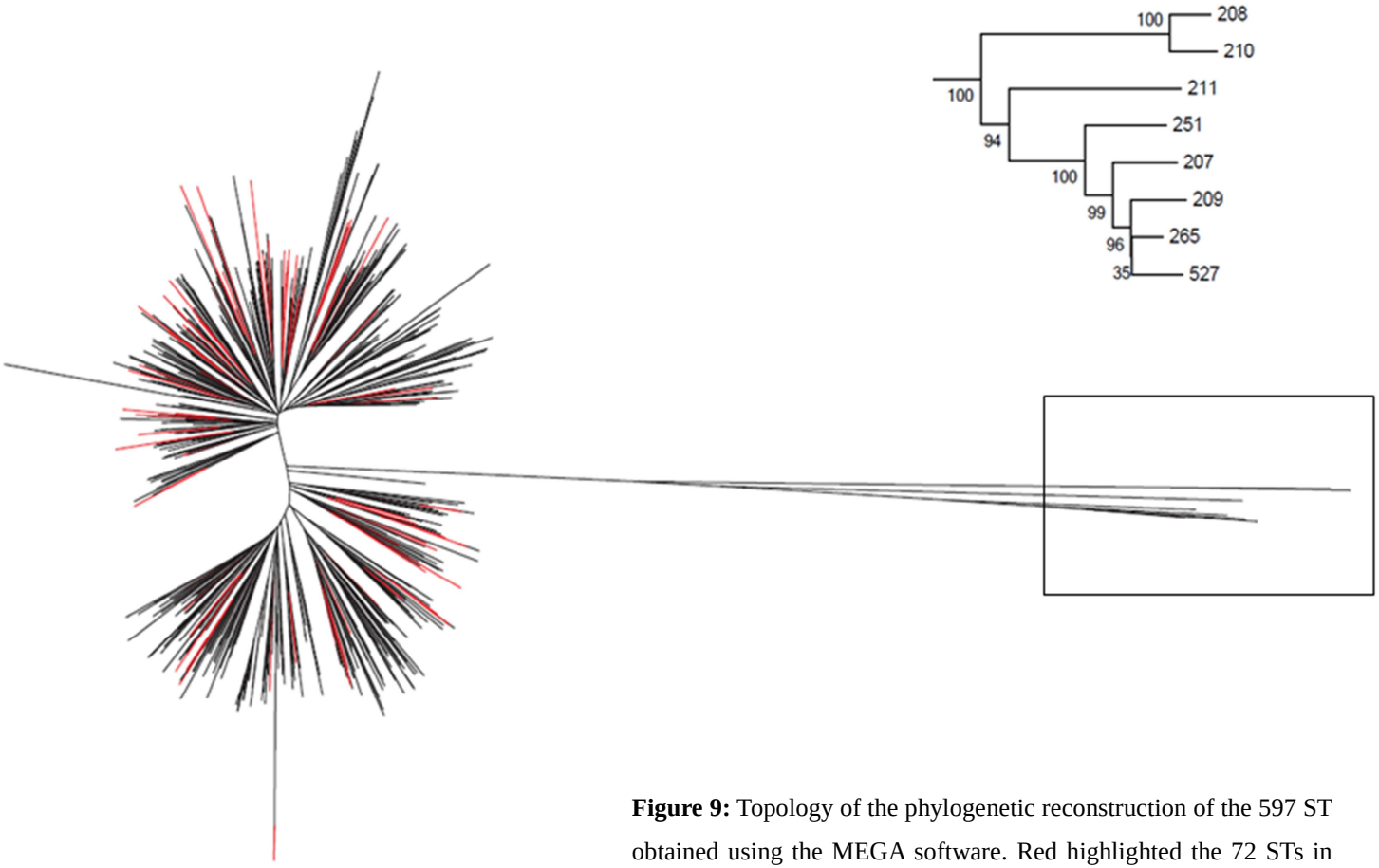

Figure 9: Topology of the phylogenetic reconstruction of the 597 ST obtained using the MEGA software. Red highlighted the 72 STs in this study. The special portrays highlighted the 8 STs away from the main scheme.

3.3.4. Evidence of recombination and strains relationships

3.3.4.1. Identification of Clonal complexes

The eBURST software and goeBURST, implemented in Phyloviz made it possible to identify the clonal complexes among the strains isolated from mollusks using STs of the dataset; and the source of isolation displayed using different colors (Fig. 13). A total of 54 clonal groups highlighted of which 17 are clonal complex.

With regard to the strains analyzed in this study, according to the SLV (Single Locus Variant) analysis, it is possible to observe how there is a clonal complex represented by ST 481, 564, 585 and 6. This clonal complex was defined as the ST 564, 585 and 6 differ from 481 by only one allele. The ST 481 is therefore identified as the founder genotype (or "ancestor").

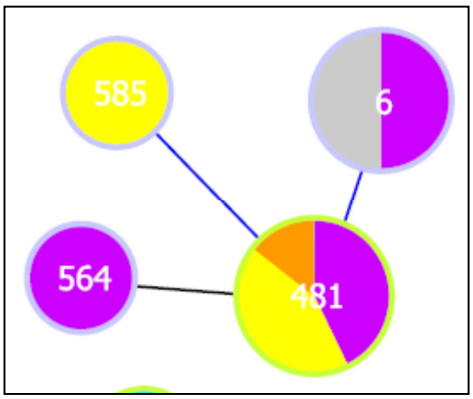

Figure 10: ST 481 as founder genotype (or "ancestor"), differ from ST 564, 585 and 6 by only one allele. Detail in Figure 13.

It was also shown that seven clonal groups share the same allelic profile in 6 of the 7 loci, identified by gray lines, formed by ST: 540-371, 557-547, 323-550, 544-78, 551-143 , 592-417, 536-539, 411-122. Some of these groups relate only strains from the North Adriatic (ST 557-547, 536-539); while the others are already in the database.

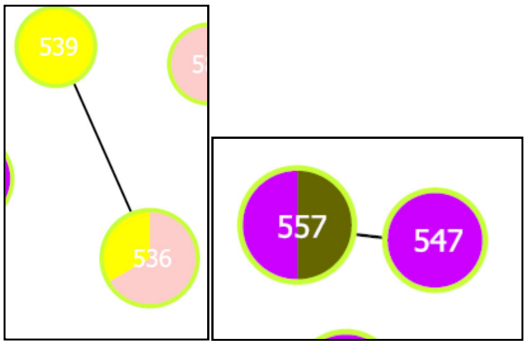

Figure 11: Clonal groups of STs isolated only from the North Adriatic in our study (ST 557-547, 536-539). Detail in Figure 13.

The majorities of ST has found no clonal relationship with other strains and are visible as isolated points (singletons).

None of the strains analyzed was part of the large clonal complex isolated from clinical cases whose ancestor is ST3. It should also be noted that the ST 592 isolated from Manila clam (*Ruditapes philippinarum*) and trh + was associated with ST 417 that came from the case of gastroenteritis in USA in 2007 (Fig. 12).

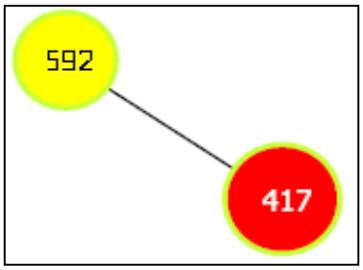

Figure 12: Clonal group of ST 592 isolated from clam in this study and the ST 417 (in database) isolated from a clinical case. Detail in Figure 13.

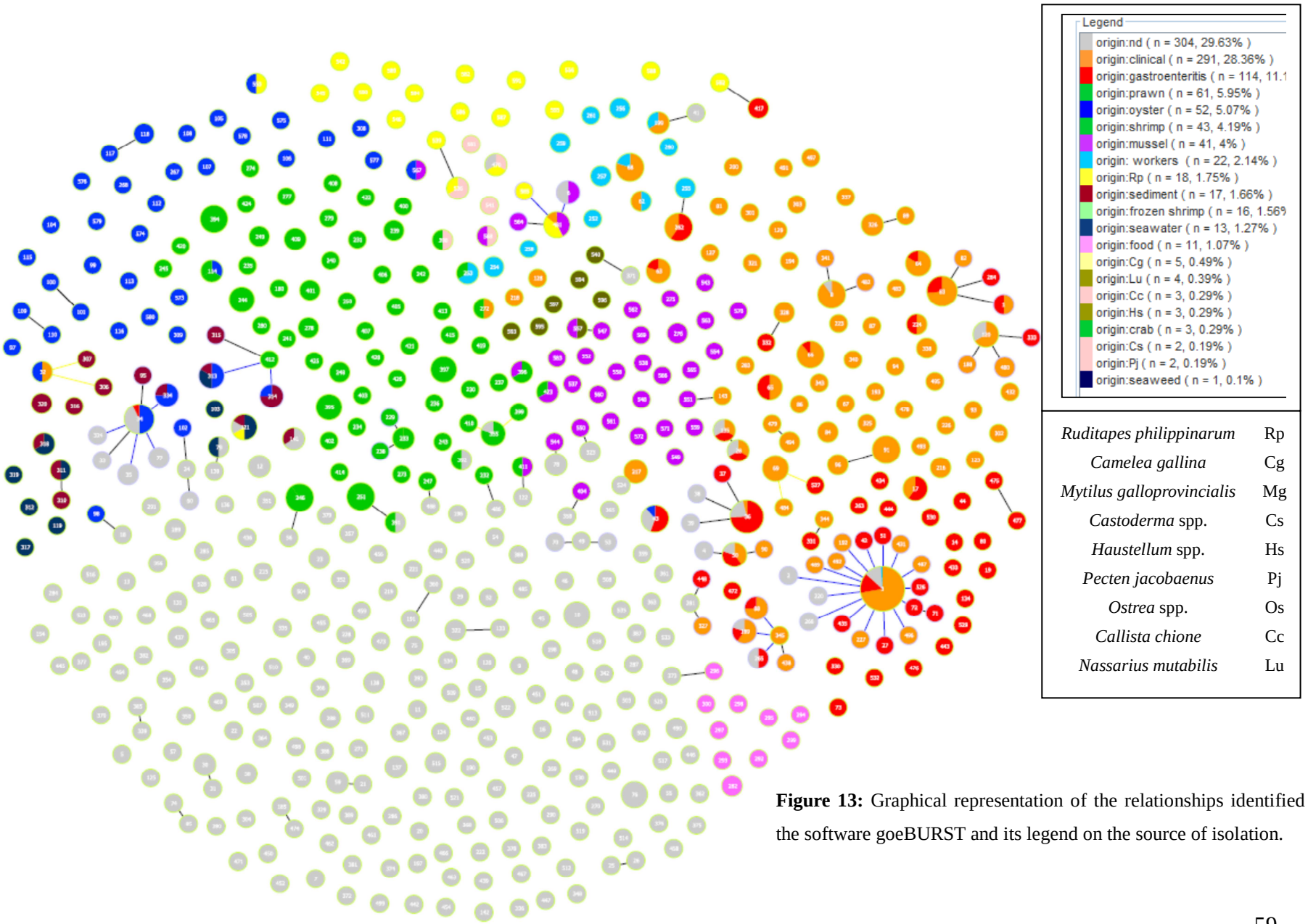

Figure 13: Graphical representation of the relationships identified by the software goeBURST and its legend on the source of isolation.

