

# Phylogenetic analysis and serotyping of *Vibrio splendidus*-related bacteria isolated from salmon farm cleaner fish

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**ABSTRACT:** Cleaner fish, i.e. various wrasse (Labridae) species and lumpsucker *Cyclopterus lumpus*, are to an increasing extent used for biocontrol of the salmon louse *Lepeophtheirus salmonis* in European salmon farming. Although efficient de-licers, cleaner fish mortality levels in salmon farms are often high. Bacterial infections are common, and *Vibrio splendidus*-related strains are frequently identified during diagnostic investigations. The population structure of 112 *V. splendidus*-related isolates, derived primarily from wrasse species, was investigated by means of multi-locus sequence analysis using 5 housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*). Most isolates were found to be closely related to the *V. splendidus* type strain, yet displayed extensive genetic microdiversity. Slide agglutination testing using polyclonal rabbit antisera further indicated O-antigen variability. Intra-outbreak genetic and antigenic diversity suggests direct infection from seawater, rather than fish-to-fish transmission, as the main route of infection. The variable nature of isolates involved complicates qualified selection of representative candidate strains, e.g. for infection and vaccine trials.

**KEY WORDS:** *Vibrio splendidus* · Molecular characterisation · Multilocus sequence analysis · MLSA · Serotyping · Cleaner fish · Biocontrol · Salmon louse

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## INTRODUCTION

The use of cleaner fish for biocontrol of the salmon louse *Lepeophtheirus salmonis* has become increasingly popular in European salmon farming in recent years. In Norway, wild-caught wrasse (goltsinny *Ctenolabrus rupestris*, ballan *Labrus bergylta* and corkwing *Symphodus melops*) make up the bulk of cleaner fish used, with over 15 million captured annually for this purpose (Norwegian Directorate of Fisheries, [www.fiskeridir.no](http://www.fiskeridir.no)). Both lumpsucker *Cyclopterus lumpus* and ballan wrasse are now being farmed to meet the growing demand. However, considerable health and welfare problems are related to

the use of cleaner fish, with high mortality levels amongst both wild-caught and farmed fish following transfer to salmon cages. Over a period of 6 mo, a recent study registered 33% cumulative mortality of cleaner fish, a figure which was almost certainly an underestimate (Nilsen et al. 2014). This leads to a significant turnover of cleaner fish in salmon farms.

The causes of cleaner fish losses are often unclear, but bacterial infections are amongst the most common diagnostic findings in Norway (Nilsen et al. 2014, Bornø & Lie Linaker 2015). Recognised bacterial fish pathogens regularly found include atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *V. ordalii*, *Pseudomonas anguilliseptica* and a recently

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described *Pasteurella* sp. (Poppe et al. 2012, Alarcón et al. 2015, Bornø & Lie Linaker 2015, Gulla et al. 2015), although perhaps the most frequently isolated bacteria from such investigations are *V. splendidus*-related strains (Johansen 2013, Hjeltnes 2014, Bornø & Lie Linaker 2015).

Bacterial diseases depend not only on the virulence of the bacterium involved, but also on the environment and the immunological status of the host. In this regard, the pathogenic role (if any) of *V. splendidus* in relation to disease in wrasse and lumpsucker remains poorly understood. Although some strains have previously been associated with disease in marine animals, including wrasse (Bergh & Samuelsen 2007), the term '*V. splendidus*' is often (imprecisely) used with reference to a range of phenotypically (Thompson et al. 2004), genetically (Thompson et al. 2005) and antigenically (Wildschutte et al. 2010) diverse bacteria, commonly dominating the marine bacterioplankton (Le Roux & Austin 2006). In recent years, several *V. splendidus*-related taxa have been validly designated as distinct species, and this complex collection of strains/species has collectively, at the sub-genus level, been named the Splendidus-clade (Sawabe et al. 2013).

Sustainable culture of many fish species is dependent on the development and use of effective vaccines against bacterial diseases. Similarly, comprehensive vaccination programs must presumably also be developed for cleaner fish if the industry is to remain sustainable. In order to shed light on the population structure of *V. splendidus*-related bacteria from fish, and hopefully identify suitable candidate strains for eventual vaccine development, we subjected a representative collection of *V. splendidus*-related isolates from cleaner fish to multilocus sequence analysis (MLSA). The scheme utilises 5 housekeeping genes previously used in phylogenetic studies of *Vibrio* spp. We also assessed O-antigen variability by slide agglutination testing with polyclonal antisera.

