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Molecular epidemiology of Vibrio nigripulchritudo, a pathogen of cultured penaeid shrimp (Litopenaeus stylirostris) in New Caledonia

Cyrille Goarant[a,], Yann Reynaud[a, b], Dominique Ansquer[a], Sophie de Decker[a], Denis Saulnier[b] and Frédérique le Roux[b]

[a]IFREMER, Département Aquaculture en Nouvelle-Calédonie, BP 2059, 98846 Nouméa cedex, Nouvelle-Calédonie
[b]IFREMER, Laboratoire de Génétique et Pathologie BP 133, 17390 La Tremblade, France

Corresponding author : Cyrille.Goarant@ifremer.fr

Abstract:

A collection of 57 isolates of *Vibrio nigripulchritudo* from either diseased or healthy shrimp and from shrimp farms environment was studied in order to gain a better understanding of the epidemiology of this pathogen, notably isolated from two distinct shrimp disease complexes. Molecular typing using two different techniques, arbitrarily primed PCR (AP-PCR) and multi-locus sequence typing (MLST), studied together with experimental pathology data allowed a relevant epidemiological insight into this possibly emerging pathogen. Additionally, results obtained with the two molecular typing techniques were congruent and allowed discriminating the strains associated with the "Summer Syndrome" from strains isolated from other contexts, especially the other shrimp vibriosis "Syndrome 93". These results highlight that the "Summer Syndrome" is most probably caused by an emergent clonal pathogen that therefore deserves surveillance and that AP-PCR can satisfactorily be used for that purpose

Keywords: Vibriosis; Mariculture; AP-PCR; MLST; Typing; Epidemiology; Virulence

INTRODUCTION

Shrimp aquaculture has been constantly increasing over the last decades, providing now half of the world shrimp supplies [30]. However, this rapid increase in culture has often been impeded by very severe epizootics [19, 34]. Worldwide, viruses are major problems, being responsible for the most spectacular losses among shrimp [23, 33]. However, vibriosis is also an important disease among penaeid shrimp [3, 21, 22, 26, 36], but this has yet only received little research attention. Therefore, limited knowledge has been gained on these pathologies and little is known concerning the epidemiology of *Vibrio* spp that are pathogenic to farmed shrimp.

New Caledonia (a 450 x 50 km wide island in the South Pacific between 19°S and 23°S), is a small producer regarding world global trade. However this industry has gained considerable economic importance in the last three decades, now representing the second major export sector of the country. Actually, the shrimp farming industry has major advantages, namely a tropical oceanic climate, a domesticated Litopenaeus stylirostris line reared in closed cycle for almost 25 years, an almost virus-free status, IHHNV (infectious hypodermal and hematopoietic necrosis virus) being the only known virus present and the domestic stock of L. stylirostris being resistant to it [40]. Yet this industry is based on a unique domesticated but non-indigenous species, L. stylirostris. So the emergence of an infectious disease would threaten its profitability and sustainability. In such a context, it is of prime importance to detect the emergence of pathogenic infectious agents as early as possible and to understand the mechanisms of infection in order to be able to control the disease.

Actually, New Caledonian shrimp farming has been affected since 1993 by a cool season vibriosis causing high mortalities in juvenile shrimp reared in earthen ponds, that was shown to be caused by Vibrio penaeicida and was named Syndrome 93 [6, 12, 25, 31]. During Syndrome 93 epizootics, a few other Vibrio strains could be isolated from moribund shrimp septicemic hemocultures, including V. nigripulchritudo [6] that demonstrated to be highly pathogenic by experimental infection in healthy L. stylirostris [15]. These pathogenic strains were, at that time, geographically restricted to two adjoining farms within one bay [14] and therefore, zoosanitary recommendations were given in order to minimize the risk of disease spread. The industry's strategy has since been to avoid winter crops, thus limiting the impact of Syndrome 93. However V. nigripulchritudo was yet isolated in late December 1997 from moribund shrimp obtained from two ponds at one farm located 50 km south of the original isolation. The organism was also causing an epizootic due to systemic vibriosis [11], but in high water temperature conditions that did not fit the classical Syndrome 93 epidemiology, which typically occurs at lower water temperatures [13, 25]. This new disease, which was named "Summer Syndrome" has affected all summer crops on this farm ever since. Since 2002, it also affected a new farm built in the close proximity of the affected one. If it was to affect all shrimp farms, the profitability of the industry would be seriously threatened.

The facts that (i) V. nigripulchritudo is associated with two distinct shrimp diseases and (ii) that one of these ("Summer Syndrome") is possibly an emergent disease highlight the need for accurate epidemiological data to gain appropriate knowledge and to propose adequate sanitary surveillance. Phenotypic identification of V. nigripulchritudo is easily conducted by traditional methods, but lacks a sufficient discriminating power for epidemiological studies. Therefore, the genetic structure of a New Caledonian V. nigripulchritudo collection has been studied using two molecular typing methods over a selection of 58 V. nigripulchritudo strains. The two methods used were Multi Locus Sequencing Typing, MLST, [24] and Arbitrarily Primed PCR, AP-PCR, [39]. Results were analysed together with virulence patterns of the strains that had been previously determined [15]. Here, we describe the results of this study, compare the two molecular typing techniques used, and propose a surveillance scheme of the potential emergence of this new pathogen for the shrimp aquaculture industry of New Caledonia.