Figure 14 highlighted the strains belonging to the pandemic serotype O3: K6 present in the database, ST 6 and 121 isolated from mussels and clams were attributable to the pandemic serotype O3: K6.

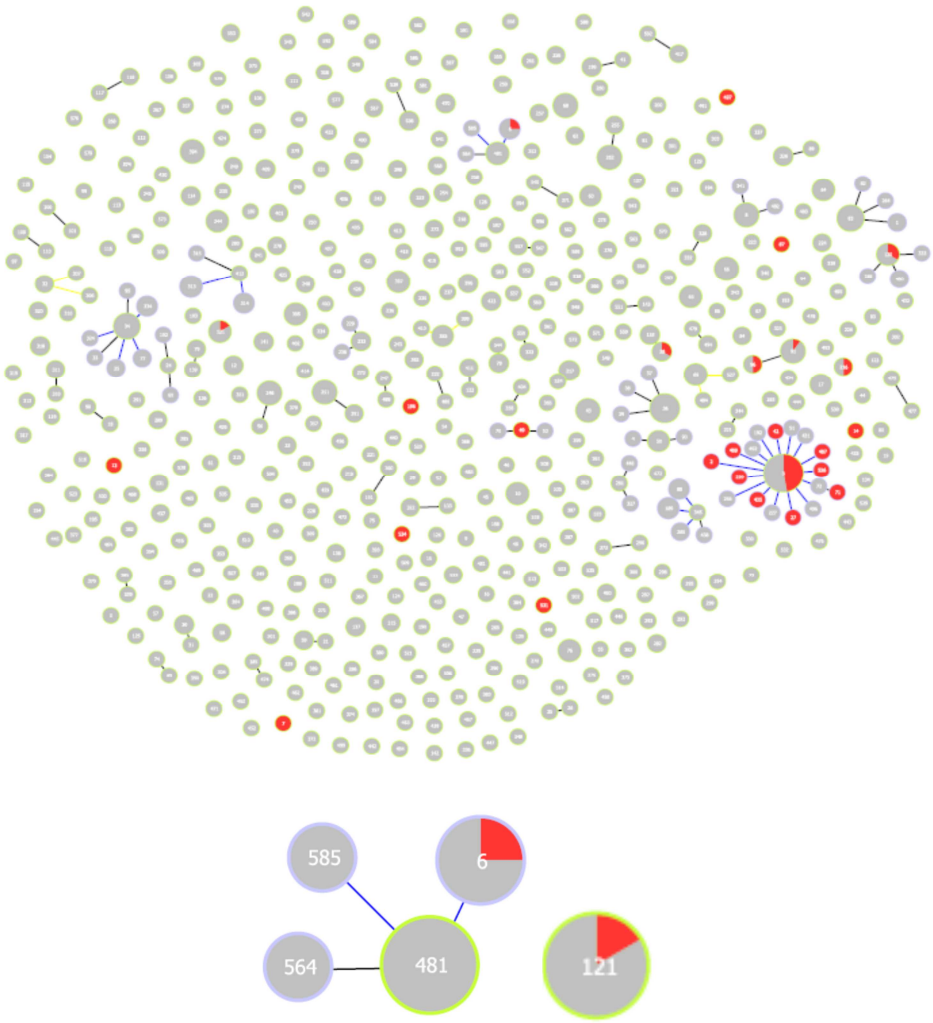

Figure 14: Graphical representation of the relationships identified by the software goeBURST. Red strains belonging to the pandemic serotype O3: K6. The strains of this study (ST6 and ST121) related to O3: K6 were highlighted separately.

The correlation with environmental parameters has not provided interpretable results with the analysis via Phyloviz since most of the strains isolated consisted of singletons.

3.3.4.2. Analysis with Structure

The concatenated 72 non-redundant STs suitably aligned in eXtended Multi-Fasta (XMFA) format using MAUVE software (Darling *et al*., 2006) and then converted into the input file str using the software xmfa2struct has been used to obtain information

regarding the population structure of our dataset using Structure software.

The processing of the results was carried out through the online software Structure Harvester with which it appears that the strains formed two populations (for K = 2, DeltaK = 330.66).

Figure 15 have shown two distinct populations and strains that belong to both. The population marked by the red part, in order, the ST 551, 537, 539, 577; in green, ST 481, 564, 585, 562, 6, 567, 575, 544 and 595. The other 59 STs are strains that have in varying percentages of components from one population or the other.

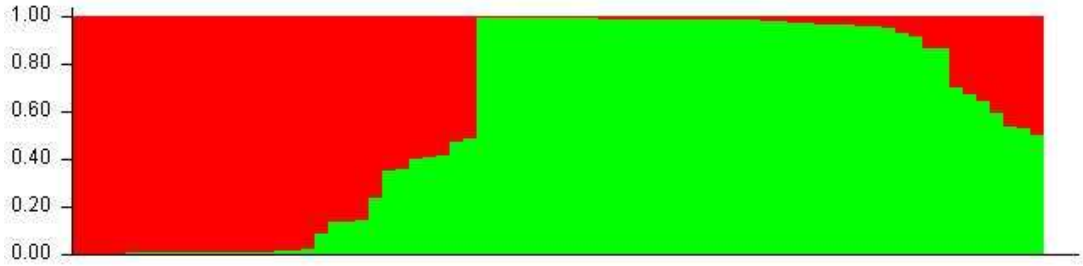

Figure 15: Graphical representation of the population identified by the software Structure. The mixed color shows the percentage of membership of ST to one or the other population.

3.3.4.3. Analysis of Recombination

The recombination rate (r / m, r = recombination; m = mutation) processed by the software ClonalFrame for the 72 STs provides a value of 0.38.

Analyses of concatenated sequences with the RDP software detected 12 probable events of recombination, as confirmed by the analysis with ClonalFrame, which mainly concern with *rec*A and *pnt*A genes.

With the software SplitsTree, the recombinations of the strains under study were visualized (Fig. 16). It has been shown that 72 STs of our dataset have a significant recombination (P = 0.001), although the various branches are well-defined. Strains that are member of a clonal groups and clonal complex were highlighted with a red circle.

It should also be noted that the arrangement of the strains reflects the division into two populations evident in Structure and MEGA tree.

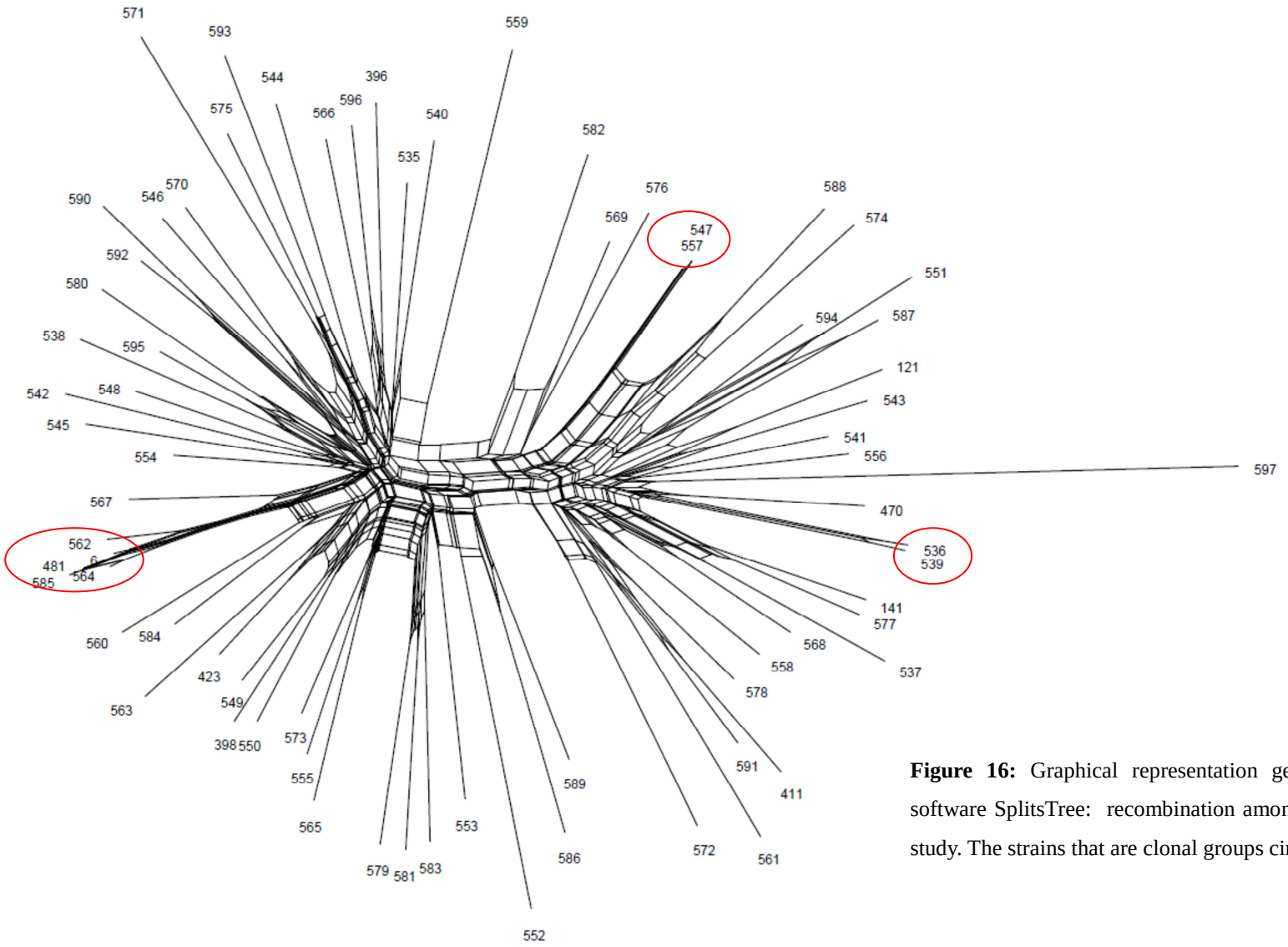

Figure 16: Graphical representation generated by the software SplitsTree: recombination among 72 STs of the study. The strains that are clonal groups circled in red.