## MATERIALS AND METHODS

### Bacterial strains and culture

The studied isolates (112) were obtained primarily through the Norwegian Veterinary Institute's (NVI) diagnostic service between 2004 and 2014. A variety of host species were represented, although the majority of isolates were recovered from dead or moribund cleaner fish in Norwegian salmon farms, sampled during periods of increased mortality. Primary

cultures were mainly obtained by sowing from aseptically exposed head kidneys onto 5% bovine blood agar with 2% NaCl (BA2%), followed by incubation at 15°C for up to 1 wk. Phenotypic characterisation is often too indiscriminate/versatile to differentiate *Vibrio splendidus*-related species (Thompson et al. 2004). Unsurprisingly, therefore, investigated cleaner fish isolates differed to some extent from the *V. splendidus* biochemical profile offered by the Bergey's Manual (Garrity et al. 2005), and they were on this basis identified as *V. splendidus*-related (Table 1). The Splendidus-clade affiliation was confirmed for a random selection of isolates through partial 16S rRNA gene sequencing (see below). *V. splendidus* NCIMB1<sup>T</sup> and 14 *V. splendidus* reference

Table 1. Typical phenotypic profile of *V. splendidus*-related isolates subjected to biochemical assessment in the present study. This profile differs with regards to some parameters from the *V. splendidus* biochemical profile offered by the Bergey's Manual (Garrity et al. 2005). O/F: aerobic/anaerobic glucose fermentation; A/L/O: arginine, lysine and/or ornithine metabolism

<b>Culture conditions</b>		
Grows on 5% bovine blood agar with 2% NaCl within 2 d when incubated at 15°C		
<b>General morphology</b>		
1–4 mm, grey/beige, opaque, usually β-haemolytic, colonies, often with production of green diffusible pigment and a strong characteristic smell		
<b>Microscopy</b>		
Motile rods, often curved or pleomorphic, Gram-negative		
Biochemical test	Typical reaction	Isolates displaying reaction (%)
O/F	+/+	100
Vibriostat O/129	Sensitive <sup>a</sup>	97
A/L/O	-/-/-	84
Production of:		
Cytochrome c oxidase	+	100
Alginase	+	90
Gelatinase	+	95
Indole	+	94
Acid production from:		
Arabinose	-	100
Cellobiose	+	97
Lactose	-	100
Mannitol	+	99
Mannose	+	99
Sucrose	-	76
Trehalose	+	100
<sup>a</sup> Several isolates designated 'sensitive' displayed small inhibitory zones and/or colonies appearing within the zones		

strains were also acquired for investigation. Stock cultures (maintained at  $-80^{\circ}\text{C}$ ) were sub-cultured on BA2% at  $15^{\circ}\text{C}$  for 24 to 48 h prior to DNA extraction. MLSA sequence data for an additional 9 strains were retrieved from GenBank. For details on all isolates/strains, see Table S1 in the Supplement at [www.int-res.com/articles/suppl/d117p121\\_supp.pdf](http://www.int-res.com/articles/suppl/d117p121_supp.pdf).

### DNA extraction, PCR and sequencing

Genomic DNA was obtained by boiling bacterial cells in  $\text{dH}_2\text{O}$  for 7 min, followed by centrifugation and use of the supernatant as PCR template. For some isolates, DNA was isolated using a QiaCube (Qiagen) according to the manufacturer's instructions. Partial sequences of the genes encoding RNA polymerase  $\sigma$ -factor (*rpoD*), cell division protein (*ftsZ*), uridylyate kinase (*pyrH*), RNA polymerase  $\alpha$ -subunit (*rpoA*) and  $\alpha$ -subunit of bacterial ATP synthase (*atpA*) were amplified using the primers specified in Table 2. Each PCR reaction volume consisted of  $2.5\ \mu\text{l}$   $10\times$  ThermoPol Reaction Buffer (New England BioLabs),  $0.2\ \text{mM}$  dNTP (VWR),  $0.4\ \mu\text{M}$  of both forward and reverse primers (Invitrogen), 1 unit *Taq* DNA polymerase (New England BioLabs),  $3\ \mu\text{l}$  (boil-extracted) or  $1\ \mu\text{l}$  (QiaCube-extracted) DNA template and a final addition of Milli-Q water to reach a total reaction volume of  $25\ \mu\text{l}$ . PCR was conducted on a Dyad Dual 96-Well Thermal Cycler (MJ Research) under conditions specified in Table 2. From selected isolates (Table S1), approximately the first third of the 16S rRNA gene (442 bp) was obtained as described by Suau et al. (1999). Following visual confirmation of PCR products of the ex-

pected size by gel electrophoresis (1.5% agarose gel with GelRed staining) and purification (ExoSAP-IT; Amersham Biosciences), products were sequenced using BigDye Terminator v3.1 (protocol: Platt et al. 2007) and an Avant 3130xl Genetic Analyzer (Applied Biosystems).

### Sequence analysis

Contigs were assembled and manually corrected (Geneious v7.1; Biomatters), and nucleotide sequences trimmed and (for housekeeping genes) adjusted to reading frame (MEGA6; Tamura et al. 2013) prior to alignment (ClustalX; Larkin et al. 2007). The 5 partial sequences (trimmed: *rpoD* = 573–576 bp; *ftsZ* = 480 bp; *pyrH* = 456 bp; *rpoA* = 492 bp; *atpA* = 639 bp) were examined individually and as a concatenated sequence (MLSA = 2640–2643 bp) in synteny with chromosome 1 of *V. tasmaniensis* LGP 32 (GenBank acc. no. FM954972). Upon identification of identical concatenated sequence types in multiple isolates from individual clinical cases (presumably clones), only single representatives from each case were included for further analysis. Eventual selection pressures operating on the housekeeping genes were evaluated with the Codon-based Z-test in MEGA6. The scope was set to 'overall average', and an alternative hypothesis of purifying selection—substitution rate at nonsynonymous sites ( $d_N$ ) < substitution rate at synonymous sites ( $d_S$ )—was employed with otherwise default settings. Maximum likelihood (ML) trees were constructed using PhyML v3.0 (Guindon et al. 2010) with default settings, except for the 'proportion of invari-