MATERIALS AND METHODS

Bacterial strains and geographical data

Both the type strain and wild-type isolates of Vibrio nigripulchritudo were used in this study. V. nigripulchritudo CIP 103192^T (= ATCC 27043) was provided by Collection de l'Institut Pasteur. Fifty seven wild-type isolates, from both clinical and environmental origins, isolated between May 1995 and November 2003 in 13 different farms and 3 hatcheries along almost 300 kilometers of the New Caledonian West Coast were included in this study (Table 1). The origin, identification and virulence of these V. nigripulchritudo isolates towards healthy L. stylirostris were determined previously [6, 14, 15], (see table 1).

The shrimp farms and hatcheries included in the study are located on the southwest coast of New Caledonia (Fig. 1).

Clinical V. nigripulchritudo isolates originated from septicemic hemocultures associated with Syndrome 93 (= cool season vibriosis; 3 strains), Summer Syndrome (7 strains), and opportunistic vibriosis, *i.e*. affecting only a few shrimp under adverse pond conditions and lasting only a limited time (9 strains). Other strains were isolated when found (one or very few colonies) in hemocultures from either healthy shrimp (24 isolates) or moribund shrimp dying from a non bacterial cause (4 strains). Environmental isolates were isolated from pumping water in a Summer Syndrome affected farm (2 isolates), pond water or sediment pore water in the two Summer Syndrome affected farms (7 strains) and from a healthy crab (Portunus pelagicus) carapace swab (1 isolate). Identification to the species level was achieved on the basis of phenotyping tests and specific PCR confirmation as described elsewhere [15]. Additionally, V. penaeicida strain AM101 [6, 13, 14], isolated in a Syndrome 93 context was included in this study, as an outgroup for phylogenetic analysis.

Out of these 59 strains, 25 were used for the MLST approach, including V. nigripulchritudo type strain and V. penaeicida AM101 as an outgroup (see Table 1). All of these 25 strains are deposited in the bacterial collection of the CRB (Centre de Ressources Biologiques, Laboratoire de Génétique et Pathologie, Institut Français de Recherche pour l'Exploitation de la MER [IFREMER], La Tremblade, France).

Culture conditions and extraction of bacterial genomic DNAs

Vibrio strains stored frozen at -80°C in Marine Broth 2216E (Difco) with 17% glycerol were cultured in accordance with standard procedures [1] i.e. grown in Marine Broth 2216E (Difco) at 30°C with continuous shaking until the stationary phase of growth was reached, i.e. usually 18 h. DNAs were extracted and purified as described elsewhere [18].

AP-PCR

Fingerprinting was performed as described previously [14]. Primers RSP and SP (see Table 2) were purchased from Proligo (Singapore). Fifty-microliter reaction mixtures were prepared with 100 ng of DNA–1 X *Taq* polymerase buffer–$MgCl_2$ 4 mM–0.2 mM each deoxynucleoside triphosphate (Sigma laboratories)–1.25 U of DNA polymerase (Qiagen). Amplification reactions were cycled twice in a 60-well Gene-Amp 9700 thermocycler (Applied BioSystems) through a low-stringency temperature profile and then 40 times through a high-stringency temperature profile as previously described [29]. Ten microliters of each reaction mixture was combined with 2 µl loading buffer and loaded onto a 2% agarose gel with 1X TBE (90 mM Tris-borate, 2 mM EDTA), and electrophoresis was performed at 40 V overnight. After ethidium bromide staining, the gel was then photographed under UV trans-illumination. In accordance with Welsh and McClelland [39], only major bands were considered in the analysis as share-derived characters, and this allowed the construction of a key for type grouping of the strains according to the amplicons produced with a given primer.

Major bands were considered as present (1) or absent (0) in each fingerprint. All major bands for both primers were put together in a single analysis. The resulting matrix was analyzed using the PopGene 16 freeware [41] to build a dendrogram based on Nei's genetic distances [27].

Multi Locus Sequence Typing

Six genes were considered for the MLST approach: *16S rDNA*, *gyrB*, *rpoD*, *rctB*, *toxR* and *recA* [20, 37]. Primers sequences and annealing temperatures according to gene are given in Table 2. The amplicons with the expected size were purified using a Qiaex II Gel Extraction Kit (Qiagen). PCR product were then denatured for 2 min at 96°C and mixed (final volume 10 µl) with 0.4 µl ABI Prism Big Dye Terminator ready reaction mix (Applied Biosystems), forward or reverse primer 0.6µM and buffer 5x. Cycle sequencing reaction was performed using a Gene Amp PCR System 2700 (Applied Biosystem), following instruction of the manufacturer. Separation of the DNA fragments was carried out in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Concatemers of sequences were aligned with BioEdit® [16], phylogenetic trees were built using Phylo-win program [9] applied to Neighbour Joining method and Kimura's 2-parameter distances [10]. Reliability of topologies was assessed by the bootstrap method with 1000 replicates.