4. DISCUSSION

4.1. MLSA approach and it's comparison with Alsina's scheme

With the development of sequencing methods, analysis of concatenated sequences of several housekeeping genes defined as Multilocus Sequence analysis (MLSA) became a very common and practical method for genotypic characterization and on the way to be a new standard in microbial molecular systematic for species delineation (Kämpefer and Glaeser, 2012). The use of MLSA is proven to be very useful to describe new species (Chimetto *et al*., 2011, Yoshizawa *et al*., 2011), to relocate the taxonomic positions of strains (Urbanczyk *et al*., 2007, Thompson *et al*., 2011) or differentiate very closely related species (Thompson *et al*., 2007b, 2008) of *Vibrio*.

Most of the publications based on MLSA use only the reference strains of *Vibrio* spp., but including a representative number of environmental isolates could enhance the applicability and reliability of the analysis, giving a better discrimination among strains and an overview on the real ecology and distribution of *Vibrio* spp. In the present analysis, 154 *Vibrio* strains isolated from mollusks of the Venice lagoon and Sea were used.

The developed MLSA demonstrated to be a very fast and accurate analysis to discriminate *Vibrio* spp.. The distribution and clustering of the analyzed species achieved a high supported degree of discrimination that confirmed the results of previous analyses conducted on *Vibrio* spp. (Thompson *et al*., 2007b). These cases, however, implied the use of a higher number of genes, demonstrating that the four genes used in this study are sufficient to give the similar results and represent of course a faster way to analyze *Vibrio* species. Moreover, many MLSA studies mostly included reference strains, while this work analyzed several environmental strains, giving a wider characterization and an overview on the presence of *Vibrio* species in mollusks from Venice lagoon and North Adriatic Sea.

The MLSA allowed to easily discriminating the Higher risk vibrios from the Lower risk species. Most of the strains isolated from mollusks were identified as Lower risk species: *V. alginolyticus*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. mimicus* were the most frequently isolated species. Only 25 out of 154 isolates (16.23%) analyzed by MLSA, were clustered in the Higher risk organisms group which includes three taxa (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) repeatedly involved in disease outbreaks or having the potential to do so (Austin, 2010). In this sampling, no *V. cholerae* has been identified neither by biochemical methods nor with MLSA approach.

Regarding *V. parahaemolyticus* and *V. vulnificus*, we achieved a precise clustering of both of them: in particular, it is clear a high presence of *V. parahaemolyticus* among the mollusks sampled in the Venice lagoon. The MLSA identification of *V. vulnificus* and *V. parahaemolyticus* demonstrated to be more precise and reliable than the Alsina's scheme results that overestimated the two species (Table 13). Although 16.23% represent a little part of total *Vibrio* species isolated in this study, it still represent a worrying data about the safety of mollusks in Venice lagoon, thus highlighting the need of precise and severe quality/safety controls on these products.

The MLSA also pointed out the controversial relation among V. *alginolyticus* and *V. diabolicus* species that, together, comprised the 21.4% of the isolates. The concatenated gene sequence tree revealed two subclusters within the two groups, also supported by SplitsTree analysis, while STRUCTURE showed a unique group. This result is also confirmed by the phylogenetic analysis resulted from *rec*A and *atp*A genes, in which the two groups seem more distantly related (Fig S1). This result suggest that the two species could have been affected by different mechanisms of genetic modifications which could be explained by an original and overall genetic similarity between the two species (supported by the phylogenetic data of the concatenated sequences), but in some cases they could have been subjected to recombination events with other species that could explain their distance when some single genes were studied. In one study, Oberbeckmann *et al*., (2011) reported two distinct groups of *V. alginolyticus* (Group I and II) during *rpo*B gene sequence analysis; they found that group I didn't contain any reference strain but due to close phylogenetic similarity to group II, they assumed that group I belonged to the species *V. alginolyticus.* It should be noted that they didn't include *V. diabolicus* sequence in that study.

Regarding the *V. harveyi* group, it is known that it comprises four species (*V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*). However, the resolution given by the MLSA with 4 genes is not good enough to distinguish the four species, especially *V. harveyi* and *V. campbellii*. One recent study used 5 genes MLSA (*rpo*A-*pyr*H-*top*A-*fts*Z-*mre*B) and they revealed well supported clusters to identify these four species (Cano-Gomez *et al*., 2011). Hoffmann *et al*., (2011) also described six-genes MLSA to correctly identify *Vibrio* strains of *harveyi* clade. However, the precise differentiation of the species belonging to *V. harveyi* group was not the aim of the present study that was instead directed to a general species definition and, above all, to the discrimination between "higher risk" and the "lower risk" species.

The MLSA results proved again the higher reliability of biomolecular methods than traditional microbiological techniques to identify *Vibrio* species. In a study conducted on *V. parahaemolyticus*, Croci and colleagues (Croci *et al*., 2007) concluded that among the biochemical methods, the Alsina's scheme gave the most reliable results but the biochemical identifications should be confirmed by molecular methods to avoid false-positive results. In our analyses, we also demonstrated the need of a biomolecular method to confirm microbiological identification techniques to avoid false-positive and false-negative species attributions of Alsina's scheme.

We verified our developed MLSA scheme with *Vibrio* isolates from Crustacean samples to identify *Vibrio* species. The aim was also to understand which probable enteropathogenic *Vibrio* species are present in the crustacean products sold in Veneto region, choosing the fish market in Venice as origin of the samples. Although there are several reports that *Vibrio* spp. can cause disease via crustaceans, there are currently no data on their distribution in the final product or detailed information on their potential pathogenicity to the consumer. So we wanted to investigate the presence of human pathogenic *Vibrio* in shellfish, which consumed raw or undercooked may cause food poisoning.

The only "Higher risk" *Vibrio* species identified in crustacean samples was *V. parahaemolyticus* (43.48% of total *Vibrio* strains), while there were no *V. cholerae* or *V. vulnificus*. The most represented "Lower risk" vibrios were *V. alginolyticus* (17.39%) followed by *V. diabolicus* (8.69%) and *V. splendidus* (5.43%).

In one study by Traoré *et al*., (2012) to assess the risk of *Vibrio* spp. transmission from crustaceans to humans, they identify 40% of the isolates were *V. alginolyticus*, 36% were *V. parahaemolyticus*, and 24% were nontoxigenic *V. cholerae*. Similar to our study they didn't found any *V. parahaemolyticus* strains with *tdh* or *trh* positive but did not exclude the possibility of exposure to pathogenic strains. Another study by Koralage *et al*., (2012) in shrimp farm to investigate the prevalence of *Vibrio* spp., they 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%). They also didn't found any *tdh* or *trh* positive *V. parahaemolyticus* strains. To assess the occurrence of *Vibrio* spp. in fish and shellfish collected from the Swiss market, Schärer *et al*., (2011) found none of the *V. parahaemolyticus* strains harbored species-specific virulence factors.

4.2. MLST characterization of *V. parahaemolyticus*

In this study of 133 mollusks sampled, 38 samples (28.5%) were positive for *Vibrio parahaemolyticus* of which 41.6% are isolated from *Mytilus galloprovincialis* and 27.7% from clam (*Ruditapes philippinarum* and *Chamelea gallina*) (table S3).

The positivity rate of *V. parahaemolyticus* is among the highest when put in relation to some work on the Adriatic Sea. Croci and colleagues (2001) found 4.68% positive over a period of 2 years from shellfish and water samples of Cesenatico and Goro; in the Marchigiane coast shellfish samples were positive for 24.3% between May and September 2003 (Ottaviani *et al*., 2005); between April and September 2007 in the Veneto coast 14.6% mollusks were positive, 15.1% in the Marche, 7.6% in Puglia (Ottaviani *et al*., 2010a).

As reported in the cited works, the greater presence of *V. parahaemolyticus* is found in warmer months, in our sample 78.4% of the isolates comes between June and August.

The results concerning the identification of the strains analyzed carried out by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) and subsequent molecular studies show some inconsistencies. Focusing only on the species of *V. parahaemolyticus*, biochemical identification was denied for 11 strains (6 false positives, 5 false negatives) in comparison to MLSA analysis and amplification of *tox*R and *tlh* genes. These make the molecular approach much more reliable and provide an objective fact that is not susceptible to operator error and overcome the limits of biochemical approach. This is also shown in the article by Croci *et al*., (2007) in which reference and environmental strains were analyzed by the methods API 20E, API 20NE, Alsina and the amplification of the genes *tox*R, *tl*, pR72H and concluded that between biochemical methods Alsina gives more reliable results, but at the end to avoid false positive results all the biochemical identifications should be confirmed by molecular methods.

Table 21: Information of origin, month of isolation, sampling location and risk zone of the *trh* positive strains.

Strains number	ST	Mollusks species	Month	Lagoon (L) or Sea (S)	Risk level zone
31	470	*P. jacobaenus*	7	S	A
40	544	*R. philippinarum*	6	L	B
41	545	*R. philippinarum*	7	L	B
121	-	*Chamelea gallina*	8	S	A
124	470	*Chamelea gallina*	8	S	A
150	592	*R. philippinarum*	9	L	B

With regard to the virulence, in this study no strain gives positive result for the *tdh* gene but six strains were found *trh* positive (Table 16 & 21).

From the Table 21, it has been shown that virulent strains arising mainly from clams during the summer period irrespective of growing area (lagoon or sea) or risk zone. The absence of the *tdh* gene does not mean that the strain is less virulent, being such a molecular marker gene. As evidenced by Ottaviani *et al*., (2010b), two Italian cases of gastroenteritis for consumption of bivalve mollusks in 2008 were caused by *tdh*- and *trh*+ strains belonging to the pandemic serotype O1: KUT resulting from O3: K6.

Similar to the results of the study by Leoni *et al*. (2011), strains 31 and 124 belong to ST 470 that is already in the database because isolated in Thailand in 2003 from the environmental matrix with serotype O1: KUT.

There should also be noted that in a comprehensive overview of the ST 6 and 121 of this dataset, even they do not possess the virulence genes, are linked to the pandemic serotype O3: K6 isolated in Chile in 2004 and China in 2007 (Fig. 14).

All this would lead to think that the strains linked to bivalve mollusks leading to a real risk to the consumer.

For some strains it was not possible to complete the MLST analysis since they did not amplified with some genes (1 for *dna*E, 1 for *gyr*B, 15 for *rec*A, 1 for *dtd*S). The most likely explanation is that the genome of these samples may have undergone recombination events, or polymorphisms are present in the sequence of attachment of the primer, such as to prevent the progress of amplification. Some strains in the database also did not have a ST due to lack on some alleles.

Phylogenetic analyzes and clustering show that the 72 STs represented in our isolates can be divided into two main groups. Comparison with sampling data (season, depth of sampling etc.) and virulence does not show, however, other relationships that can combine these strains into distinct groups.

Observing the global overview of the phylogenetic relationships among all 597 STs (Fig. 9), it is noted that the ST 207, 208, 209, 210, 211, 251, 265, 527 (taken from the database) resulting in a completely separate third cluster.