Table 2. Primers designed and used for PCR and sequencing of partial housekeeping genes, with product sizes and PCR thermal cycle conditions listed

Gene	Primer		Product size (bp)		PCR conditions		
	Orient.	Sequence (5'–3')	Pre-trim	Post-trim	Initial denat.	Amplific.	Final ext.
<i>rpoD</i>	Forward	TATTGCGAAACGCATTGAAG	744–747	573–576	95°C, 3 min;	30× [95°C, 1 min; 68°C, 4 min; 51°C, 1 min; 68°C, 1 min]	4°C, ∞
	Reverse	CCGATAGACATWCGACGGCT					
<i>ftsZ</i>	Forward	CAGYGTRATYCAAATTGGTGG	522	480			
	Reverse	ATRCSGCTRCCCATTGTC					
<i>pyrH</i>	Forward	GAAGCDCTWCAAGGYGAWGAAG	502	456			
	Reverse	TATCATACARCTCTGCRTCWG					
<i>rpoA</i>	Forward	GTAACTCTTGAGCCATTAGGC	545	492	95°C, 3 min;	30× [95°C, 1 min; 68°C, 4 min; 53°C, 1 min; 68°C, 1 min]	4°C, ∞
	Reverse	TCCATATCGATAACAAGCTTGTC					
<i>atpA</i>	Forward	GTTGGTGCTGTTGTAATGGGC	683	639			
	Reverse	AGTAGACGAGAGTGAAGGTAG					

able sites' (changed to estimated) and 'number of substitution rate categories' (raised to 8), as previously recommended (Hall 2007). Branch support was evaluated with the approximate likelihood ratio test (Anisimova & Gascuel 2006). Resulting ML trees were subsequently edited in MEGA6. For each unique concatenated sequence type, the complete set of partial housekeeping gene sequences from a single candidate isolate was submitted to GenBank (Table S1; accession nos. KT026600–KT026964).

Each unique housekeeping gene sequence was assigned an allele type number, and these numbers (1 for each of the 5 separate loci) were used to assign each isolate with a concatenated sequence type number (not shown). These were used as input for eBURST analysis (Feil et al. 2004), with the minimum number of identical loci required for group definition relaxed to 3.

In order to detect putative recombination events significantly influencing the phylogenetic signal, colour-coded concatenated and single housekeeping gene trees were visually checked for conflicting clustering patterns.

#### Antisera production and serotyping

Two clinical *V. splendidus* isolates (NVI-6762 and NVI-7628 from Atlantic salmon *Salmo salar* and ballan wrasse, respectively) were cultured as previously described. Bacterial cells were harvested into 50 ml phosphate-buffered saline (PBS), supplemented with 350  $\mu$ l 37% formaldehyde and refrigerated overnight. Following 2 wash cycles with PBS and centrifugation  $34 \times g$ , pelleted cells were re-suspended in PBS and diluted to a McFarland standard of 3–4. Sterility was checked by sowing onto blood agar (2% NaCl) with 1 wk incubation at 15°C. Adult New Zealand white rabbits were immunised by subcutaneous injection (4 injections, 1 wk apart) of 0.5 ml antigen solution supplemented with Freund's incomplete adjuvant. Terminal bleeding was conducted 2 wk after the final injection.

Slide agglutination testing (Sørensen & Larsen 1986) was conducted in order to assess O-antigen variability amongst selected isolates. Briefly, bacterial cells were suspended in formalin buffer and heat-inactivated by boiling for 1 h. Following cooling to room temperature, 15  $\mu$ l of the suspension were then mixed with 15  $\mu$ l of antiserum on glass slides and observed against a dark background for 2 min with gentle rocking. The agglutination reaction of each tested isolate against each antiserum was cate-

gorised as strong (agglutination within 1 min), weak (agglutination after 1 min) or absent. Auto-agglutination was tested using naïve rabbit antiserum.

## RESULTS

### Sequence analysis

Genetic variability in the housekeeping genes examined was dominated by synonymous substitutions, and purifying selection pressures were identified for all 5 loci ( $p < 0.01$ ). Average  $d_S - d_N$  values were 10.6 (*rpoD*), 6.6 (*ftsZ*), 9.7 (*pyrH*), 5.2 (*rpoA*) and 8.5 (*atpA*). The partial 16S rRNA gene tree showed no particular clustering pattern for the 24 isolates examined. In contrast, individual housekeeping gene trees (112 isolates) displayed more or less consistent topologies, revealing essentially 1 major and several minor clusters, as well as 1 or more singletons (Fig. S1).