RESULTS

AP-PCR typing:

Both primers permitted the amplification of all tested DNA's, giving complex profiles with up to 18 different bands for each strain, over a total of 28 and 26 different amplicon sizes for primers SP and RSP respectively over the V. nigripulchritudo selection. Each strain gave a unique reproducible profile when using the correct amount of genomic DNA for the amplification using the same thermocycler. The complex fingerprint profiles permitted an intra-specific discrimination between V. nigripulchritudo strains (Fig. 2, as an example). When considering only major bands, each fingerprint was scored with 6 to 12 bands out of 16 for each primer. In order to characterize the outgroup (V. penaeicida strain AM101) 6 additional bands were necessary for primer SP and 8 for primer RSP. The analysis, thus relying on a matrix of presence/absence of 46 bands for 59 bacterial strains resulted in constructing a dendrogram, which is presented in figure 3.

V. nigripulchritudo wild type isolates could be grouped into 4 major clusters (A, B, C and D, see Fig. 3), out of which 2 clusters (A and B) grouped all virulent isolates. Though sub-clusters could be considered in these two clusters, they evidenced a low genetic diversity compared to clusters C and D that contained only non-virulent isolates.

Multi Locus Sequence Typing:

A total of 3,430 bp corresponding to partial sequences of the 6 genes *recA*, *rpoD*, *toxR*, 16S rDNA, *gyrB* et *rctB* were aligned. This permitted the construction of a concatemer phylogenetic tree by Neighbour joining Method and Kimura's 2-parameter distances (Fig. 4). The concatemer resulted in at least 97% overall homology between nucleotidic sequences within the 24 V. nigripulchritudo strains studied. The most polymorphic gene proved to be *gyrB* with 95% gene sequence similarity, then 96% for *recA*, 97% for *toxR*, 98% for *rpoD* and *rctB* and 99% for 16S rDNA. Regarding genetic structuring of the sub-selection of strains, one cluster with very low genetic variability groups only strains being virulent to L. stylirostris (cluster A'), another cluster, with higher genetic variability whatever gene being considered, groups virulent together with avirulent strains (cluster B'). Lastly, all other non-virulent strains belong to none of these two main clusters.

Genospecies identification:

The sequence similarities obtained in 16S rDNA being all above 99% confirm that all of the 24 strains studied in MLST belong to the species *V. nigripulchritudo*, considering Stackebrandt and Embley [35] who advise a level of intraspecific similarity of ≥98% in 16S rDNA gene sequences. Because at least one strain from each AP-PCR cluster was used for the MLST approach and therefore its 16S rDNA gene sequenced, it confirmed the belonging of these isolates to the species *V. nigripulchritudo* [14, 15].

DISCUSSION

Both techniques permitted discriminations and groupings between isolates. MLST and AP-PCR are very powerful DNA typing tool, which have shown a high degree of intraspecies discriminatory power for bacterial pathogens [14, 28, 29, 38]. The overwhelming advantage of MLST is that sequence data are portable and cumulative between laboratories allowing to exchange data for multi-laboratory epidemiological studies. Nevertheless this technique is time consuming and expensive. AP-PCR is easier to develop, requires no prior knowledge of the sequences of the organism to be studied and is an easy and rapid technique. However, it has been shown to present a lack of reproducibility depending on laboratory equipments and practices [2, 4, 5, 8].
Interestingly, the clusters that were evidenced by either technique were consistent. This provides an inter-validation of the results, one technique being based on the sequence analysis of major house-hold genes while the other technique relies on a global examination of the complete genome. Therefore, the epidemiological interpretation can be considered as relying on very solid genomic basis. Thus the AP-PCR, because being easily implemented and thanks to this "inter-validation" with MLST results, could be a reliable tool for molecular epidemiological studies of both shrimp diseases caused by *V. nigripulchritudo* in New Caledonia.

Actually, this study gives very informative insights into the epidemiology of V. nigripulchritudo in New Caledonian shrimp culture. One cluster (B) groups all Syndrome 93 isolates, from one single geographical origin [14] together with strains from an opportunistic vibriosis outbreak that occurred in a farm from the same bay during the summer 2002-2003. This demonstrates that this cluster, though being originally involved in winter mortalities (Syndrome 93) can also possibly be involved in opportunistic outbreaks during summer months.
The major cluster (A) includes 20 wild type isolates and puts together all Summer Syndrome isolates from diseased animals, from both affected farms, whatever their date of isolation, over more than 3 years covering 4 culture cycles. Together with these strains, a few environmental isolates from the affected farms (sediment pore water or pond water) demonstrate that they may be environmentally-transmitted. This evidences that the Summer Syndrome is most probably attributable to a single pathogenic clone, surviving from one year to the next in the shrimp farm environment then re-developing inside the grow out system at the next crop. Also within this cluster, other isolates originate from opportunistic mortality outbreaks from other farms or hatcheries or from healthy juvenile shrimp from both Summer Syndrome-affected and non-affected farms all along the West coast of New Caledonia. This not only highlights the major role of environmental or zootechnical factors in the dynamics of the Summer Syndrome in the affected farms but also demonstrates that this pathogenic V. nigripulchritudo cluster already has a wide geographical distribution. Therefore, it can be assumed that the emergence of Summer Syndrome does not correspond to the geographical extension of pathogenic V. nigripulchritudo strains associated with Syndrome 93, but merely corresponds to the selection of another pathogenic clone within a shrimp farm environment.

