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RESEARCH ARTICLE

MLVA genotyping of *Moritella viscosa* reveals serial emergence of novel, host-specific clonal complexes in Norwegian salmon farming

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Abstract

A Multi-Locus Variable number of tandem repeat Analysis (MLVA) genotyping scheme was developed for the epidemiological study of *Moritella viscosa*, which causes 'winter ulcer' predominantly in sea-reared Atlantic salmon (*Salmo salar* L.). The assay involves multiplex PCR amplification of six Variable Number of Tandem Repeat (VNTR) loci, followed by capillary electrophoresis and data interpretation. A collection of 747 spatiotemporally diverse *M. viscosa* isolates from nine fish species was analysed, the majority from farmed Norwegian salmon. MLVA distributed 76% of the isolates across three major clonal complexes (CC1, CC2 and CC3), with the remaining forming minor clusters and singletons. While 90% of the salmon isolates belong to either CC1, CC2 or CC3, only 20% of the isolates recovered from other fish species do so, indicating a considerable degree of host specificity. We further highlight a series of 'clonal shifts' amongst Norwegian salmon isolates over the 35-year sampling period, with CC1 showing exclusive predominance prior to the emergence of CC2, which was later supplanted by CC3, before the recent re-emergence of CC1. Apparently, these shifts have rapidly swept the entire Norwegian coastline and conceivably, as suggested by typing of a small number of non-Norwegian isolates, the Northeast Atlantic region as a whole.

KEYWORDS
aquaculture, Atlantic salmon (*Salmo salar* L.), genotyping, MLVA, *Moritella viscosa*, winter ulcer

1 | INTRODUCTION

Moritella viscosa-associated winter ulcer affects intensively sea-farmed Atlantic salmon (*Salmo salar* L.) during periods of low water temperature. The clinical presentation varies but often involves prolonged low-grade mortality, with affected fish displaying clearly demarcated lesions on lateral body surfaces, which may in severe cases cover large areas, penetrate deep into underlying muscle tissues and result in terminal septicaemia (Benediktsdóttir et al., 1998; Lunder et al., 1995). The disease is a particular problem in Norwegian aquaculture but also impacts the salmon farming industries of Scotland, Ireland, Iceland, the Faroe Isles and the eastern seaboard of the United States/Canada (Benediktsdóttir et al., 2000; Bruno et al., 1998; Whitman et al., 2001). Winter ulcer remains, despite vaccination against *M. viscosa*, probably the most financially significant bacterial disease in Norwegian

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salmon farming and also represents a severe animal welfare issue (Sommerset, 2022).

Moritella viscosa was initially described as a phenotypically and genetically homogenous species based on the study of isolates originating almost exclusively from farmed salmon in Norway (Lunder et al., 2000). A degree of genetic (Benediktsdóttir et al., 2000) and antigenic (Heidarsdóttir et al., 2008) heterogeneity was, however, quickly identified following comparison of geographically disparate isolates from different fish species. Later work identified a specific genotype, termed 'typical' *M. viscosa*, exclusively associated with salmon displaying winter ulcer (Grove et al., 2010; Karlsen et al., 2014) within a restricted geographical area, while genetically diverse strains predominated amongst other fish species. More recently, whole-genome sequencing (WGS) confirmed the existence of several distinct phylogenetic lineages amongst 12 *M. viscosa* isolates examined (Karlsen et al., 2017).

In light of the continued and recently increasing problems associated with winter ulcer in Norway, and against a background of established heterogeneity amongst the *M. viscosa* strains involved, the need for an affordable, high-resolution typing system for this bacterium became apparent. The present study was therefore initiated to develop and utilize Multi-Locus Variable number of tandem repeat Analysis (MLVA) for *M. viscosa*. By MLVA typing a large number of *M. viscosa* isolates recovered over a 35-year period, predominantly from Norwegian salmon, we aimed to shed light on the genetic diversity and population dynamics of this important bacterial fish pathogen.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolates and DNA extraction

A total of 747 clinical *M. viscosa* isolates, recovered between 1988 and 2022 from nine different fish species and seven countries, were included in the present study (see metadata summary in Table 1 and details in Table S1). Stock cultures, cryopreserved at −80°C, were revived on 5% bovine blood agar supplemented to a final concentration of 2% NaCl (BAS) and incubated at 15°C for 1–3 days prior to further processing. For PCR, genomic DNA was extracted by boiling bacterial cells from a single colony in 50 µL MQ-water for 7 min, followed by centrifugation and use of supernatant as template (or storage at −20°C until such use).