Analysis of concatenated sequences reinforced the patterns observed in single gene analyses (Fig. 1). The major cluster included the *Vibrio splendidus* type strain, almost all of the assessed wrasse isolates (59/63) and just under half of the lumpsucker isolates (5/12). Isolates within and outside this 'type strain cluster' are therefore, for the purposes of the present study, referred to as *V. splendidus sensu stricto* and *V. splendidus*-related, respectively. Notably, several *V. splendidus* reference strains and GenBank accessions designated as *V. splendidus* fell outside the main cluster, and many of these showed high sequence identity with type strains of other validly published (Euzéby 1997, Parte 2014) *V. splendidus*-related species (Fig. 1, Table S1). These type strains could not be included in the concatenated analysis presented here, however, as full sequence coverage for all 5 analysed gene regions could not be obtained from public sequence databases.

Pairwise sequence identities (PIDs) for concatenated housekeeping gene sequences for the material as a whole were  $\geq 93.6\%$ , which increased to  $\geq 99.3\%$  when the analysis was restricted to the fish-associated *V. splendidus sensu stricto* cluster (disregarding putative recombinants; see below). Both major and minor clusters displayed considerable microdiversity, with 51 different concatenated sequence types identified amongst the 78 *V. splendidus sensu stricto* isolates (Fig. 2). While only single isolates were examined from individual fish, intra-outbreak sequence variability was identified in the majority of diagnostic cases from which more than 1 isolate was sequenced

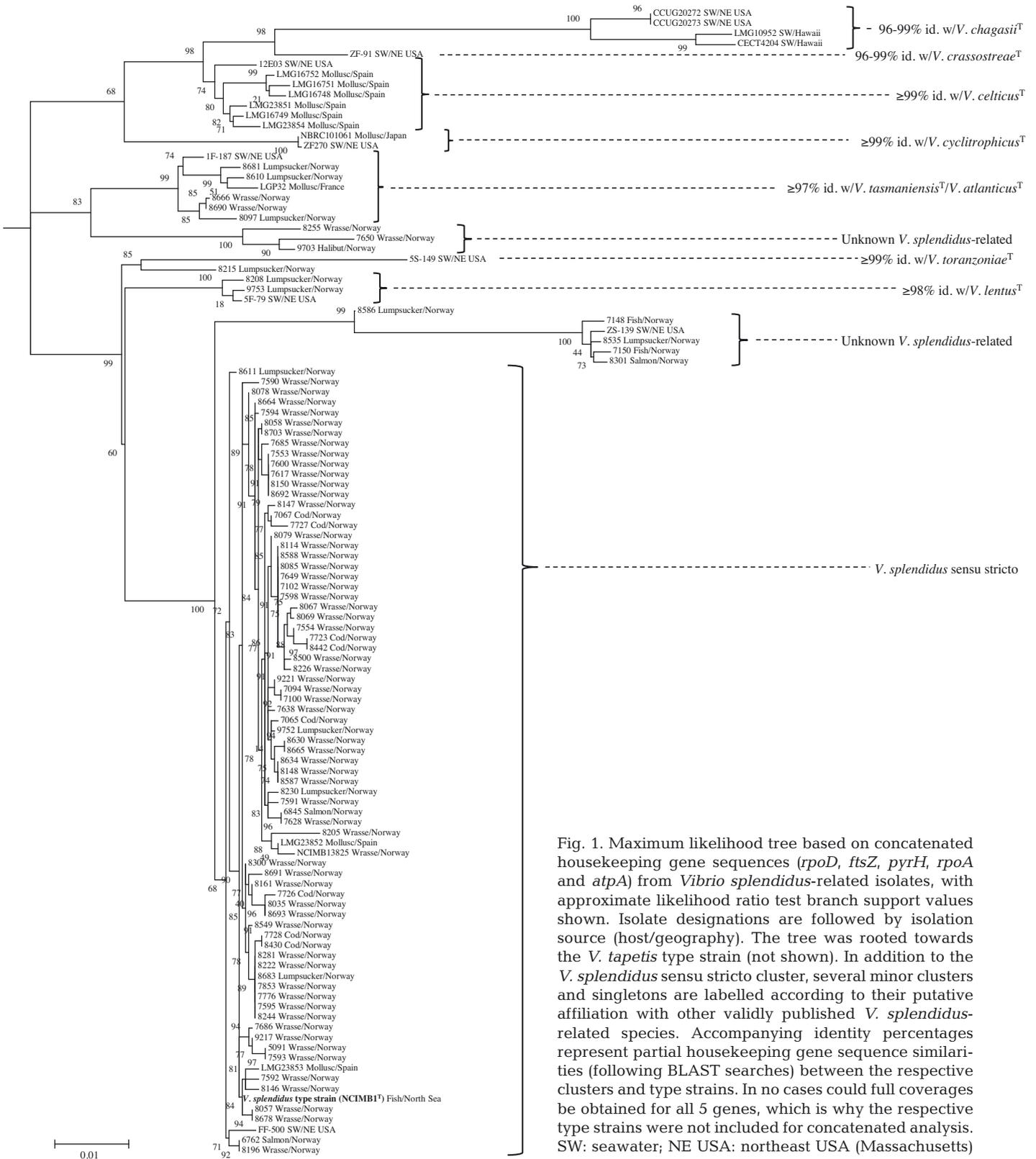


Fig. 1. Maximum likelihood tree based on concatenated housekeeping gene sequences (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*) from *Vibrio splendidus*-related isolates, with approximate likelihood ratio test branch support values shown. Isolate designations are followed by isolation source (host/geography). The tree was rooted towards the *V. tapetis* type strain (not shown). In addition to the *V. splendidus* sensu stricto cluster, several minor clusters and singletons are labelled according to their putative affiliation with other validly published *V. splendidus*-related species. Accompanying identity percentages represent partial housekeeping gene sequence similarities (following BLAST searches) between the respective clusters and type strains. In no cases could full coverages be obtained for all 5 genes, which is why the respective type strains were not included for concatenated analysis. SW: seawater; NE USA: northeast USA (Massachusetts)