The selection of bacterial strains used in this study was composed of a single strain conserved for each individual shrimp, this isolate being considered representative of clonal V. nigripulchritudo being present in the shrimp hemolymph, except in one moribund shrimp (2 strains Wn13 and Wn14) and one healthy carrier shrimp (3 strains AgMn1, AgMn2 and AgMn3) [15]. So it was considered that one infected shrimp would carry V. nigripulchritudo cells from a single clonal origin. However, it was demonstrated that other marine invertebrates, namely oysters, could carry non-clonal (both human-pathogenic and non-pathogenic) V. parahaemolyticus cells at the same time [7]. Even though within the few strains originating from the same animals that we included in this study, there was no evidence of multi-clonal carriage of V. nigripulchritudo in shrimp, further work could consider verifying our assumption, notably study if shrimp can carry both virulent and non-virulent strains at the same time.

When comparing the molecular typing with experimental pathology results, it must be noted that some strains genetically close to the Syndrome 93 isolates (cluster B) demonstrated no experimental virulence towards L. stylirostris, whereas strains from the "Summer Syndrome" cluster (A) all demonstrated virulence. Still, even within this latter cluster, various levels of virulence could probably be evidenced [15] with more discriminative experimental infection models [32].
Still, V. nigripulchritudo from both Syndrome 93 and Summer Syndrome clusters could be isolated from opportunistic vibriosis episodes or even from apparently healthy shrimp in non-diseased farms. This demonstrates that the disease is the result of complex interactions between the pathogen, the shrimp (host) and their environment. As a response, IFREMER (the French Institute for Marine Sciences) has developed a specific research program, named DeSanS (possibly translated as Stylirostris Health Challenge), that is based on a multidisciplinary approach (rearing technology, pond ecosystem studies, shrimp eco-physiology and immunology, nutrition, pathology and genetics). It aims at gaining a global explanation scheme of both seasonal vibriosis as a contribution to strengthen the sustainability of New Caledonian aquaculture [17]. Summer syndrome being currently a research priority within DeSanS, a representative strain of this disease was chosen in order to study virulence determinants pathogenesis and host damages. Comparatively with a non-virulent isolate, these strains are being studied for their production of extracellular toxic products, infection capacity and shrimp response. Lastly, a genomic subtraction using Suppressive Subtractive Hybridization is currently in progress.

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Table 1: Vibrio nigripulchritudo type strain and field isolates used in this study

Bold: Isolates from farms which are affected by the Summer Syndrome.

Italics: Isolates collected during surveys specifically dedicated to the isolation of V. nigripulchritudo strains.

Virulence as determined in text: V stands for virulent and N for Non-virulent.

The names of wild type isolates refer to the initials of the grow out farms and hatcheries as mentioned on the map in Fig. 1.

* Virulence data from Goarant *et al*. [15]

Strain name	*Date of isolation*	context	Virulence to *L. stylirostris* *	MLST	Reference
CIP 103195^T	*V. nigripulchritudo* type strain		N	X	
AM102	May 1995	S93, moribund shrimp hemoculture	V	X	[6 ; 14]
AM 115	May 1995	S93, moribund shrimp hemoculture	V	X	[6 ; 14]
SO65	May 1995	S93, moribund shrimp hemoculture	V	X	[6 ; 14]
SFn1	March 2000	Summer syndrome, moribund shrimp hemoculture	V	X	[15]
SFn2	March 2000	Summer syndrome, moribund shrimp hemoculture	V		[15]
SFn27	December 2000	Sediment pore water, diseased pond	V	X	[15]
SFn48	November 2001	Summer syndrome, moribund shrimp hemoculture	V		[15]
SFn49	November 2001	Growout pond water, diseased pond	V		[15]
SFn105	February 2002	Growout pond water, diseased pond	V		[15]
SFn106	February 2002	Summer syndrome, moribund shrimp hemoculture	V		[15]
SFn111	March 2002	Carapace of a healthy crab (*Portunus pelagicus*), diseased farm			[15]
SFn115	March 2002	Lagoon water in front of pumps, diseased farm		X	[15]
SFn118	April 2002	Lagoon water in front of pumps, diseased farm	N	X	[15]
SFn127	November 2002	Healthy shrimp hemoculture, before disease outbreak	V		[15]
SFn128	December 2002	Summer syndrome, moribund shrimp hemoculture	V		[15]
SFn135	December 2002	Growout pond water, diseased pond	V	X	[15]
AgMn1	October 2003	Healthy shrimp hemoculture, before disease outbreak (same animal)	N	X	[15]
AgMn2			N		[15]
AgMn3			N		[15]
AgMn7	November 2003	Healthy shrimp hemoculture, before disease outbreak	V		[15]
AgMn8	November 2003	Summer syndrome, moribund shrimp hemoculture	V		[15]
AgMn9	November 2003	Growout pond water, diseased pond	V		[15]
AgMn10	November 2003	Summer syndrome, moribund shrimp hemoculture	V		[15]
AgMn12	November 2003	Sediment pore water, diseased pond	V		[15]