Through bioinformatics analysis we have verified that this discrepancy is caused by the nucleotide region encoding the portion of the *rec*A gene. The *rec*A alleles present in these strains showed greater homology with the sequence of other bacteria (*V. halioticoli, Photobacterium mandapamensis, V. sinaloensis, V. fortis*) compared with the other *rec*A alleles of *V. parahaemolyticus.*

That particular divergence, already noted previously by other authors (Yu *et al*., 2011) was explained by assuming that the *rec*A gene may have recombination via horizontal transfer with other bacteria, that due to the high rate of recombination of the gene (3.038).

Vibrio parahaemolyticus shows one of the highest rates of recombination (39.8) compared to other genera (Vos and Didelot, 2009). In this study for concatenated sequences the calculated rate of 0.38 is lower and contradictory to the previous MLST analysis (Gonzalez-Escalona *et al*., 2008; Yan *et al*., 2011) but is confirmed by Yu *et al*., 2011, in which with a value of 0.83 states that the differences between the various strains are more to the mutation than the recombination, bearing in mind that the rate is individually high in *rec*A gene. The analysis of recombination using RDP3 and ClonalFrame has identified *rec*A and *pnt*A genes involved in the phenomenon of recombination in the 72 strains isolated in the North Adriatic.

The strains for which SLV are found clonally related are reported in figure 17.

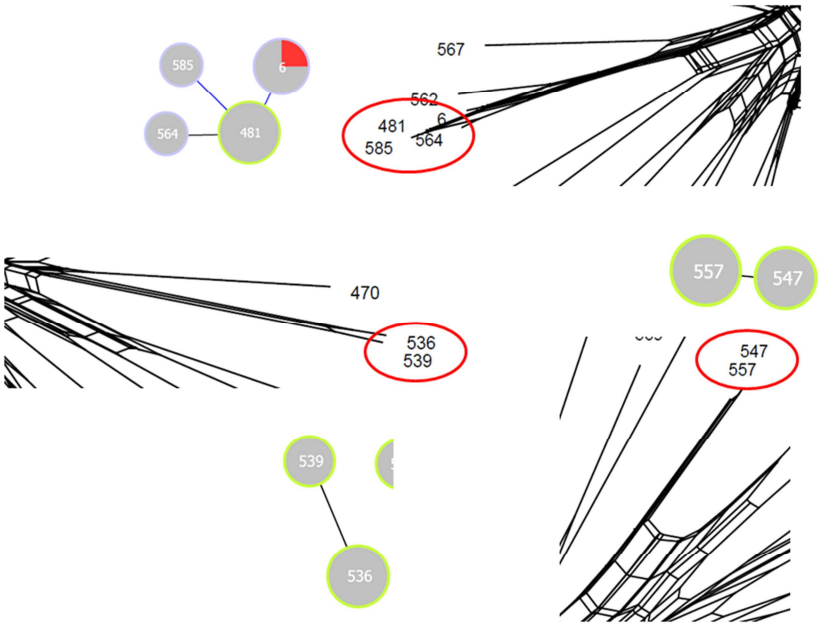

Figure 17: Interpolation of figures of Phyloviz and SplitsTree for strains with Clonal relationships and recombination events.

In Phyloviz, interpolating the data of clonal relationship with the data of isolation, sampling and virulence genes do not show any correlation between our strains, probably because they are mostly singletons. On the other hand, the STs of the online database belonging to the major clonal complex are isolated from clinical cases and many of them belong to the pandemic serotype O3: K6 (Fig. 18)

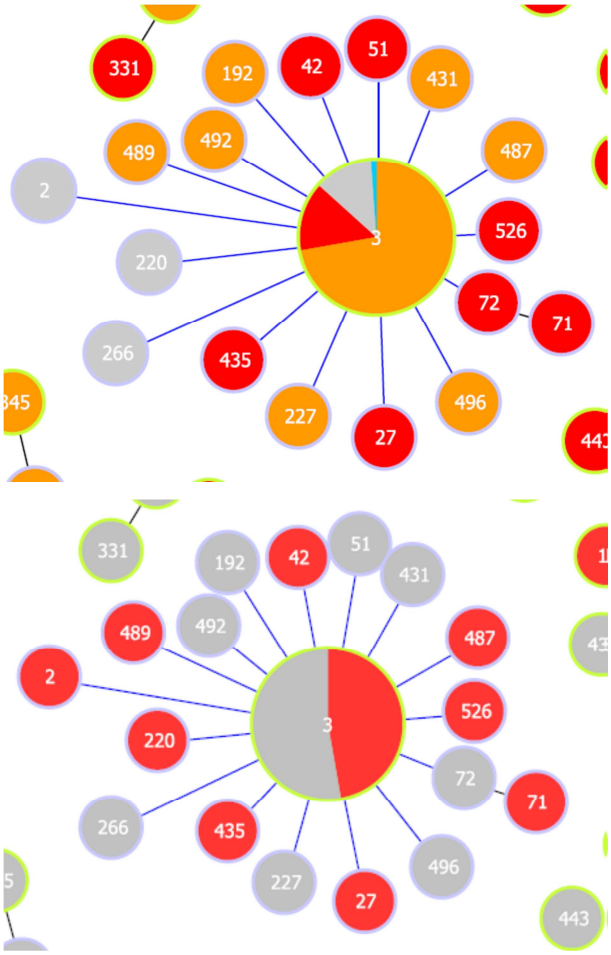

Figure 18: Redrawn from figure 13 and 14 to highlight the relationships of major clonal complex

5. CONCLUSIONS

The aim of the present study was to develop a MLSA scheme for a rapid and a reliable identification of *Vibrio* spp. in shellfish (mollusks and crustacea), not exactly for taxonomic purposes but with the aim to obtain an overview of the distribution of *Vibrio* species among mollusks sampled in the Venice lagoon and Sea. The MLSA demonstrated to recognize all the main species and to fully match with the aim in comparison to the traditional biochemical approaches.

In fact, false negative results of Alsina's scheme need to be considered as it might represent a potential public health risk. Finally, the connection of environmental information to genetic data need to be studied and characterize in order to describe potential *Vibrio* habitats, their distribution and ecology and to enhance *Vibrio* spp. characterization.

Vibrio parahaemolyticus is considered an emerging pathogen which is part of the indigenous microflora of coastal marine waters, fish products; especially the shellfish represent one of the main vehicles of transmission.

The EC Regulation 2073/2005, while not considering *V. parahaemolyticus* among the microbiological criteria applicable to food, recommended the standardization and harmonization of techniques for the isolation and characterization of this organism in order to ensure the safety of products intended for human consumption.

Second part of this study involved Biochemical and MLSA identification, survey of virulence and Multilocus Sequence Typing (MLST) of *V. parahaemolyticus* strains isolated from edible mollusks. The MLST data obtained were then analyzed for phylogenetic information of our dataset and possible correlations with worldwide clinical isolates. The continuation of this study would include an analysis of the serotypes and the virulence factors to get a more complete picture of the current situation.

In conclusion, the findings of this study could be of help in comprehensive monitoring of the different species of *Vibrio*, especially *V. parahaemolyticus* to identify the major sources of contamination and the potential risk in different types of shellfish products. The connection between the molecular data and other relevant information (area of origin, season, species of shellfish etc.) also allows formulating new hypotheses on the population dynamics of *Vibrio* associated with shellfish and provides guidance for the future food safety management.

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SUPPORTING MATERIALS

Table S1: Characteristics of the *Vibrio* strains of 2007 isolated from Mollusks of Venice Lagoon and Sea

Strain code	Sampling location	Biochemical Identification (Alsina's scheme)	MLSA identification	Origin (Species of Mollusks)	Season	Water temperature (°C)	Level risk zone	Area (Lagoon /sea)	Depth of sampling (cm)
Vi_1	12L022	*V. vulnificus*	-	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_2	12M017	*V. vulnificus*	*Vibrio* sp. Vi2	*C. gallina*	winter	6-10	B	Sea	>200
Vi_4	12M018	*V. vulnificus*	-	*Ensis* spp./ *Solen* spp.	winter	6-10	B	Sea	>200
Vi_5	19L046	*V. vulnificus*	-	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_6	19L047	*V. vulnificus*	-	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_7	19L050	*V. vulnificus*	-	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_8	14L003	*V. vulnificus*	-	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_9	19M002	*V. vulnificus*	*Vibrio* sp. Vi9	*M. galloprovincialis*	winter	6-10	A	Sea	>200
Vi_10	12M013	*V. fluvialis*	*V. anguillarum*	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_11	12L044	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_12	14L006	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	winter	6-10	B	Lagoon	101-200
Vi_13	12M017	*V. fluvialis*	*V. anguillarum*	*C. gallina*	winter	6-10	B	Sea	>200
Vi_14	12M018	*V. fluvialis*	*V. anguillarum*	*Ensis* spp./ *Solen* spp.	winter	6-10	B	Sea	>200
Vi_15	19L050	*V. mediterranei*	-	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_16	14L006	*V. mediterranei*	*V. mediterranei* like	*R. philippinarum*	winter	6-10	B	Lagoon	101-200
Vi_17	12L044	*V. pelagius* I	-	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_18	12M008	*V. pelagius* I	*V. splendidus*	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_19	12M013	*V. splendidus* II	-	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_20	12L024	*V. nereis*	*Vibrio* sp. Vi20	*Ostrea edulis*	winter	11-15	NC	Lagoon	0-100
Vi_21	12L041	*V. campbellii*	*Vibrio* sp. Vi21	*R. philippinarum*	winter	6-10	B	Lagoon	101-200
Vi_22	12M008	*V. anguillarum* like	*V. anguillarum* like	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_23	19L043	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_24	19L044	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_25	19M003	*V. fluvialis*	*V. anguillarum*	*M. galloprovincialis*	spring	6-10	A	Sea	>200