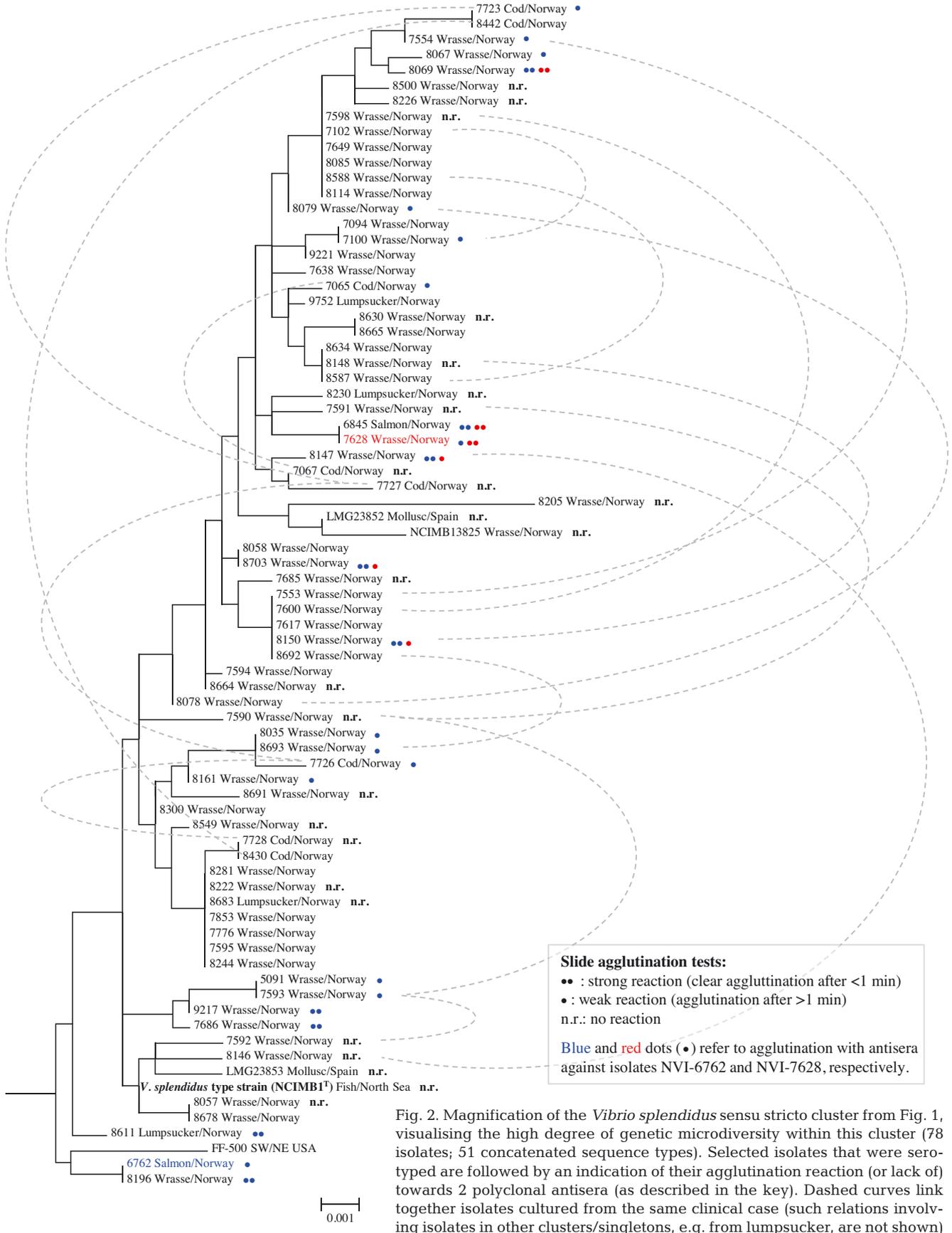


Fig. 2. Magnification of the *Vibrio splendidus* sensu stricto cluster from Fig. 1, visualising the high degree of genetic microdiversity within this cluster (78 isolates; 51 concatenated sequence types). Selected isolates that were serotyped are followed by an indication of their agglutination reaction (or lack of) towards 2 polyclonal antisera (as described in the key). Dashed curves link together isolates cultured from the same clinical case (such relations involving isolates in other clusters/singletons, e.g. from lumpsucker, are not shown)

(18/21; primarily cleaner fish cases), with as many as 4 different concatenated sequence types being identified from single investigations. No mutual founding genotype for the *V. splendidus* sensu stricto cluster could be identified using eBURST analysis (not shown), which depicted several minor clonal complexes and singletons.