AgMn13	November 2003	Sediment pore water, diseased pond	V		[15]
POn2	April 2002	Healthy shrimp hemoculture, healthy pond 2, healthy farm	V		[15]
POn3	April 2002	Healthy shrimp hemoculture, healthy pond 3, same healthy farm	V	X	[15]
POn4	April 2002	Healthy shrimp hemoculture, healthy pond 6, same healthy farm	N	X	[15]
POn10	December 2002	Moribund shrimp hemoculture, no vibriosis, healthy pond 5, same healthy farm	N		[15]
POn12	December 2002	Healthy shrimp hemoculture, healthy pond 4, same healthy farm	N	X	[15]
POn13	December 2002	Healthy shrimp hemoculture, same healthy pond 4, same healthy farm	N	X	[15]
POn19	January 2003	Healthy shrimp hemoculture, same healthy pond 4, same healthy farm	V	X	[15]
AMn3	March 2003	Moribund shrimp hemoculture, opportunistic vibriosis	V	X	[15]
AMn4	March 2003	Moribund shrimp hemoculture, opportunistic vibriosis, same episode	V		[15]
SOn1	July 2001	Moribund shrimp hemoculture, no vibriosis	N		[15]
SOn2	March 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	N		[15]
FTn1	January 2001	Moribund shrimp hemoculture, no vibriosis	N		[15]
FTn2	March 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V		[15]
SBn2	March 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	N		[15]
Wn1	January 2001	Moribund shrimp hemoculture, opportunistic vibriosis	V	X	[15]
Wn3	January 2001	Moribund shrimp hemoculture, opportunistic vibriosis	V	X	[15]
Wn13	November 2002	Moribund shrimp hemoculture, opportunistic vibriosis (same animal)	V	X	[15]
Wn14			V		[15]
BDn1	February 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V		[15]
BDn2	February 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V		[15]
Fn1	February 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V		[15]
Fn2	February 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	N	X	[15]
AQn1	March 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V	X	[15]
AQn2	March 2003	Healthy shrimp hemoculture, healthy pond, healthy farm	V		[15]
MT1	January 2000	Moribund shrimp hemoculture, opportunistic vibriosis, broodstock	V		[15]
BLFn1	March 2001	Moribund shrimp hemoculture, opportunistic vibriosis	V	X	[15]
BLFn2	December	Moribund shrimp hemoculture,	V		[15]

	2001	opportunistic vibriosis			
ENn1	June 2000	Healthy shrimp hemoculture, healthy broodstock	N		[15]
ENn2	June 2000	Healthy shrimp hemoculture, healthy broodstock	V	X	[15]
SVn2	April 2002	Moribund shrimp hemoculture, no vibriosis	N		[15]
SVn3	February 2003	Healthy shrimp hemoculture, healthy farm	N	X	[15]
ESn2	June 2000	Healthy shrimp hemoculture, healthy broodstock	N		[15]
V. penaeicida AM 101	May 1995	Moribund shrimp hemoculture, Syndrome 93	V	X	[15]

Table 2: Nucleotidic sequences of the primers.
For MLST, the sequence of the primers used in sequencing reaction is indicated in italic.
The amplified gene and the annealing temperature are also indicated for each pair of primers.

gene	Primers sequence	Annealing T°C
rrs	Forward 5'-*CAGGAAACAGCTATGACC*AGAGTTTGATCATGGCTCAGA-3' Reverse 5'-*GTAAAACGACGGCCAG*GTTACCTTGTTACGACTT-3'	50
gyrB	F 5'-*GAAGTCATCATGACCGTTCTGCA*YGCNGGNGGNAARTTYRA-3' R 5'-*AGCAGGGTACGGATGTGCGAGCC*RTCNACRTCNGCRTCNGYCAT-3'	58
rpoD	F 5'-*ACGACTGACCCGGTACGCATGTA*YATGMGNGARATGGGNACNGT-3' R 5'-*ATAGAAATAACCAGACGTAAGTT*NGCYTCNACCATYTCYTTYTT-3'	57
rctB	F 5'-*CAGGAAACAGCTATGACC*ATHGARTTYACNGAYTTYCARYTNCAY- 3' R 5'-*GTAAAACGACGGCCA*YTTNCTYTGHATNGGYTCRAAYTCNCCRT -3'	57
toxR	F 5'-*CAGGAAACAGCTATGACC*GANCARGGNTTYGARGTNGAYGAYTC-3' R 5'-*GTAAAACGACGGCCA* GTTDKKTTGNCCNCYNGTVGCDATNAC3'	57
recA	F 5'-TGGACGAGAATAAACAGAAGGC-3' R 5'-CCGTTATGCTGTACCAAGCGCCC-3'	55
	AP-PCR RSP 5'-GGAAACAGCTATGACCATGA-3'	
	AP-PCR SP 5'-TTGTAAAACGACGGCCAG-3'	