2.2 | Identification of VNTR loci

Prospective Variable Number of Tandem Repeat (VNTR) loci were initially identified by running Tandem Repeats Finder v4 (tandem.bu.edu/trf/trf.html) against a draft genome of the *M. viscosa* type strain NCIMB 13584^T available in-house (Grove et al., 2008). Subsequently, the suitability of individual candidate VNTR loci for inclusion in the MLVA assay was evaluated via a series of singleplex PCRs and Sanger sequencing (not shown) performed on a spatiotemporally diverse collection of 36 *M. viscosa* isolates (indicated in Table S1) from various fish species. Based on this, a selection was made of loci displaying ubiquitous occurrence in the tested isolates, repeat unit size uniformity and a degree of inter-strain copy number variation. In accordance with the suggested guidelines (Nadon et al., 2013), these selected VNTR loci were annotated according to their position (closest kbp) within the closed chromosome of *M. viscosa* strain MVIS1 (RefSeq accession no. NZ_LN554852.1).

2.3 | Multiplex PCR and capillary electrophoresis

A multiplex PCR assay was established containing 12 primers designed in Primer3 v0.4.0 (Untergasser et al., 2012), targeting each of the six VNTR loci selected (Table 2). Reverse primers (Applied Biosystems) were 5′ labelled with either of three fluorescent dyes (6FAM, VIC or NED) and care was taken during primer design to minimize the risk of amplicon size overlaps between loci labelled with identical dyes.

TABLE 1 Summary of the spatiotemporal and host species origins represented amongst the 747 *M. viscosa* isolates subjected to MLVA typing in this study. For detailed metadata on each isolate, see Table S1.

Host species and number of isolates	Time span	Countries represented
Atlantic salmon, *Salmo salar* (*n* = 589)	1988–2022	Norway (*n* = 563), Scotland (*n* = 12), Faroe Islands (*n* = 4), Iceland (*n* = 3), Canada (east coast; *n* = 3), Ireland (*n* = 2), unknown (*n* = 2)
Lumpfish, *Cyclopterus lumpus* (*n* = 75)	2000[a]–2021	Norway (*n* = 72), Faroe Islands (*n* = 2), Iceland (*n* = 1)
Rainbow trout, *Oncorhynchus mykiss* (*n* = 57)	1999–2020	Norway (*n* = 56), Iceland (*n* = 1)
Atlantic cod, *Gadus morhua* (*n* = 13)	2002–2011	Norway (*n* = 13)
Ballan wrasse, *Labrus bergylta*; goldsinny wrasse, *Ctenolabrus rupestris*; and corkwing wrasse, *Symphodus melops* (*n* = 10)	2010–2020	Norway (*n* = 10)
Atlantic halibut, *Hippoglossus hippoglossus* (*n* = 2)	2011–2012	Norway (*n* = 2)
Whiting, *Merlangius merlangus* (*n* = 1)	1998	Sweden (*n* = 1)

[a]Subject to some uncertainty.

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TABLE 2 Characteristics of the selected VNTR loci within the chromosome of *M. viscosa* strain MVIS1 (RefSeq accession no. NZ_LN554852.1). Primer sequences used for multiplex PCR are also shown, with the fluorescent dye label (6FAM, VIC or NED) used at the 5′ end of reverse primers in bold.

VNTR locus	Repeat unit	MVIS1 chromosome position	Position relative to MVIS1 annotated protein(s) (GenBank acc. no.)	5′ → 3′ MLVA flanking primers (forward; reverse)
MV_525	GTTTTG[a]	524,884–524,949	*Within* peptidase, family M23 (CED58473.1)	CAGCCTTAACCCATTGCTGT **VIC**-CTAAACCGGTGCCGATAAAA
MV_2946	AACTA[a]	2,945,645–2,945,694	*Between* UDP-glucose 4-epimerase (CED60505.1) *and* putative uncharacterized protein (CED60506.1)	TTGCCATTCGAATTCCTTCT **6FAM**-CACAGAAATGCCGTTTGGTA
MV_3707	CATATT	3,707,034–3,707,045	*Between* pseudo-protein (NA) *and* putative phosphomannomutase (CED61167.1)	CCTAAATTGGTTGGGTGTGATT **NED**-TCGTGATCTACGCCTACTTCC
MV_3793	ACTAACATC[a]	3,792,641–3,792,676	*Within* polar flagellar hook-length control protein FliK (CED61247.1)	GTTGATAGCTCGTTCGTTTGC **6FAM**-CACCTGATAGTCGCGTTGC
MV_4460	GGTGTGACA[a]	4,460,011–4,460,082	*Within* pseudo-protein (NA)	ATTACGGATGAATTGCTAAAACG **NED**-CGGCATACCTTATCAAGCAA
MV_4694	ACAGAG	4,693,814–4,693,825	*Within* putative uncharacterized membrane-associated protein (CED61972.1)	CCAGGGGCATTCTTTAATAA **VIC**-TTTGAGCCCCCTCTAAATCA

[a]Varying degrees of repeat unit sequence (but not size) heterogeneity was observed amongst examined isolates.