PID-ranges for individual housekeeping genes and concatenated sequences revealed varying taxonomic resolutions both within the *V. splendidus* sensu stricto cluster (intra-cluster), and between this cluster and other *V. splendidus*-related taxa (inter-cluster). When disregarding putative recombinants (see next paragraph), intra-cluster PID ranges were  $\geq 98.4\%$  (*rpoD*),  $\geq 98.9\%$  (*ftsZ*),  $\geq 98.5\%$  (*pyrH*),  $\geq 99.4\%$  (*rpoA*),  $\geq 99.2\%$  (*atpA*) and  $\geq 99.3\%$  (MLSA), while inter-cluster PID ranges were 88.5–97.6% (*rpoD*), 96.0–99.4% (*ftsZ*), 91.9–96.3% (*pyrH*), 96.5–99.2% (*rpoA*), 92.5–99.7% (*atpA*) and 93.6–98.3% (MLSA). Only *pyrH*, *rpoD* and *rpoA* (and concatenated sequences) could thus alone distinguish *V. splendidus* sensu stricto from other *V. splendidus*-related taxa (Fig. 3; non-overlapping intra- and inter-cluster bars). The discriminatory ability of *rpoD* and the concatenated sequences disappeared, however, following the inclusion of putative recombinants. Due to its multi-copy nature and allele variability within individual strains, PID ranges for the partial 16S rRNA gene sequences could not be properly assessed.

Comparison of colour-coded single gene trees clearly revealed instances of conflicting clustering in the *rpoD*, *atpA* and *ftsZ* trees (Fig. 4), presumably due to recombination. Such events in *rpoD* (and to some degree *ftsZ*) alone significantly affected concatenated tree topology. This was in part due to the relatively large contribution of *rpoD* to the combined genetic diversity (Fig. 3), and partly to large distances between donor and recipient clusters of the involved sequences (*ftsZ* and *rpoD*; Fig. 4).

### Slide agglutination

Polyclonal rabbit antisera raised against 2 genetically distinct *V. splendidus* sensu stricto isolates yielded positive agglutination (strong or weak) in only around half of the tested can-

didates in the *V. splendidus* sensu stricto cluster (Fig. 2). The majority of isolates displaying positive agglutination reacted with anti-NVI-6762 serum, while few isolates reacted with anti-NVI-7628 serum. No isolates reacted exclusively with anti-NVI-7628 serum. Curiously, anti-NVI-6762 serum yielded only weak/delayed agglutination with the autologous isolate despite several repeated attempts with fresh cultures. No auto-agglutination was observed.

## DISCUSSION

In the present study, sequencing of core housekeeping genes (*rpoD*, *ftsZ*, *pyrH*, *rpoA* and *atpA*) was used to investigate the phylogenetic population structure of 112 *Vibrio splendidus*-related isolates, primarily cultured in relation to increased cleaner fish (wrasse and lumpsucker) mortality in Norwegian salmon farms. The majority of wrasse isolates proved to be very closely related to *V. splendidus* NCIMB1<sup>T</sup> ( $\geq 99.3\%$  PID), and presumably represent true examples of this species (i.e. *V. splendidus* sensu stricto). However, we found a considerable degree of genetic microdiversity amongst such isolates, often even within individual clinical investigations, and O-antigen variations were also detected. No 2 lumpsucker

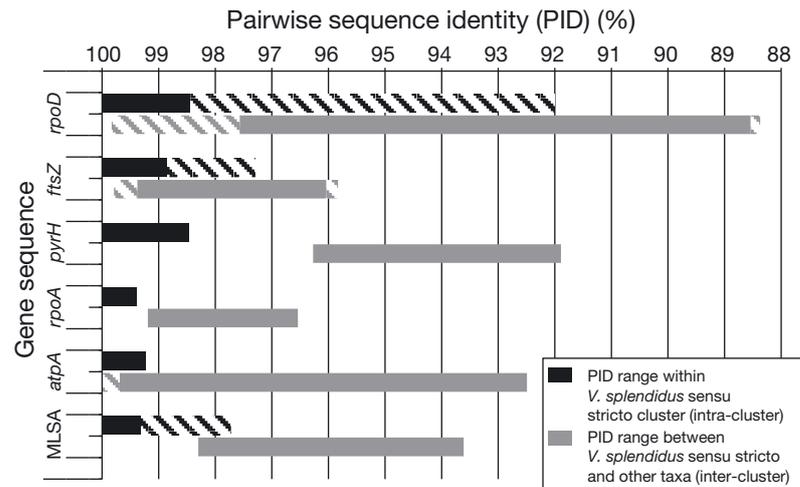


Fig. 3. Intra-cluster (amongst *Vibrio splendidus* sensu stricto isolates; black bars) and inter-cluster (*V. splendidus* sensu stricto vs. *V. splendidus*-related; grey bars) taxonomic resolution of individual genes and concatenated sequences (multilocus sequence analysis, MLSA), as determined by pairwise sequence identity (PID) ranges. Upper and lower bar values represent maximum and minimum PIDs, respectively. Partial diagonal striping in lateral bar segments represent proportions of the observed PID ranges that can be attributed to putative recombinational events (see 'Results' and Fig. 4). The presentation form was modified after Martens et al. (2007)