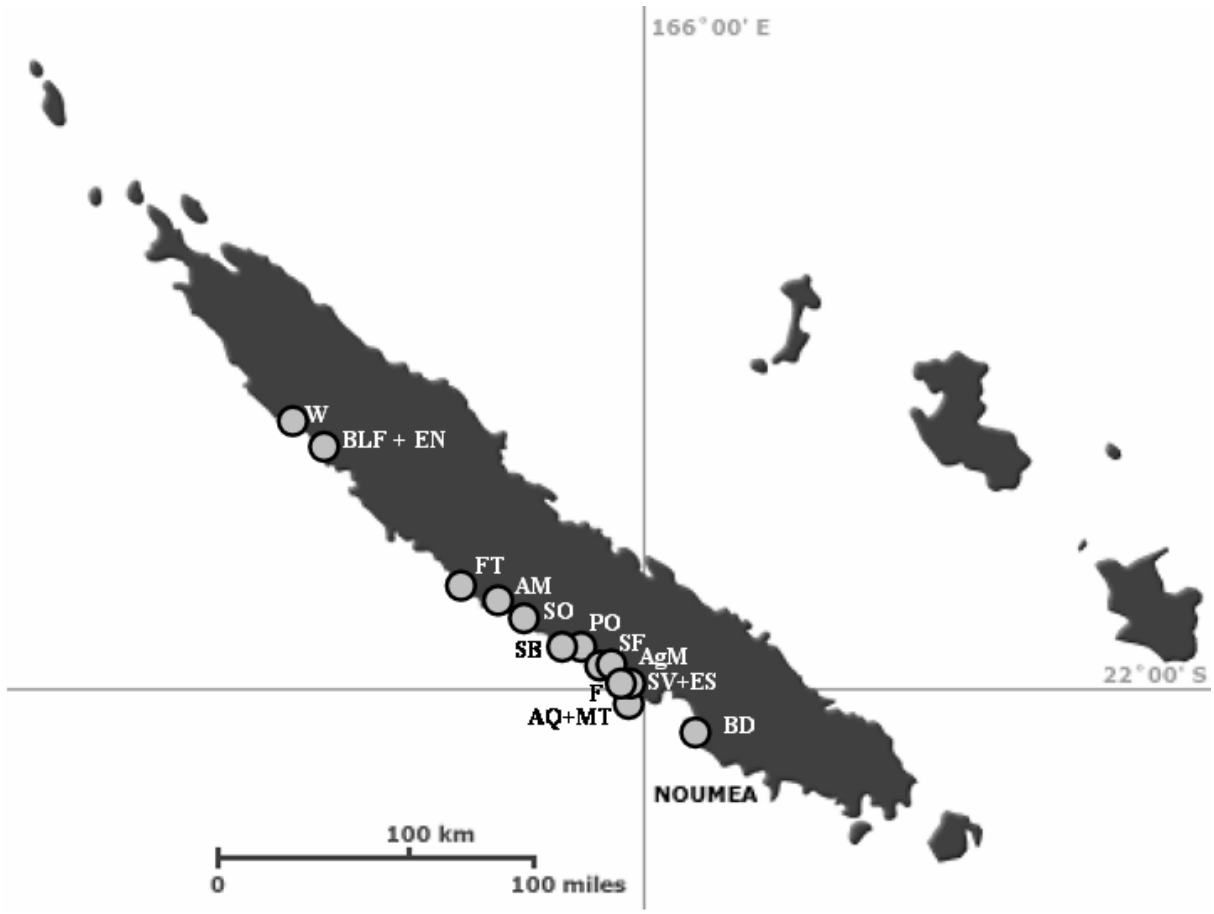

Figure 1: Map of New Caledonian shrimp farms and hatcheries mentioned in this study. Initials ES, EN and MT refer to hatcheries, whereas all the others refer to grow out farms.