Each multiplex PCR reaction contained 12.5 μL 2x Multiplex PCR Master Mix (Qiagen), 0.2 μM of each primer, 2 μL undiluted DNA template and a volume of RNase-free water amounting to a total reaction volume of 25 μL. PCR cycles involved (i) 15 min at 95°C, (ii) 35 cycles of 0.5 min at 94°C, 1.5 min at 57°C, 1.5 min at 72°C and (iii) 10 min at 72°C, followed by cooling to 4°C indefinitely. PCR products were verified by gel electrophoresis and then diluted 1:10 (vol:vol) in Milli-Q water. From the diluted samples, 0.5 μL was added to 9 μL Hi-Di Formamide (Applied Biosystems) and 0.5 μL GeneScan 600 LIZ dye size standard v2.0 (Applied Biosystems). Samples were denatured for 3 min at 95°C prior to capillary electrophoresis on an Avant 3500xl Genetic Analyser (Applied Biosystems) utilizing POP-7 polymer (Applied Biosystems) and the following settings: 5 s injections at 1.6 kV (32 V/cm) and 32 min run time at 15 kV (300 V/cm) and 60°C.

2.4 | MLVA profiling and cluster analysis

Following capillary electrophoresis, electrophoretic peaks were size called in GeneMapper 5 (Applied Biosystems) and assigned to VNTR loci according to fluorescent labelling and size. Due to the common phenomenon of biased amplicon mobility patterns in capillary electrophoresis machines (Lista et al., 2006; Pasqualotto et al., 2007), some disagreement was observed between amplicon sizes as determined by Sanger sequencing and the capillary electrophoresis size calls. These were therefore subjected to locus-specific corrections and converted to VNTR repeat counts as previously described (Gulla et al., 2018). Briefly, for each VNTR locus, linear regression was used to compare the capillary electrophoresis size call to verified amplicon size (as inferred from Sanger sequencing) in a collection of isolates known to cover a spectrum of allelic diversity. This allowed the calculation of line-of-best-fit equations, which were in turn employed to correct capillary electrophoresis size calls (see Figure S1).

Each isolate was thus awarded a string of six integers (MLVA profile) reflecting the number of whole repeats identified at each VNTR locus. MLVA profiles for all 747 isolates were then imported into BioNumerics v7.6 (Applied Maths NV, Saint-Martens-Latem, Belgium), and Minimum spanning tree cluster analyses were performed with default settings.

2.5 | Allelic diversity and VNTR stability

Based on the observed allelic diversity, the discriminatory capacity of the studied VNTR loci, both individually and in combination, was evaluated by calculating Simpson's diversity index (Simpson, 1949). In vitro stability of the VNTR loci was assessed in two *M. viscosa* representatives, i.e. the type strain NCIMB 13584^T from Atlantic salmon and isolate NVI-5450 from rainbow trout (*Oncorhynchus mykiss*, Walbaum), following 40 serial passages with subcultures taken at 1–2 day intervals. For each passage, single colonies were

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re-sown onto fresh BAS plates and incubated at 15°C. MLVA profiles were obtained as described from colonies harvested after 0, 10, 20, 30 and 40 passages.

2.6 | Core genome phylogeny

Twelve *M. viscosa* genome assemblies available via the NCBI RefSeq repository (accession nos. GCF_000953735.1, GCF_900119925.1, GCF_900119985.1, GCF_900120015.1, GCF_900120025.1, GCF_900120035.1, GCF_900120065.1, GCF_900120075.1, GCF_900120105.1, GCF_900120115.1, GCF_900120145.1 and GCF_900120305.1) were downloaded and subjected to phylogenetic recreation using the in-house pipeline ALPPACA v1.0.0 (Kaspersen & Fiskebeck, 2022). Briefly, this involved core genome alignment with ParSNP v1.6.1 (Treangen et al., 2014), recombination identification with Gubbins v3.1.3 (Croucher et al., 2015) and alignment masking with maskrc-svg v0.5 (github.com/kwongj/maskrc-svg), all run on default settings. Snp-dists v0.8.2 (github.com/tseemann/snp-dists) was then used to produce a pairwise Single Nucleotide Polymorphism (SNP) distance matrix, while the phylogeny was recreated in IQtree v2.1.4 (Minh et al., 2020) with 1000 ultrafast bootstrap replicates (Hoang et al., 2018) on a GTR+I+F model. Physical isolates cryopreserved in our laboratory, from which the 12 analysed genome assemblies had been generated, were also MLVA genotyped for comparison.