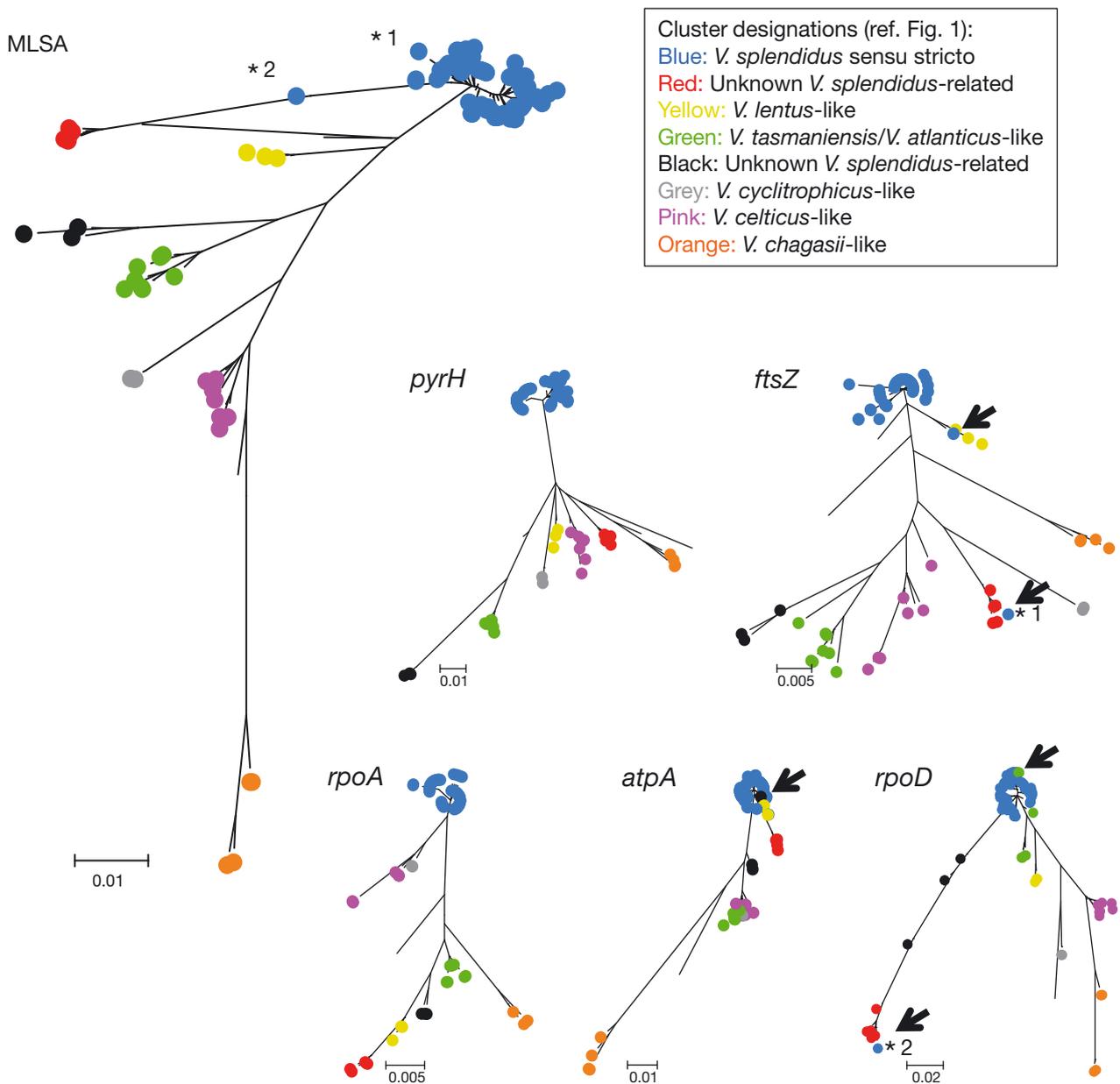


Fig. 4. Concatenated- and single housekeeping gene radial trees (unrooted) aimed at highlighting isolates with conflicting clustering across loci due to putative recombination. Non-singleton isolates have been coloured consistently according to clustering in the concatenated tree in Fig. 1 (see key). Although asterisked isolates (\*<sup>1</sup>: NVI-8205 and \*<sup>2</sup>: NVI-8586) on average displayed relatively low concatenated pairwise sequence identity (PID) in relation to other isolates in the *Vibrio splendidus* sensu stricto cluster, they have been included as only 1 of 5 genes fell outside this cluster. These incidents, and some other clear examples of discrepancies between genes (putative recombinants), are indicated by arrows in the individual housekeeping gene trees

isolates displayed identical concatenated sequence types, and a lesser proportion of these isolates clustered close to the *V. splendidus* type strain.