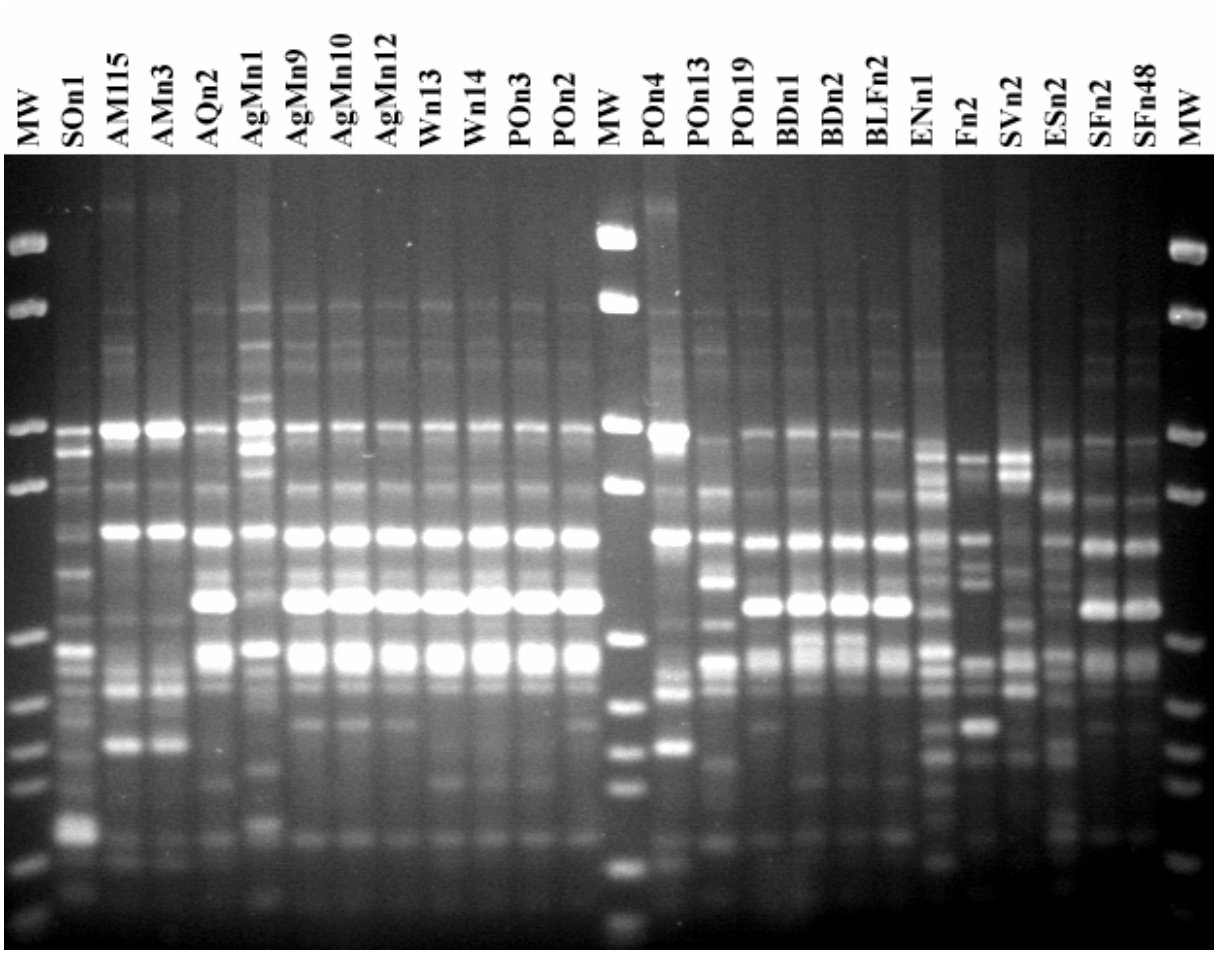

Figure 2: AP-PCR fingerprints obtained with primer SP from a few <u>Vibrio nigripulchritudo</u> wild type isolates.