3 | RESULTS

3.1 | MLVA development and deployment

Following the identification and examination of putative VNTR regions, six informative loci were selected for inclusion in a single-tube multiplex MLVA. For all 747 *M. viscosa* isolates thus examined, capillary electrophoresis performed on PCR amplicons produced peaks corresponding to all six VNTR loci. The amplicons were readily distinguished based on fluorescent labelling and size (Figure S2), and no size overlap was observed between identically labelled loci.

For some of the loci subjected to Sanger sequencing for comparison towards capillary electrophoresis amplicon size calls, a varying degree of repeat unit sequence heterogeneity was observed. More importantly, however, repeat count predictions (based on corrected size calls) consistently deviated ≤10% from a full repeat, indicating size uniformity in both repeat units and flanks. One notable exception to the latter nevertheless exists for locus MV_525, where a 30 bp fragment located immediately downstream of the repeat region was found to be duplicated in two of the Sanger sequenced isolates. Being devisable by the repeat unit size (6 bp) of MV_525, this duplication did not interfere with the typing scheme's ability to produce integer MLVA profiles, but some rare instances of isolates being incorrectly identified as identical in this particular locus may conceivably occur.

Table 3 summarizes the most central technical metrics recorded for each of the six VNTR loci. A total of 154 distinct MLVA profiles were detected amongst the 747 *M. viscosa* isolates typed.

3.2 | Assay evaluation

The allelic diversity within individual VNTR loci varied from 6 to 24 alleles, with Simpson's diversity index values ranging from 0.443 to 0.884 (Table 3). The Simpson's index of diversity for all six loci combined was 0.941, indicating the very high probability of separating non-clonal isolates. No repeat unit copy number changes were observed in any of the VNTR loci amongst the two *M. viscosa* (NCIMB 13584^T and NVI-5450) MLVA-typed after 0, 10, 20, 30 and 40 subcultures.

3.3 | MLVA cluster analysis

Using a relatively stringent cluster partitioning threshold (>4/6 identical loci), Minimum spanning tree cluster analysis grouped most of the *M. viscosa* isolates within either of three clonal complexes (CC1, CC2 and CC3) while remaining isolates formed minor clonal complexes or singletons (Figure 1a). CC1 encompasses the isolates historically designated as typical (Grove et al., 2010), including the type strain NCIMB 13584^T.

TABLE 3 Metrics for each VNTR locus across the studied dataset, as inferred from MLVA typing.

VNTR locus	Repeat size (bp)	Repeat count range	Amplified flanks (bp)	Amplicon size range (bp)	No. unique alleles	Simpson's index of diversity
MV_525	6	4–16	378[a]	402–474	13	0.687
MV_2946	5	3–27	289	304–424	24	0.884
MV_3707	6	1–14	229	235–313	13	0.443
MV_3793	9	2–17	73	91–226	12	0.687
MV_4460	9	3–17	443	470–596	14	0.699
MV_4694	6	2–7	155	167–197	6	0.648

[a]A 30 bp duplication located immediately downstream of the repeat region was observed in two of the sequenced isolates (see Results).

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FIGURE 1 (a) Minimum spanning tree based on the MLVA profiles of 747 *Moritella viscosa* isolates, with thick and thin connective branches representing 5/6 and 4/6 identical VNTR loci, respectively (connections of lower similarity are hidden). The three main clonal complexes are delimited by coloured shading (CC1, CC2 and CC3), while variable shades of grey reflect different host species origins (Atlantic salmon and other, respectively). (b) Stacked bar chart showing the relative representation of these two host groups across MLVA genotypes. (c) Stacked bar chart indicating (for Norwegian salmon isolates only) the relative proportion (left vertical axis) of annual genotype detections since 1988, overlaid with a line indicating the total number (right vertical axis) of Norwegian salmon isolates represented per year.

The distribution of isolates across the tree further displays a strong correlation towards host species, with CC1, CC2 and CC3 showing a predilection for Atlantic salmon, whereas other genotypes originate primarily from other fish species (Figure 1b). While the majority of isolates examined originated from Norway, this trend is irrespective of geographic origin, with 23/24 non-Norwegian salmon isolates (all North Atlantic; see Tables 1 and S1) also belonging to either of these three major clonal complexes. Moreover, looking at the Norwegian salmon isolates ($n = 563$) specifically, CC1, CC2 and CC3 show an uneven detection prevalence over time (Figure 1c). A comparable trend is also observed for non-Norwegian isolates in regard to overrepresentation of CC1 (7/12) and CC3 (8/12) amongst older (1992–2008) and newer (2015–2019) salmon isolates, respectively. It should be noted, however, that both CC2 and CC3 were evidently present internationally at a date prior to their first known Norwegian isolations. Specifically, this pertains to Icelandic CC2 and CC3 isolates from 1992 and two Canadian CC3 isolates from 2005. Notably, all the three main clonal complexes have essentially been detected wherever extensive salmon farming occurs along the Norwegian coastline (Figure 2).