Vi_26	19L043	*V. campbellii*	*V. splendidus*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_28	19L046	*V. alginolyticus*	*Vibrio* sp. Vi2	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_29	12M010	*V. alginolyticus*	*V. alginolyticus*	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_30	12M015	*V. alginolyticus*	-	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_31	14L007	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	winter	6-10	B	Lagoon	101-200
Vi_32	12M017	*V. alginolyticus*	*V. alginolyticus*	*C. gallina*	winter	6-10	B	Sea	>200
Vi_33	14L003	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_34	12M008	*V. alginolyticus*	*V. diabolicus*	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_35	12M008	*V. alginolyticus*	*V. diabolicus*	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_36	19M002	*V. alginolyticus*	*V. alginolyticus*	*M. galloprovincialis*	winter	6-10	A	Sea	>200
Vi_37	19L049	*V. parahaemolyticus*	*V. diabolicus*	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_38	19L046	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	winter	6-10	B	Lagoon	0-100
Vi_39	14L006	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	winter	6-10	B	Lagoon	101-200
Vi_41	14L003	*Vibrio spp.*	Harveyi group	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_42	12L024	*Vibrio spp.*	Harveyi group	*Ostrea edulis*	winter	11-15	NC	Lagoon	0-100
Vi_43	12M018	*V. alginolyticus*	*V. diabolicus*	*Ensis* spp./ *Solen* spp.	winter	6-10	B	Sea	>200
Vi_44	12L022	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_45	12M008	*Vibrio spp.*	Harveyi group	*M. galloprovincialis*	winter	11-15	A	Sea	>200
Vi_46	12L044	*V. parahaemolyticus*	Harveyi group	*R. philippinarum*	winter	11-15	B	Lagoon	>200
Vi_47	12M017	*V. fluvialis*	*V. anguillarum*	*C. gallina*	winter	6-10	B	Sea	>200
Vi_48	12L093	*V. fluvialis*	Harveyi group	*R. philippinarum*	spring	11-15	B	Lagoon	>200
Vi_49	14L004	*V. fluvialis*	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_50	14L004	*V. fluvialis*	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_51	19L044	*V. nereis*	*Vibrio* sp. Vi20	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_52	19M003	*V. nereis*	*V. brasiliensis*	*C. gallina*	spring	6-10	A	Sea	>200
Vi_53	19L043	*V. splendidus*	*V. splendidus*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_54	14L004	*V. splendidus*	*Vibrio* sp. Vi20	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_55	19M003	*V. anguillarum* like	*V. anguillarum*	*C. gallina*	spring	6-10	A	Sea	>200
Vi_56	12L093	*V. parahaemolyticus*	Harveyi group	*R. philippinarum*	spring	11-15	B	Lagoon	>200
Vi_57	14L004	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200

Vi_58	12L093	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	spring	11-15	B	Lagoon	>200
Vi_59	14L004	*V. fluvialis*	*V. anguillarum*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_60	19L020	*V. splendidus* II	*Vibrio* sp. Vi60	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_61	19L020	*V. parahaemolyticus*	*V. splendidus*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_62	19M003	*V. anguillarum* like	*Vibrio* sp. Vi20 like	*M. galloprovincialis*	spring	11-15	A	Sea	>200
Vi_63	19M003	*V. vulnificus* B2	*V. alginolyticus*	*M. galloprovincialis*	spring	11-15	A	Sea	>200
Vi_64	12L040	*V. parahaemolyticus*	Harveyi group	Ricci di Mare	spring	16-20	B	Lagoon	>200
Vi_65	12L040	*V. alginolyticus*	-	Ricci di Mare	spring	16-20	B	Lagoon	>200
Vi_66	12L039	*V. parahaemolyticus*	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_67	12L039	*V. anguillarum like*	*V. orientalis* like	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_68	19L023	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	6-10	B	Lagoon	0-100
Vi_69	14L008	*V. harveyi*	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_70	14L008	*V. alginolyticus*	*V. alginolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_71	12L025	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	11-15	B	Lagoon	0-100
Vi_72	12L025	*V. alginolyticus*	*V. anguillarum*	*R. philippinarum*	spring	11-15	B	Lagoon	0-100
Vi_73	12L025	*V. harveyi*	*Vibrio* sp. Vi20	*R. philippinarum*	spring	11-15	B	Lagoon	0-100
Vi_74	19L027	*V. logei*	Harveyi group	*R. philippinarum*	spring	6-10	B	Lagoon	101-200
Vi_79	14L008	*V. anguillarum* like	*V. splendidus*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_80	14L008	*V. fischeri*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_81	12L020	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_1a	12L020	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_2a	19L018	*V. mimicus*	*Vibrio* sp. Vi2a	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_3a	19M003	*V. parahaemolyticus*	*V. parahaemolyticus*	*M. galloprovincialis*	spring	11-15	A	Sea	>200
Vi_4a	19M003	*V. parahaemolyticus*	*V. parahaemolyticus*	*M. galloprovincialis*	spring	11-15	A	Sea	>200
Vi_5a	14L001	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	>200
Vi_6a	19L018	*V. parahaemolyticus*	*V. parahaemolyticus* like	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_7a	14L003	*V. fluvialis*	*V. orientalis*	*R. philippinarum*	spring	16-20	B	Lagoon	>200
Vi_8a	14L003	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	>200
Vi_9a	19M003	*V. anguillarum* like	*Vibrio* sp. Vi20	*M. galloprovincialis*	spring	11-15	A	Sea	>200
Vi_10a	14L001	*V. vulnificus* B2	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	>200

Vi_11a	14L003	*V. vulnificus* B2	Harveyi group	*R. philippinarum*	spring	16-20	B	Lagoon	>200
Vi_12a	14L001	*V. vulnificus* B2	Harveyi group	*R. philippinarum*	spring	11-15	B	Lagoon	101-200
Vi_13a	14L001	*V. vulnificus* B2	*V. diabolicus*	*R. philippinarum*	spring	11-15	B	Lagoon	101-200
Vi_14a	14L001	*V. vulnificus* B2	*V. alginolyticus*	*R. philippinarum*	spring	11-15	B	Lagoon	101-200
Vi_15a	10L021	*V. vulnificus* B2	*V. mediterranei* like	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_16a	10L021	*V. splendidus* II	*Vibrio* sp. Vi20	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_17a	19L039	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_18a	12L028	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	21-30	B	Lagoon	0-100
Vi_19a	12L028	*V. alginolyticus*	*V. alginolyticus*	*R. philippinarum*	spring	21-30	B	Lagoon	0-100
Vi_20a	12L028	*V. alginolyticus*	*V. diabolicus*	*Cerastoderma* spp.	spring	21-30	B	Lagoon	0-100
Vi_21a	12L028	*V. parahaemolyticus*	*V. parahaemolyticus*	*Cerastoderma* spp.	spring	21-30	B	Lagoon	0-100
Vi_22a	10L021	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_23a	14L009	*V. mediterranei*	*V. mediterranei* like	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_24a	12L041	*V. mimicus*	*Vibrio* sp. Vi24a	*R. philippinarum*	spring	21-30	B	Lagoon	101-200
Vi_25a	12L028	*V. parahaemolyticus*	*V. parahaemolyticus*	*Cerastoderma spp.*	spring	21-30	B	Lagoon	0-100
Vi_26a	12L028	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	21-30	B	Lagoon	0-100
Vi_27a	12L041	*V. parahaemolyticus*	*V. parahaemolyticus* like	*R. philippinarum*	spring	21-30	B	Lagoon	101-200
Vi_28a	12L041	*V. alginolyticus*	*V. diabolicus*	*R. philippinarum*	spring	21-30	B	Lagoon	101-200
Vi_29a	14L009	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_30a	14L009	*V. marinus*	*V. diabolicus*	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_31a	14L009	*V. mediterranei*	*V. mediterranei* like	*R. philippinarum*	spring	16-20	B	Lagoon	0-100
Vi_32a	12L041	*V. vulnificus*	Harveyi group	*M. galloprovincialis*	spring	21-30	B	Lagoon	101-200
Vi_33a	12L041	*V. alginolyticus*	*V. alginolyticus*	*M. galloprovincialis*	spring	21-30	B	Lagoon	101-200
Vi_34a	12L040	*V. alginolyticus*	*V. alginolyticus*	*M. galloprovincialis*	spring	21-30	B	Lagoon	>200
Vi_35a	12L040	*V. fischeri*	*V. alginolyticus*	*M. galloprovincialis*	spring	21-30	B	Lagoon	>200
Vi_36a	19L049	*V. alginolyticus*	*V. alginolyticus*	*M. galloprovincialis*	spring	21-30	B	Lagoon	0-100
Vi_37a	12L023	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	spring	21-30	B	Lagoon	>200
Vi_38a	19L047	*V. nereis*	*V. brasiliensis* like	*M. galloprovincialis*	spring	21-30	B	Lagoon	0-100
Vi_39a	19L046	*V. vulnificus*	Harveyi group	*M. galloprovincialis*	spring	21-30	B	Lagoon	0-100

Vi_40a	12L023	*V. anguillarum* like	*V. alginolyticus*	*C. gallina*	spring	21-30	B	Lagoon	>200
Vi_41a	19L049	*V. vulnificus*	Harveyi group	*M. galloprovincialis*	spring	21-30	B	Lagoon	0-100
Vi_42a	19L047	*V. logei*	Harveyi group	*M. galloprovincialis*	spring	21-30	B	Lagoon	0-100
Vi_43a	14L006	*V. alginolyticus*	*V. alginolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_44a	14L006	*V. campbellii*	*V. parahaemolyticus*	*R. philippinarum*	spring	16-20	B	Lagoon	101-200
Vi_45a	14L009	*V. vulnificus*	Harveyi group	*R. philippinarum*	spring	21-30	B	Lagoon	101-200
Vi_46a	12L022	*V. parahaemolyticus*	*V. parahaemolyticus*	*C. gallina*	spring	16-20	B	Lagoon	>200
Vi_47a	12L023	*V. parahaemolyticus*	*V. parahaemolyticus*	*C. gallina*	spring	21-30	B	Lagoon	>200
Vi_48a	12L023	*V. fischeri*	*V. alginolyticus*	*C. gallina*	spring	21-30	B	Lagoon	>200
Vi_49a	19L022	*V. mediterranei*	*V. mediterranei*	*R. philippinarum*	spring	21-30	B	Lagoon	0-100
Vi_50a	19M001	*V. harveyi*	*V. chagasii*	*M. galloprovincialis*	spring	16-20	A	Sea	>200
Vi_51a	19L022	*V. pelagius* I	*V. chagasii*	*R. philippinarum*	spring	21-30	B	Lagoon	0-100
Vi_52a	14L005	*V. parahaemolyticus*	*V. parahaemolyticus*	*M. galloprovincialis*	spring	16-20	B	Lagoon	>200
Vi_53a	14L010	*V. mediterranei*	*Vibrio* sp. Vi24a	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_54a	14L010	*V. parahaemolyticus*	Harveyi group	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_55a	14L010	*V. parahaemolyticus*	Harveyi group	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_56a	12M006	*V. parahaemolyticus*	Harveyi group	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_57a	12M006	*V. mediterranei*	*V. mediterranei* like	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_58a	12M006	*V. pelagius* I	*Vibrio* sp. Vi58a	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_59a	12L025	*V. mediterranei*	*V. mediterranei*	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_60a	19M002	*V. splendidus* II	*V. chagasii*	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_61a	19M002	*V. pelagius* II	-	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_62a	19M002	*V. harveyi*	Harveyi group	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_63a	14L002	*V. splendidus* II	*V. mediterranei*	*R. philippinarum*	summer	21-30	B	Lagoon	>200
Vi_64a	19M002	*V. mediterranei*	*V. mediterranei*	*M. galloprovincialis*	summer	21-30	A	Sea	>200
Vi_65a	14M001	*V. mediterranei*	*V. mediterranei*	*Callista chione*	summer	21-30	A	Sea	>200
Vi_66a	14M001	*V. alginolyticus*	-	*Callista chione*	summer	21-30	A	Sea	>200
Vi_67a	14L002	*V. mediterranei*	*V. mediterranei* like	*M. galloprovincialis*	summer	21-30	B	Lagoon	>200
Vi_68a	14L002	*V. fluvialis*	*V. furnissii*	*M. galloprovincialis*	summer	21-30	B	Lagoon	>200
Vi_69a	14L002	*V. anguillarum* like	*V. orientalis*	*M. galloprovincialis*	summer	21-30	B	Lagoon	>200