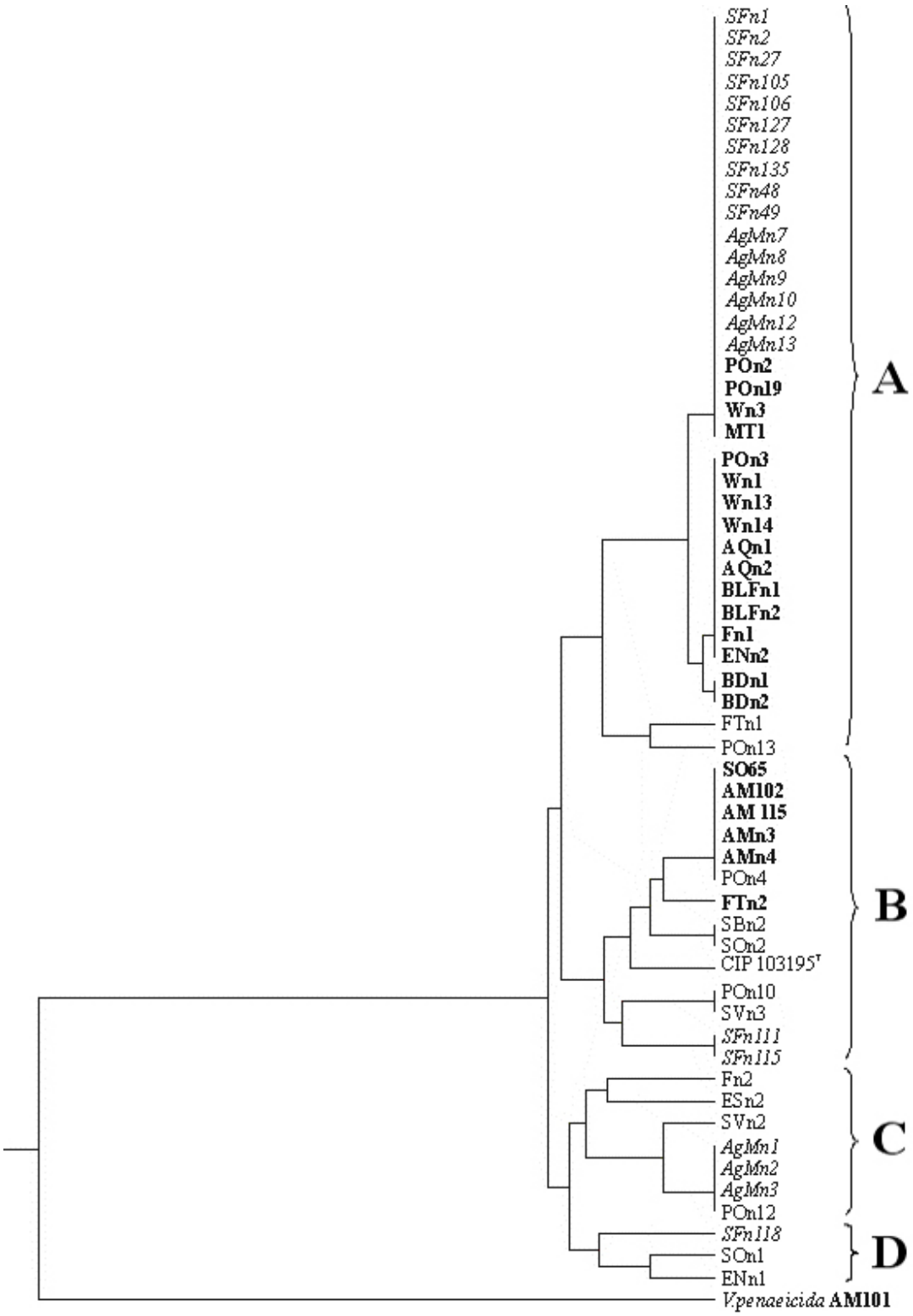

Figure 3: Dendrogram drawn from the presence / absence of major amplicons in each individual AP-PCR fingerprints obtained with primer RSP and SP.

Bold: Virulent isolates (as determined experimentally, see text).

Italics: Isolates collected from farms which are affected by the Summer Syndrome.

Cluster names as mentioned in the text.

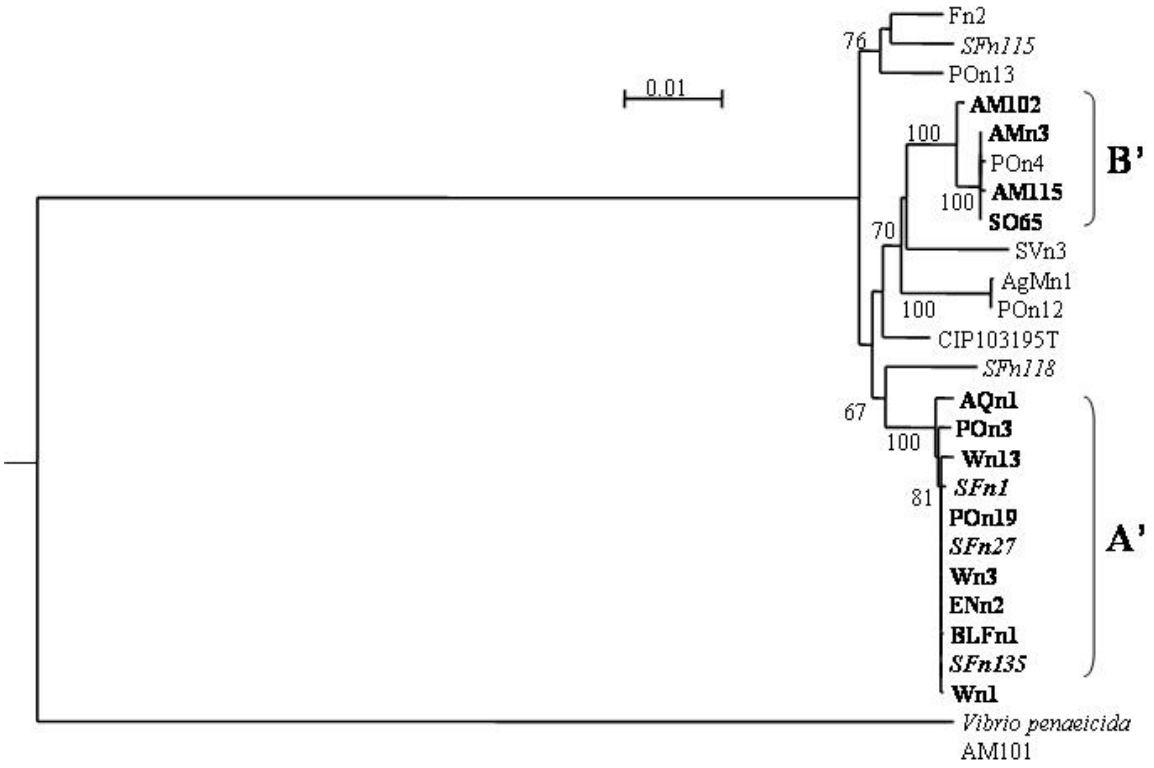

Figure 4: Dendrogram constructed from the alignment of 3430 bp sequences of the six genes *recA*, *rpoD*, *toxR*, 16S rDNA, *gyrB* and *rctB* over a sub-selection of the strains.

Cluster names as mentioned in the text.