From most investigated winter ulcer cases, only a single *M. viscosa* isolate per case was included for MLVA, but from 64 cases, multiple isolates recovered simultaneously at the same farm were analysed in order to assess the possibility for intraoutbreak strain diversity. This survey revealed MLVA diversity in 41% (17/41) of the cases involving the analysis of two isolates only. Amongst cases involving analysis of three or more isolates, however, differing MLVA profiles were detected in 96% (22/23) of the cases, with 70% (16/23) even implicating more than one clonal complex. Particular scrutiny of three winter ulcer outbreaks in Norwegian farmed salmon in 2021, from each of which eight *M. viscosa* isolates had been recovered, demonstrates this phenomenon while also highlighting the possibility of individual fish being simultaneously colonized by multiple genotypes (Figure 3).

Amongst the eight non-salmon host species included in the dataset (Tables 1 and S1), only lumpfish (*Cyclopterus lumpus*, L.) and

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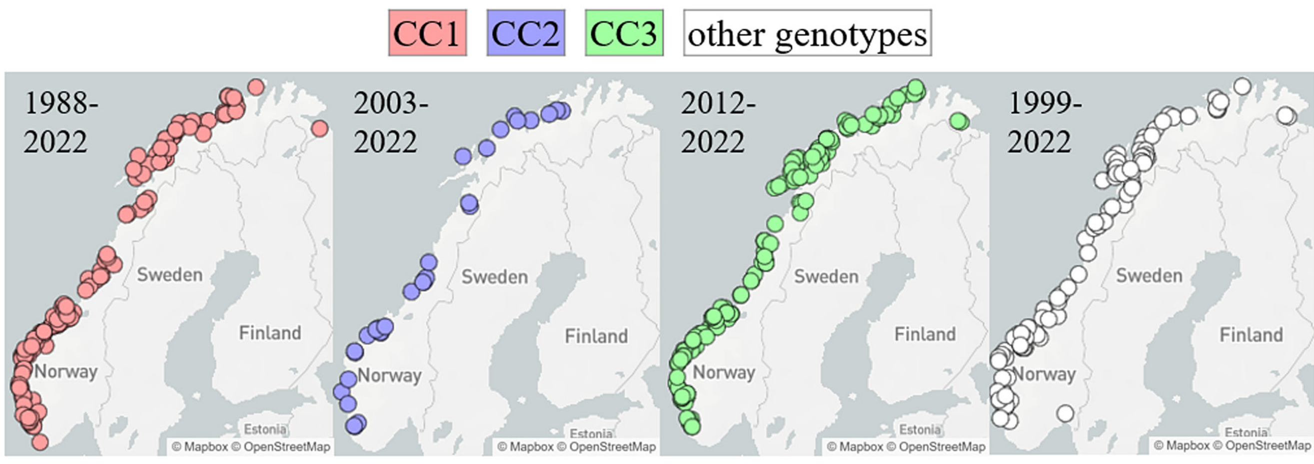
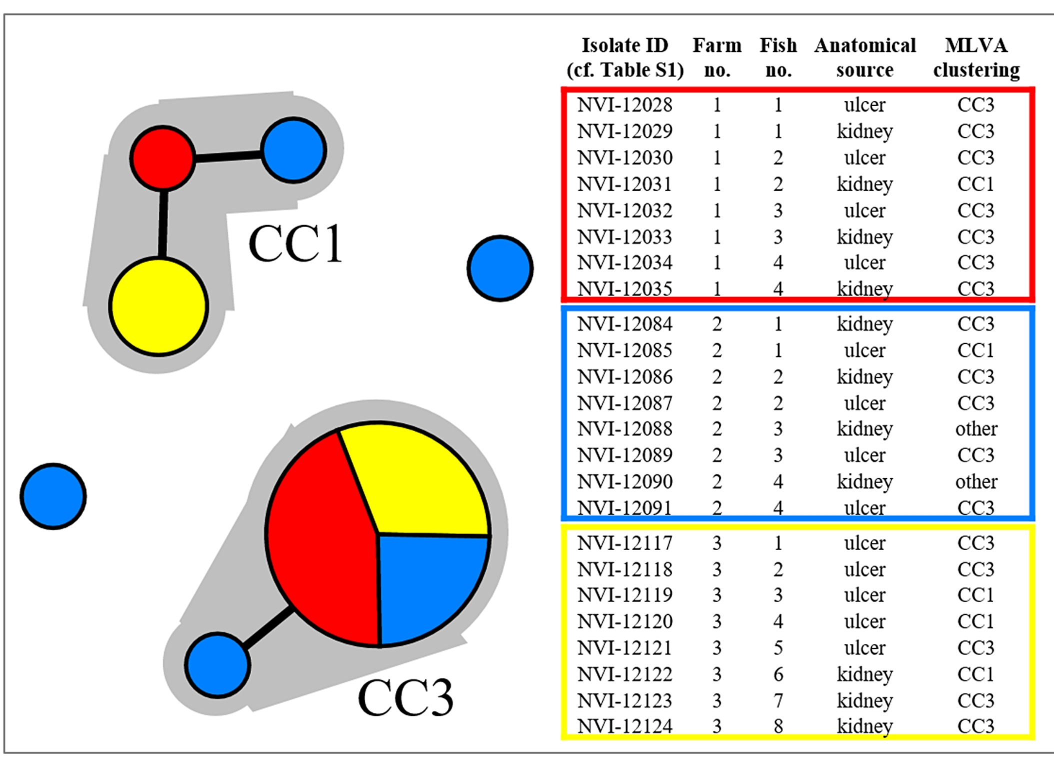