Vi_70a	14L009	*V. mediterranei*	*Vibrio* sp. Vi70a	*R. philippinarum*	summer	21-30	B	Lagoon	101-200
Vi_71a	14L009	*V. mediterranei*	*V. mediterranei*	*R. philippinarum*	summer	21-30	B	Lagoon	101-200
Vi_72a	19L007	*V. harveyi*	Harveyi group	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_73a	19L008	*V. harveyi*	Harveyi group	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_74a	12L044	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	summer	21-30	B	Lagoon	101-200
Vi_75a	19L009	*V. parahaemolyticus*	*V. parahaemolyticus*	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_76a	12L028	*V. parahaemolyticus*	*V. parahaemolyticus*	*Crassostrea gigas*	summer	21-30	B	Lagoon	0-100
Vi_77a	12M028	*V. parahaemolyticus*	*V. parahaemolyticus*	*Cerastoderma spp.*	summer	21-30	B	Sea	0-100
Vi_78a	19M001	*V. vulnificus*	*V. vulnificus*	*R. philippinarum*	summer	21-30	A	Sea	>200
Vi_79a	19L019	*V. mediterranei*	*Vibrio* sp. Vi70a	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_80a	12M017	*V. logei*	Harveyi group	*Ensis* spp./ *Solen* spp.	autumn	16-20	B	Sea	>200
Vi_81a	19L023	*V. mediterranei*	*V. mediterranei*	*R. philippinarum*	summer	21-30	B	Lagoon	0-100
Vi_2b	14L008	*V. mimicus*	*V. chagasii*	*R. philippinarum*	autumn	16-20	B	Lagoon	0-100
Vi_3b	14L008	*V. splendidus* II	*V. chagasii*	*R. philippinarum*	autumn	16-20	B	Lagoon	0-100
Vi_4b	12L026	*V. anguillarum*	Harveyi group	*R. philippinarum*	autumn	16-20	B	Lagoon	0-100
Vi_5b	19M002	*V. splendidus* II	*Vibrio* sp. Vi58a	*M. galloprovincialis*	autumn	16-20	A	Sea	>200
Vi_6b	19L021	*V. pelagius* I	*V. chagasii*	*R. philippinarum*	autumn	16-20	B	Lagoon	101-200
Vi_7b	19L021	*V. splendidus* II	*V. chagasii*	*R. philippinarum*	autumn	16-20	B	Lagoon	101-200
Vi_8b	19L021	*V. logei*	Harveyi group	*R. philippinarum*	autumn	16-20	B	Lagoon	101-200
Vi_9b	12L018	*V. anguillarum* like	*V. mediterranei*	*Cerastoderma spp.*	autumn	16-20	B	Lagoon	0-100
Vi_10b	19M003	*V. alginolyticus*	*V. diabolicus*	*M. galloprovincialis*	autumn	11-15	A	Sea	>200
Vi_3		*V. vulnificus*	-						
Vi_27		*V. alginolyticus*	*V. diabolicus*						
Vi_40		*V. parahaemolyticus*	Harveyi group						
Vi_1b		*V. parahaemolyticus*	*V. parahaemolyticus*						

(1, 3-8) these 7 strains didn't grow in broth or plate during the revitalization process.
(15, 17, 19, 30, 65, 61a and 66a) these 7didn't amplify with *gyr*B, *pyr*H or *rec*A;
and among the remaining 154, 3 strains (27, 40, 1b) don't have the environmental information.

Table S2: Characteristics of the *Vibrio* strains of 2011 isolated from Crustacean samples in collaboration with IZSVe Adria (Italy)

Strain number	Code by IZS Adria	Sampling date	Species of crustaceans	Conservation	Origin	% of NaCl in Alkaline peptone water	Incubation temperature	Biochemical Identification	MLSA Identification
1A	234/lit 1	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	2	37°C	*V. harveyi*	Harveyi group
2A	234/lit 2	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	2	37°C	*V. harveyi*	Harveyi group
3A	234/lit 3	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	2	37°C	*V. alginolyticus*	*V. alginolyticus*
4A	234/lit 4	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	2	37°C	*V. alginolyticus*	*V. alginolyticus*
5A	234/lit 5	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	0	37°C	*V. parahaemolyticus*	*P. profundum* like
6A	234/lit 6	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	0	37°C	*V. alginolyticus*	*V. diabolicus*
7A	234/lit 7	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	2	37°C	*V. alginolyticus*	*V. alginolyticus*
8A	234/lit 8	7/18/2011	*Palaemon elegans*	Frozen	Malamocco	0	37°C	*V. parahaemolyticus*	*P. profundum* like
9A	268/ITT 1	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*P. damselae damselae*	didn't amplify with *gyr*B and *atp*A
10A	268/ITT 2	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*V. agarivorans*	didn't amplify with *atp*A
11A	268/ITT 3	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*V. tubiashii*	*V. brasiliensis*
12A	268/ITT 4	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*V. alginolyticus*	*V. alginolyticus*
13A	268/ITT 5	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	0	37°C	*V. alginolyticus*	didn't amplify with *atp*A
14A	268/ITT 6	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	0	37°C	*V. alginolyticus*	didn't amplify with *atp*A
15A	268/ITT 7	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*V. alginolyticus*	*V. alginolyticus*
16A	268/ITT 8	9/8/2011	*Squilla mantis*	Fresh	Alto Adriatico-Grado	2	37°C	*V. alginolyticus*	*Vibrio* sp. 16A
17A	269/ITT 1	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. alginolyticus*	*V. parahaemolyticus*
18A	269/ITT 2	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*

19A	269/ITT 3	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
20A	269/ITT 4	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
21A	269/ITT 5	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. alginolyticus*	*V. alginolyticus*
22A	269/ITT 6	9/8/2011	*Crangon crangon*	Fresh	Laguna Nord di venezia	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
23A	270/ITT 1	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*V. parahaemolyticus*	Harveyi group
24A	270/ITT 2	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
25A	270/ITT 3	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	0	37°C	*V. parahaemolyticus*	*P. profundum like*
26A	270/ITT 4	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	0	37°C	*V. parahaemolyticus*	*Vibrio* sp. 26A
27A	270/ITT 5	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*V. parahaemolyticus*	Harveyi group
28A	270/ITT 6	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*Vibrio spp.*	didn't amplify with *gyr*B and *rec*A
29A	270/ITT 7	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*V. alginolyticus*	*V. parahaemolyticus*
30A	270/ITT 8	9/8/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*P. damselae damselae*	Harveyi group
31A	271/ITT 1	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di venezia (Campalto)	2	37°C	*V. parahaemolyticus*	Harveyi group
32A	271/ITT 2	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di venezia (Campalto)	2	37°C	*Vibrio spp.*	*V. diabolicus*
33A	271/ITT 3	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di venezia (Campalto)	2	37°C	*V. alginolyticus*	*V. alginolyticus*
34A	271/ITT 4	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di venezia (Campalto)	2	37°C	*V. alginolyticus*	*V. alginolyticus*
35A	271/ITT 5	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di venezia (Campalto)	2	37°C	*Vibrio spp.*	Harveyi group
36A	271/ITT 6	9/8/2011	*Carcinus aestuarii*	Fresh	Laguna Nord di	2	37°C	*Vibrio spp.*	*V.*

					venezia (Campalto)				*parahaemolyticus*
37A	277/ITT 1	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*V. alginolyticus*	*V. diabolicus*
38A	277/ITT 2	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*Vibrio sp.*	*V. diabolicus*
39A	277/ITT 3	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*Vibrio sp.*	*V. diabolicus*
40A	277/ITT 4	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*V. alginolyticus*	*V. alginolyticus*
41A	277/ITT 5	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*V. alginolyticus*	*V. alginolyticus*
42A	277/ITT 6	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	2	37°C	*V. alginolyticus*	*V. alginolyticus*
43A	278/ITT 1	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.8	2	37°C	*V. alginolyticus*	*V. alginolyticus*
44A	278/ITT 2	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.9	2	37°C	*V. alginolyticus*	*V. diabolicus*
45A	278/ITT 3	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.8	2	37°C	*V. alginolyticus*	*V. alginolyticus*
46A	278/ITT 4	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.8	2	37°C	*Vibrio sp.*	*V. alginolyticus*
47A	278/ITT 5	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.8	2	37°C	*V. alginolyticus*	*Vibrio* sp. 26A
48A	278/ITT 6	9/8/2011	*Penaeus vannamei*	Thawed	Oceano pacifico FAO n.10	2	37°C	*Vibrio sp.*	didn't amplify with *atp*A
49A	279/ITT 2	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.41	0	37°C	*Vibrio sp.*	*Vibrio* sp. 49A
50A	279/ITT 3	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.43	0	37°C	*Vibrio sp.*	didn't amplify with *pyr*H and *rec*A
51A	279/ITT 4	9/8/2011	*Hymenopenaeus*	Thawed	Atlantico sud	0	37°C	*Vibrio sp.*	*Vibrio* sp. 49A

			muelleri		occidentale FAO n.44				
52A	279/ITT 5	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.44	2	37°C	*Vibrio sp.*	*Vibrio* sp. 52A
53A	279/ITT 9	9/8/2011	*Hymenopenaeus muelleri*	Thawed	Atlantico sud occidentale FAO n.45	2	37°C	*Vibrio sp.*	*Vibrio* sp. 52A
54A	314/ITT 5	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	0	37°C	*Vibrio sp.*	*Shewanella* spp. as *atp*A
55A	314/ITT 6	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	0	37°C	*Vibrio sp.*	*Shewanella* spp. as *atp*A
56A	314/ITT 7	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	0	37°C	*Vibrio sp.*	*V. diabolicus*
57A	314/ITT 9	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
58A	314/ITT 10	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	37°C	*Vibrio sp.*	*V. diabolicus*
59A	314/ITT 18	9/23/2011	*Palaemon elegans*	Fresh	Comacchio-Goro	2	22°C	*V. parahaemolyticus*	*V. parahaemolyticus*
60A	341/ITT 1	10/12/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
61A	341/ITT 2	10/13/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
62A	341/ITT 4	10/14/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
63A	341/ITT 6	10/15/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
64A	341/ITT 7	10/16/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
65A	341/ITT 8	10/17/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
66A	341/ITT 9	10/18/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
67A	341/ITT 10	10/19/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
68A	341/ITT 11	10/20/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di	0	37°C	*V. parahaemolyticus*	*Shewanella* spp.