All 5 housekeeping genes investigated have previously been extensively used for phylogenetic analysis of vibrios (e.g. Thompson et al. 2007, Pascual et al. 2010, Sawabe et al. 2013). In the present

study, they were all shown to be under purifying selection ( $p < 0.01$ ), corroborating their suitability for use in phylogenetic studies (Stackebrandt et al. 2002). The taxonomic resolution (PID ranges) of individual genes varied (Fig. 3), and putative recombination events identified in 3 of the genes (Fig. 4) affected PID values. Nevertheless, the MLSA

seemed to efficiently resolve and depict the overall phylogeny of our material (Fig. 1). Unsurprisingly, analysis of a variable region of the 16S rRNA gene from selected isolates was unable to sensibly resolve phylogeny amongst these closely related bacteria at any level (Fig. S1). This method can therefore only be used to identify isolates as members of the Splendidus-clade, pending further investigation. The applicability of this multi-copy gene for subtyping of *V. splendidus*-related bacteria is further complicated by the fact that intra-cell allele heterogeneity may surpass that observed between distinct strains (Le Roux et al. 2004, Jensen et al. 2009).

Isolates in most minor clusters/singletons in the concatenated 5-gene tree showed high sequence identity with type strains of distinct *V. splendidus*-related species (e.g. *V. chagasii*, *V. celticus* and *V. cyclitrophicus*; Fig. 1), and most probably belong to these species. However, genetic microdiversity was observed within all clusters, including the major *V. splendidus* sensu stricto cluster (Fig. 2). *V. splendidus*-related taxa are extremely common members of the marine bacterioplankton, and Thompson et al. (2005) estimated >1000 distinct *V. splendidus*-related genotypes to be present within a geographically restricted area. Unsurprisingly, therefore, considering the limited number of isolates examined in the present study, no mutual founding genotype for the major cluster could be identified using eBURST (not shown).

Slide agglutination using polyclonal antisera raised against 2 *V. splendidus* sensu stricto isolates from ballan wrasse and Atlantic salmon, respectively, and with a relatively low PID (99.4%), yielded ambiguous results (Fig. 2). While elucidation of the antigenic relationships was undoubtedly limited by the low number of sera used, the results do show that considerable O-antigen diversity (which apparently cannot be linked to MLSA tree topology) exists within this genetically compact, and presumably conspecific, cluster. This is consistent with the findings of Wildschutte et al. (2010) amongst environmental and animal-associated *V. splendidus*-related isolates with up to 100% MLSA identity.

Interestingly, both genetic and antigenic (micro-)diversity was often (18/21 cases) also observed amongst *V. splendidus* sensu stricto isolates cultured from different fish specimens during individual episodes of increased mortality (Fig. 2; dashed curves). Similar findings have previously been reported following investigation of *V. splendidus*-related losses in molluscs (Gay et al. 2004). The

apparent lack of clonal expansion within cleaner fish mortality episodes suggests that fish-to-fish transmission is perhaps not the main route of infection during clinical outbreaks, and direct infection from seawater may be more prevalent.

Nevertheless, most wrasse isolates fell into a single cluster (*V. splendidus* sensu stricto; Fig. 1), representing only 1 sub-taxon within the comprehensive group of environmental *V. splendidus*-related bacteria (Le Roux & Austin 2006). While this may reflect an intrinsic predilection for these fish, perhaps based on pathogenicity, *V. splendidus* infection trials on wrasse have produced ambiguous results (Bergh & Samuelsen 2007, Ø. Vågnes unpubl. data). Moreover, *V. splendidus*-related strains have also been identified as natural members of the intestinal flora in healthy wrasse larvae (Birkbeck & Treasurer 2014).

In addition to virulence, infection and eventual disease progression will, for all infective agents, also depend upon the health and immune status of the host. Capture, storage, transport and salmon-cage stocking of wild wrasse undoubtedly entails a range of physical and mental stressors, and although farmed cleaner fish are bred in captivity, transfer to the alien environment of the salmon cages will represent a stressful transition. This presumably increases the susceptibility of these fish to infectious disease. The ability of *V. splendidus*-related bacteria to cause disease in cleaner fish, and the extent of an eventual outbreak, may thus result from a complex interplay between bacterioplankton composition, host predilection and immunocompetence.

As most of the isolates examined in the present study originated from wrasse in Norwegian waters, the occurrence of 1 dominating cluster (*V. splendidus* sensu stricto) could conceivably also be explained by geographical sampling bias. This seems unlikely, however, as Massachusetts (USA) seawater isolates were present in most clusters, and isolates from Norwegian lumpsucker primarily belonged to minor clusters or appeared as singletons (Fig. 1).

In summary, the 5-gene MLSA used in the present study identified a relatively high degree of microdiversity within distinct *V. splendidus*-related taxa. This was most evident amongst the numerous *V. splendidus* sensu stricto isolates primarily cultured in relation to episodes of increased wrasse (cleaner fish) mortality in Norwegian salmon farms, which also displayed O-antigen dissimilarities. The lack of dominance by 1 or a few virulent clones amongst infected specimens, even within individual 'out-

breaks', indicates that direct infection from seawater may be more prevalent than fish-to-fish transmission. Furthermore, mechanisms underlying *V. splendidus* infection and eventual disease progression in wrasse are probably complex, and may depend both upon the strain in question and not least on the health status of the fish (i.e. through opportunism). While vaccination against such infection may conceivably help limit *V. splendidus*-related losses, the variable nature of these bacteria complicates selection of representative candidate strains for infection trials and vaccine development.

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