FIGURE 2 Maps created in Microreact (Argimón et al., 2016) displaying the geographic distribution of *Moritella viscosa* MLVA genotypes (see colour legend) isolated along the Norwegian coastline.



Isolate ID (cf. Table S1)	Farm no.	Fish no.	Anatomical source	MLVA clustering
NVI-12028	1	1	ulcer	CC3
NVI-12029	1	1	kidney	CC3
NVI-12030	1	2	ulcer	CC3
NVI-12031	1	2	kidney	CC1
NVI-12032	1	3	ulcer	CC3
NVI-12033	1	3	kidney	CC3
NVI-12034	1	4	ulcer	CC3
NVI-12035	1	4	kidney	CC3
NVI-12084	2	1	kidney	CC3
NVI-12085	2	1	ulcer	CC1
NVI-12086	2	2	kidney	CC3
NVI-12087	2	2	ulcer	CC3
NVI-12088	2	3	kidney	other
NVI-12089	2	3	ulcer	CC3
NVI-12090	2	4	kidney	other
NVI-12091	2	4	ulcer	CC3
NVI-12117	3	1	ulcer	CC3
NVI-12118	3	2	ulcer	CC3
NVI-12119	3	3	ulcer	CC1
NVI-12120	3	4	ulcer	CC1
NVI-12121	3	5	ulcer	CC3
NVI-12122	3	6	kidney	CC1
NVI-12123	3	7	kidney	CC3
NVI-12124	3	8	kidney	CC3

FIGURE 3 The minimum spanning tree on the left is based on the MLVA profiles of 24 *Moritella viscosa* isolates, collected from winter ulcer outbreaks in three Norwegian salmon farms (separated by colour) in 2021. Origin (farm no., fish no., anatomical source) and genotype (MLVA clustering) are listed for each isolate on the right. Genetic diversity, as occurs in all three outbreaks, can further be observed for four out of eight individual fish from which both ulcer and kidney isolates were examined (see farms 1 and 2). No compelling trend can be inferred linking anatomical source to genotype.

rainbow trout are represented by a considerable number of isolates (75 and 57, respectively). Despite this, no prominent patterns could be interpreted for either of these fish species with regard to MLVA clustering, with isolates presenting as randomly scattered across the minimum spanning tree (Figure 1a; not indicated specifically). It is, however, worth noting that amongst the 57 rainbow trout isolates examined, only a single (CC1) isolate (NVI-6191 from a Norwegian rainbow trout sea farm in 2008) belongs to either of the three clonal complexes dominating in Atlantic salmon. The same is true for 27/75 lumpfish isolates, but these 27 had all been collected from lumpfish