					Venezia (Fusina)				as *atp*A
69A	341/ITT 12	10/21/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
70A	341/ITT 14	10/23/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	0	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
71A	341/ITT 15	10/24/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
72A	341/ITT 16	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
73A	341/ITT 19	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
74A	341/ITT 20	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
75A	341/ITT 21	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
76A	341/ITT 22	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
77A	341/ITT 24	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
78A	341/ITT 25	10/25/2011	*Carcinus aestuarii*	Fresh	Laguna centrale di Venezia (Fusina)	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
79A	378/ITT 1	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	2	37°C	*Vibrio sp.*	didn't amplify with *rec*A
80A	378/ITT 2	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*L. anguillarum*	*V. anguillarum*
81A	378/ITT 3	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*V. splendidus*	*Vibrio* sp. 81A
82A	378/ITT 4	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*Vibrio sp.*	*V. splendidus*
83A	378/ITT 5	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	2	37°C	*Vibrio sp.*	*V. splendidus*
84A	378/ITT 6	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*L. pelagius*	*V. splendidus*
85A	378/ITT 7	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	2	37°C	*Vibrio sp.*	*V. anguillarum*
86A	378/ITT 8	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*Vibrio sp.*	*V. anguillarum*
87A	378/ITT 9	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*Vibrio sp.*	*V. splendidus*

88A	378/ITT 10	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	0	37°C	*Vibrio sp.*	*V. splendidus*
89A	144/ITT 1	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
90A	144/ITT 2	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*Shewanella* spp. as *atp*A
91A	144/ITT 4	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*Shewanella* spp. as *atp*A
92A	144/ITT 5	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
93A	144/ITT 7	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
94A	144/ITT 8	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*Vibrio* sp. 94A
95A	144/ITT 9	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*Shewanella* spp. as *atp*A
96A	144/ITT 11	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*Shewanella* spp. as *atp*A
97A	144/ITT 12	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
98A	144/ITT 13	6/8/2011	*Carcinus aestuarii*	Fresh	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
99A	156/ITT 1	6/8/2011	*Carcinus aestuarii*	Frozen	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
100A	156/ITT 2	6/8/2011	*Carcinus aestuarii*	Frozen	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
101A	156/ITT 3	6/8/2011	*Carcinus aestuarii*	Frozen	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
102A	156/ITT 4	6/8/2011	*Carcinus aestuarii*	Frozen	laguna Ca'Ravagnan	2	37°C	*V. parahaemolyticus*	*V. alginolyticus*
103A	215/ITT 1	7/20/2011	*Palaemon elegans*	Fresh	Malamocco	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
104A	215/ITT 5	7/20/2011	*Palaemon elegans*	Fresh	Malamocco	2	37°C	*V. parahaemolyticus*	*V. alginolyticus*
105A	215/ITT 7	7/20/2011	*Palaemon elegans*	Fresh	Malamocco	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*

106A	215/ITT 8	7/20/2011	*Palaemon elegans*	Fresh	Malamocco	2	37°C	*V. parahaemolyticus*	*V. parahaemolyticus*
107A	365/ITT 3	12/20/2011	*Squilla mantis*	Fresh	Nord Adriatico Caorle	2	37°C	*L. anguillarum*	*V. parahaemolyticus*

(9A, 10A, 13A, 14A, 28A, 48A, 50A and 79A) these 8 didn't amplify with *gyr*B, *pyr*H, *rec*A or *atp*A;
and (54A, 55A, 68A, 90A, 91A, 95A and 96A) these 7 strains amplified only with *atp*A gene and identified as *Shewanella* spp. by BLAST search.

Table S3: Sampling information of *Vibrio parahaemolyticus* strains used in this study isolated from mollusks of North Adriatic Sea in 2011

Strains number	Sampling location	Month of 2011	Origin (species of mollusks)	Lagoon (L)/ Sea (S)	Open Fishery/ Farming	Level Risk Zone	Air Temperature	Water temperature	Depth of Sampling
1	12L049	1	*R. philippinarum*	L	-	-	-	-	1.1
2	12L052	2	*M. galloprovincialis*	L	Farming	-	5.7	6.1	6
3	19L018	2	-	L	-	-	-	9	1.2
4	19L018	2	-	L	-	-	-	9	1.2
5	19L018	2	-	L	-	-	-	9	1.2
6	19L018	2	-	L	-	-	-	9	1.2
7	14L007	3	*R. philippinarum*	L	Open Fishery	B	12	9	8
8	19L019	3	-	L	-	-	5	1.8	0.5
9	19L023	4	*R. philippinarum*	L	Farming	B	-	-	-
10	19L023	4	*R. philippinarum*	L	Farming	B	-	-	-
11	14L009	4	*Castoderma* spp.	L	Open Fishery	B	12	10	0.8
12	14L009	4	*Castoderma* spp.	L	Open Fishery	B	12	10	0.8
13	14L009	4	*Castoderma* spp.	L	Open Fishery	B	12	10	0.8
14	14L010	4	*Castoderma* spp.	L	Open Fishery	B	12	10	1
15	14L010	4	*Castoderma* spp.	L	Open Fishery	B	12	10	1
16	14L010	4	*Castoderma* spp.	L	Open Fishery	B	12	10	1
17	14L004	4	*M. galloprovincialis*	L	Farming	B	14	9	2.5
18	14L004	4	*M. galloprovincialis*	L	Farming	B	14	9	2.5
19	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
20	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
21	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
22	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
23	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
24	14L010	6	*R. philippinarum*	L	Open Fishery	B	12	10	0.9
25	19M001	5	*Haustellum* spp.	S	Open Fishery	A	12	8	8

26	19M001	5	*Haustellum* spp.	S	Open Fishery	A	12	8	8
27	19M001	5	*Haustellum* spp.	S	Open Fishery	A	12	8	8
28	19M001	5	*Haustellum* spp.	S	Open Fishery	A	12	8	8
29	19M001	5	*Haustellum* spp.	S	Open Fishery	A	12	8	8
30	12M001	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	10
31	12M001	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	10
32	12M001	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	10
33	12M001	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	10
34	12M002	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	-
35	12M002	7	*Pecten jacobaenus*	S	Open Fishery	A	22	14	-
36	14L001	6	*R. philippinarum*	L	Open Fishery	B	25	-	3
37	14L001	6	*R. philippinarum*	L	Open Fishery	B	25	-	3
38	14L001	6	*R. philippinarum*	L	Open Fishery	B	25	-	3
39	14L005	6	*M. galloprovincialis*	L	Farming	B	16	10	2
40	14L005	6	*M. galloprovincialis*	L	Farming	B	16	10	2
41	14L005	7	*R. philippinarum*	L	Farming	B	25	19	1.8
42	14L009	7	*Castoderma* spp	L	Open Fishery	B	25	19	0.8
43	14L009	7	*Castoderma* spp	L	Open Fishery	B	25	19	0.8
44	14L009	7	*Castoderma* spp	L	Open Fishery	B	25	19	0.8
45	14L009	7	*Castoderma* spp	L	Open Fishery	B	25	19	0.8
46	14L007	7	*R. philippinarum*	L	Farming	B	26	16	1.8
47	14L009	7	*R. philippinarum*	L	Farming	A	18	24	5
48	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
49	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
50	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
51	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
52	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24,3	5
53	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-

54	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
55	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
56	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
57	14L009	7	*R. philippinarum*	L	Farming	A	18	24	5
58	14M001	7	*Chamelea gallina*	S	Open Fishery	A	27	16	3
59	14M001	7	*Chamelea gallina*	S	Open Fishery	A	27	16	3
60	14M001	7	*Chamelea gallina*	S	Open Fishery	A	27	16	3
61	14M001	7	*Chamelea gallina*	S	Open Fishery	A	27	16	3
62	14M001	7	*Chamelea gallina*	S	Open Fishery	A	27	16	3
63	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
64	14L009	7	*R. philippinarum*	L	Farming	A	18	24	5
65	14L009	7	*R. philippinarum*	L	Farming	A	18	24	5
66	14L009	7	*R. philippinarum*	L	Farming	A	18	24	5
67	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
68	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
69	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
70	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
71	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
72	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
73	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
74	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
75	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
76	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
77	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
78	19M002	7	*M. galloprovincialis*	S	Farming	A	-	-	-
79	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
80	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
81	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-

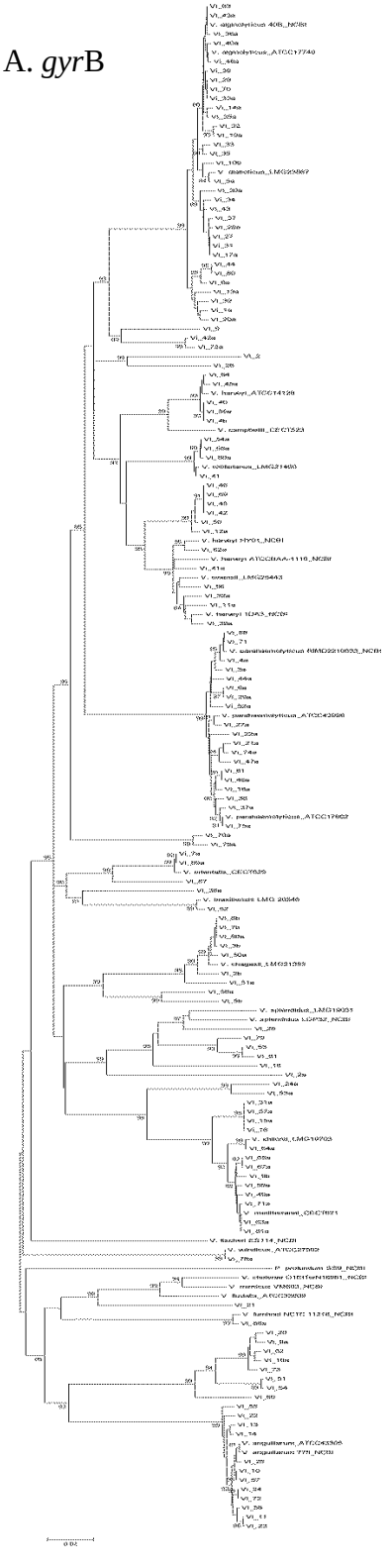
82	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
83	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
84	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
85	-	-	-	-	-	-	-	-	-
86	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
87	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
88	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
89	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
90	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
91	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
92	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
93	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
94	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
95	14L007	7	*R. philippinarum*	L	Farming	B	26	16	1.8
96	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
97	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
98	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24	10
99	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24,3	5
100	12M001	7	*M. galloprovincialis*	S	Farming	A	23	24,3	5
101	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
102	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
103	19M003	7	*M. galloprovincialis*	S	Farming	A	-	-	-
104	-	7	*Ostrea* spp.	-	-	-	-	-	-
105	-	7	*Ostrea* spp.	-	-	-	-	-	-
106	-	7	*Ostrea* spp.	-	-	-	-	-	-
107	-	7	*Ostrea* spp.	-	-	-	-	-	-
108	-	7	*Ostrea* spp.	-	-	-	-	-	-
109	-	7	*Ostrea* spp.	-	-	-	-	-	-