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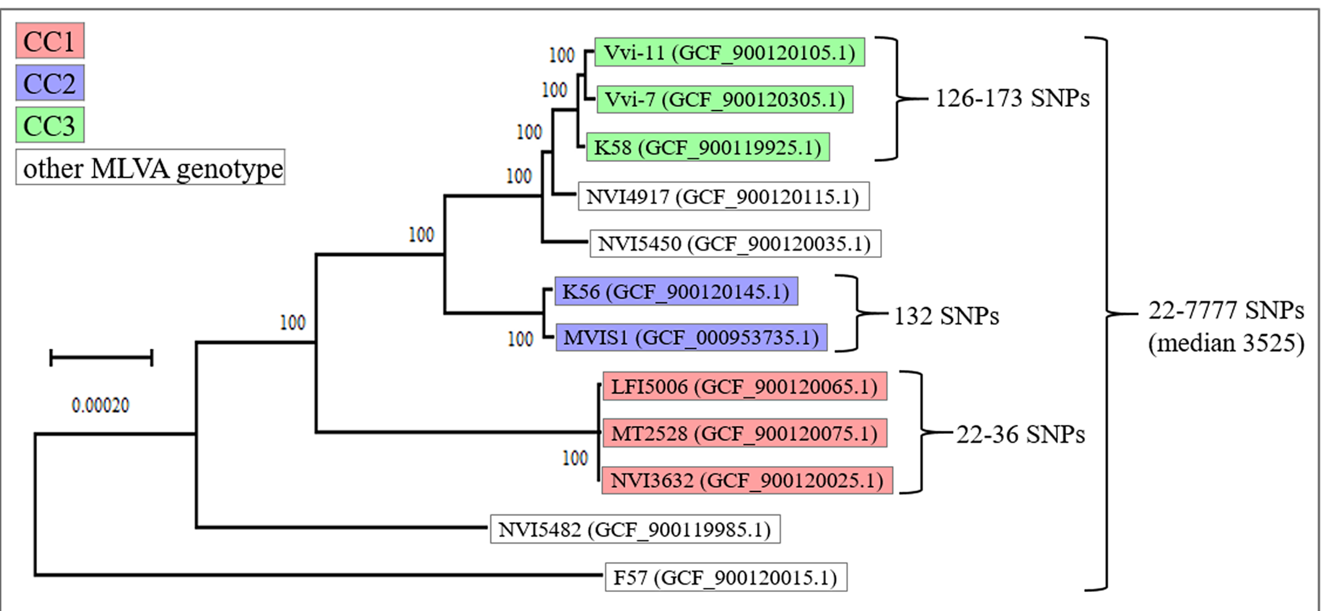

FIGURE 4 Maximum likelihood core genome phylogeny (overall coverage = 81.4%) recreated for twelve *Moritella viscosa* genome assemblies (NCBI RefSeq accession numbers in parentheses). The distribution of CC1, CC2 and CC3 isolates amongst three discrete and exclusive lineages corroborates MLVA genotyping (see colour legends on left). Intralineage and overall pairwise SNP distances are shown on the right.

already deployed in salmon farms for delousing purposes and consequently include none of the six isolates collected from lumpfish hatcheries (i.e. prior to salmon exposure).

3.4 | Phylogenetic corroboration

The twelve *M. viscosa* genome assemblies analysed included 2–3 representatives each of CC1, CC2 and CC3. This enabled the verification of compatible clustering patterns within the phylogenetic tree, where each clonal complex presents as a discrete lineage (Figure 4). With a total coverage of 81.4% across all genomes, the underlying 4,208,519 bp alignment carries a total of 13,899 SNPs (5890 parsimony informative). Each of the three salmon-associated lineages is as such distinctly separated from their closest relative by at least 419 SNPs, with intralineage SNP distances ranging no higher than 173.

4 | DISCUSSION

This study presents an MLVA assay enabling low-cost, high-resolution genotyping of *M. viscosa*, the causative agent of winter ulcer in cold-water fish farmed at sea and a particular challenge to Atlantic salmon aquaculture in Norway. Although considerable diversity is identified amongst *M. viscosa* isolates from a variety of fish species, three dominating clonal complexes (CC1, CC2 and CC3) all display an apparent predilection for Atlantic salmon (Figure 1). The relative dominance in salmon farming of each of these clonal complexes has furthermore shifted considerably over time, and these clonal shifts have seemingly swept the entire Norwegian coastline (Figure 2) and possibly the Northeast Atlantic region as a whole. Conceivably, these sequential population fluctuations may to some degree relate to the suboptimal degree of protection awarded from winter ulcer vaccines in farmed salmon (Sommerset, 2022).

A Simpson's diversity index of 0.941 (all six VNTR loci combined), together with complete MLVA conservation over 40 serial passages, suggests that this assay provides high resolution while retaining sufficient stability for epidemiological relevance. Core genome phylogeny further confirms that the three historically dominating MLVA clonal complexes do in fact represent distinct clonal lineages within *M. viscosa* (Figure 4). Further in-depth bioinformatic surveys involving genome sequencing of an extended panel of isolates will, however, be required in order to explore the genetic basis underlying historic *M. viscosa* clonal shifts and allow identification of genetic determinants of tentative importance, e.g. for host specificity and virulence.