110	-	7	*Ostrea* spp.	-	-	-	-	-	-
111	-	7	*Ostrea* spp.	-	-	-	-	-	-
112	-	7	*Ostrea* spp.	-	-	-	-	-	-
113	-	7	*Ostrea* spp.	-	-	-	-	-	-
114	-	7	*Ostrea* spp.	-	-	-	-	-	-
115	-	7	*Ostrea* spp.	-	-	-	-	-	-
116	-	7	*Ostrea* spp.	-	-	-	-	-	-
117	14L004	8	*M. galloprovincialis*	L	Farming	B	28	-	2
118	10M003	8	*Callista chione*	S	Open Fishery	A	28	-	10
119	10M003	8	*Callista chione*	S	Open Fishery	A	28	-	10
120	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
121	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
122	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
123	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
124	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
125	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
126	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
127	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
128	19M001	8	*Chamelea gallina*	S	Open Fishery	A	28	-	3
129	14M001	8	*M. galloprovincialis*	S	Open Fishery	A	27	-	4
130	14M001	8	*M. galloprovincialis*	S	Open Fishery	A	27	-	4
131	14M001	8	*M. galloprovincialis*	S	Open Fishery	A	27	-	4
132	14M001	8	*M. galloprovincialis*	S	Open Fishery	A	27	-	4
133	14M001	8	*M. galloprovincialis*	S	Open Fishery	A	27	-	4
134	12L053	8	*M. galloprovincialis*	L	Farming	B	-	-	-
135	-	-	-	-	-	-	-	-	-
136	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9

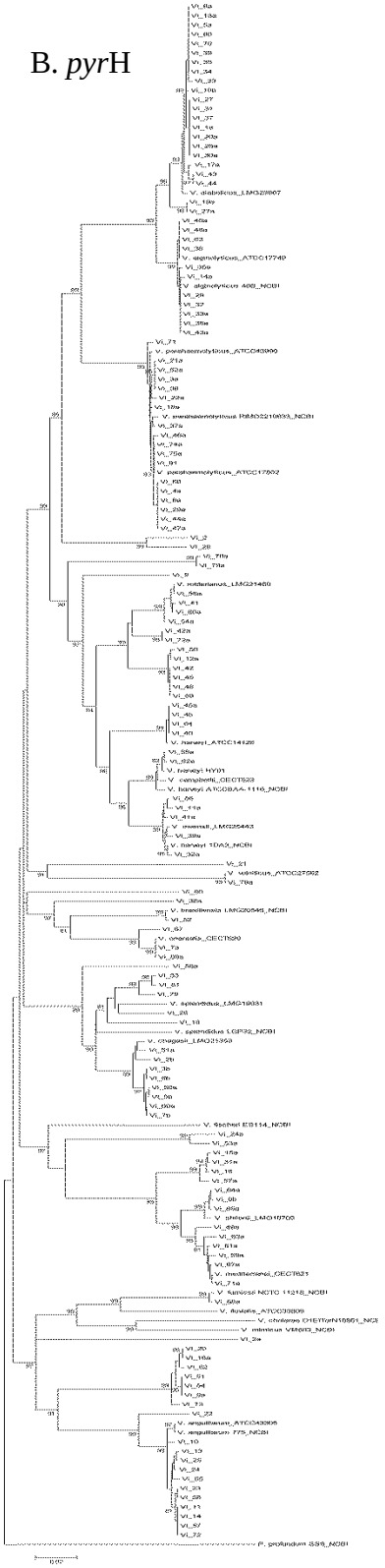
137	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
138	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
139	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
140	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
141	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
142	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
143	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
144	14L001	8	*R. philippinarum*	L	Open Fishery	B	28	22	0.9
145	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
146	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
147	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
148	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
149	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
150	14L002	9	*R. philippinarum*	L	Open Fishery	B	28	22	0.8
151	14L006	9	*Callista chione*	L	Farming	B	22	18	-
152	14L006	9	*Callista chione*	L	Farming	B	22	18	-
153	14L006	9	*Callista chione*	L	Farming	B	22	18	-
154	12M003	9	*Nassarius mutabilis*	S	Open Fishery	A	22	-	3
155	12M003	9	*Nassarius mutabilis*	S	Open Fishery	A	22	-	3
156	12M003	9	*Nassarius mutabilis*	S	Open Fishery	A	22	-	3
157	12M003	9	*Nassarius mutabilis*	S	Open Fishery	A	22	-	3
158	12M003	9	*Nassarius mutabilis*	S	Open Fishery	A	22	-	3
159	-	10	*Haustellum* spp.	-	-	-	21	18	5
160	-	10	*Haustellum* spp.	-	-	-	21	18	5

Figure S1: Neighbor-joining phylogenetic trees constructed individually for the four genes included in the MLSA analysis using strains of 2007: *gyrB* (A), *pyrH* (B), *recA* (C), *atpA* (D) and expanded tree of 4 genes concatenated (E). Bootstrap values above 80% are indicated.

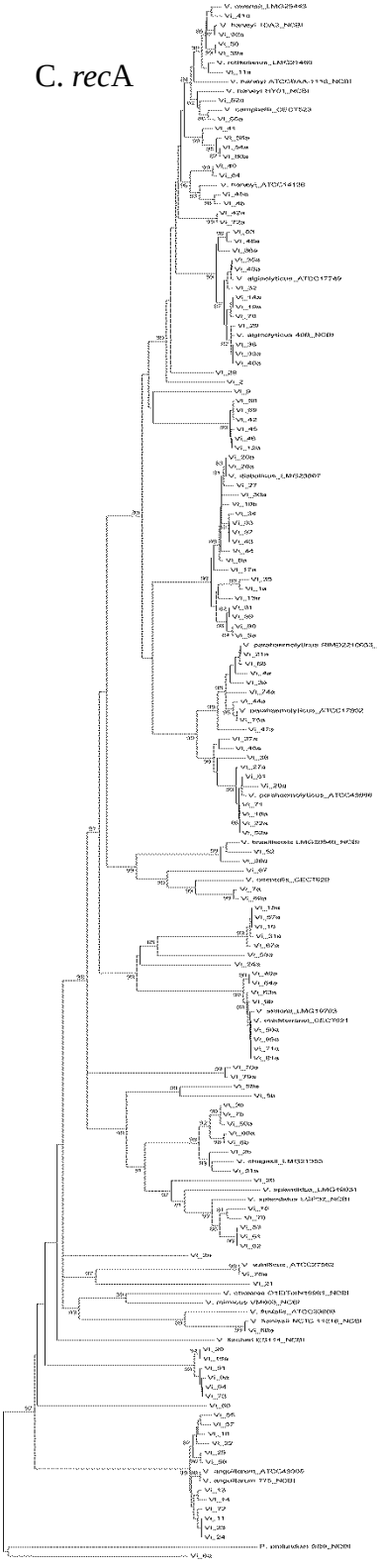
A. *gyr*B



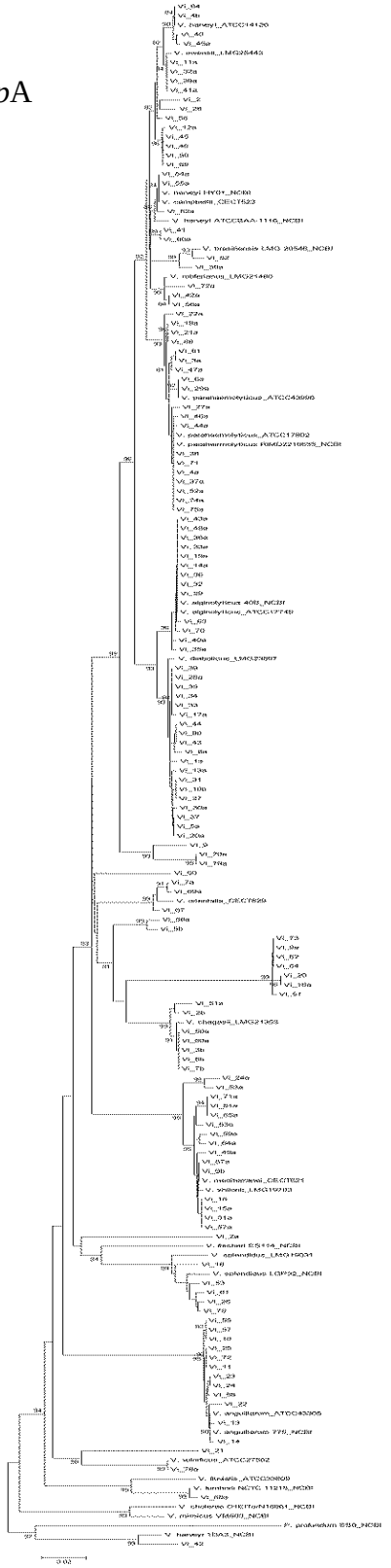
B. *pyr*H



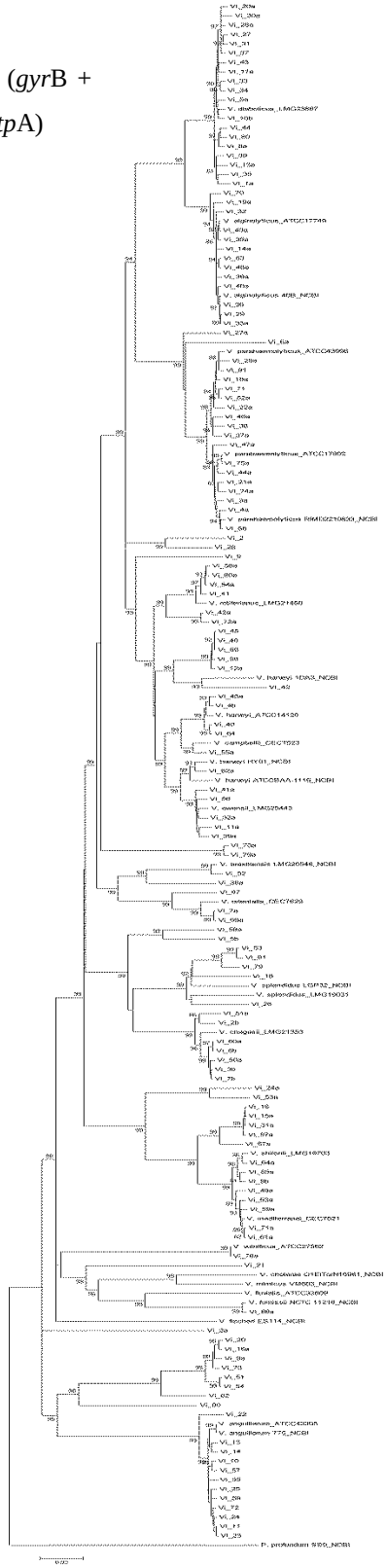
C. *rec*A



D. *atp*A



E. Concatenated (*gyr*B + *pyr*H + *rec*A + *atp*A)



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