Whereas 90% of all Atlantic salmon isolates fall into CC1, CC2 or CC3, only 20% of non-salmon isolates do the same (Figure 1b). From this skewed distribution, it is reasonable, therefore, to presume a considerable degree of host specificity for Atlantic salmon inherent to CC1, CC2 and CC3, although the biological factors underlying this phenomenon remain unknown. The almost complete lack of these three clonal complexes amongst isolates from rainbow trout (1/57) does not, however, suggest host specialization mechanisms targeting salmonid fish species in general. A higher prevalence (27/75) of CC1, CC2 and CC3 is seen amongst lumpfish used for biological delousing in salmon farms. However, given the stocking ratios commonly used (less than one lumpfish per ten salmon), it appears much more likely that this situation reflects transmission from salmon to lumpfish, rather than vice versa. This is supported by the fact that none

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of the six isolates collected from lumpfish not yet exposed to salmon belong to any of the three salmon-associated clonal complexes.

On consideration of the temporal distribution of MLVA genotypes, *M. viscosa* recovered from farmed salmon in Norway only (i.e. 563/747 examined isolates) show an exclusive predominance of CC1 from 1988 through 2002 (Figure 1c). This was followed by a period of approximately 10 years during which CC2 also accounted for a considerable proportion of the isolations before it all but disappeared coincidentally with the emergence of CC3 in 2012, which then quickly assumed dominance. Since ca. 2021, however, CC1, having been intermittently detected throughout the entire sampling time frame, has again seemingly reclaimed its footing in Norway. Although the annual number of isolates examined from earlier years is comparatively lower, the overarching trends observed here are lent credibility by their coherency over several consecutive years. Furthermore, MLVA profiles of non-Norwegian salmon isolates from the Northeast Atlantic (Iceland, Faroe Islands, Scotland and Ireland) are consistent with a historic predominance of CC1 in this region, prior to CC3's gradual emergence as the dominant genotype. These intermittent and apparently wide-ranging genotype shifts have likely occurred under the influence of multiple interdependent environmental factors, where fluctuating sea temperatures may have played some role. However, the addition of *M. viscosa* (almost certainly CC1) to the already widely used multicomponent salmon vaccines in Norway, roughly around the turn of the millennium (Håstein et al., 2005; Lillehaug et al., 2003), may conceivably also have facilitated the subsequent emergence of other genotypes.

Despite the dominance over time of specific *M. viscosa* clonal complexes in farmed Norwegian salmon, more than one clonal complex has been isolated every year since 2003 (Figure 1c). The involvement of multiple MLVA profiles in 96% (and different clonal complexes in 70%) of the clinical cases investigated by typing three or more isolates further emphasizes the complexity of the situation. Indeed, by scrutinizing 24 isolates across three separate winter ulcer outbreaks, multiple *M. viscosa* genotypes were not only identified within each outbreak but also in 50% of the individual salmon sampled at two separate anatomical locations (Figure 3). It is in this regard well known that mechanical skin injury may facilitate the development of winter ulcer (Lunder et al., 1995), and outbreaks diagnosed in recent years at the Norwegian Veterinary Institute have often manifested shortly after potentially harmful stress events (e.g. physical delousing). Such episodes could render large numbers of fish simultaneously prone to infection via damaged skin areas, which would likely become readily colonized by any *M. viscosa* strain present in the seawater and capable of infecting Atlantic salmon. It is therefore possible that the frequency of different *M. viscosa* genotypes within and between outbreaks does not only depend on their individual virulence capacities but also upon their relative and likely fluctuating prevalence in the marine bacterioplankton. Unfortunately, however, we have no concrete data relating to eventual virulence differences between members of the various clonal complexes, their demographics in the marine environment, nor the ecology of this bacterial species outside of commercial fish farming. These are all aspects that merit attention in future studies.

In summary, the MLVA typing assay developed here enabled the identification of three major clonal complexes within *M. viscosa* that each demonstrates a considerable degree of host specificity for Atlantic salmon. It is as yet unclear whether or how the unpredictable and geographically wide-ranging shifts in clonal complex predominance, as well as the often-concurrent presence of multiple genotypes within individual winter ulcer outbreaks and even single fish, may relate to external factors such as vaccination, other operational practices or environmental conditions. Research on the population structure and ecology of planktonic *M. viscosa* may cast some light on the situation and facilitate improved infection control, e.g. through informed farm management decisions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

MLVA profiling results for all *Moritella viscosa* isolates included in the study, in addition to their associated metadata, are listed in Table S1. Genomes used for the phylogenetic analysis were all previously published, and accession numbers are referenced as appropriate in the text.

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