RESEARCH ARTICLE

Multilocus sequence analysis reveals different lineages of *Pseudomonas anguilliseptica* associated with disease in farmed lumpfish (*Cyclopterus lumpus* L.)

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Abstract

The bacterium *Pseudomonas anguilliseptica* has in recent years emerged as a serious threat to production of lumpfish in Norway. Little is known about the population structure of this bacterium despite its association with disease in a wide range of different fish species throughout the world. The phylogenetic relationships between 53 isolates, primarily derived from diseased lumpfish (*Cyclopterus lumpus*) and including a number of reference strains from diverse geographical origins and fish species, were reconstructed by Multi-Locus Sequence Analysis (MLSA) using nine housekeeping genes ( *rpoB*, *atpD*, *gyrB*, *rpoD*, *ileS*, *aroE*, *carA*, *glnS* and *recA*). MLSA revealed a high degree of relatedness between the studied isolates, although seven genotypes identified formed three main phylogenetic lineages. While four genotypes were identified amongst Norwegian lumpfish isolates, a single genotype dominated, irrespective of geographic origin. This suggests the existence of a dominant genotype associated with disease in production of lumpfish in Norwegian aquaculture. Elucidation of the population structure of the bacterium has provided valuable information for potential future vaccine development.

Introduction

Introduction and widespread use of cleaner fish, such as lumpfish (*Cyclopterus lumpus*), for control of sea lice infestations (*Lepeoptheirus salmonis* and *Caligus elongatus*) in Atlantic salmon (*Salmo salar*) aquaculture in Norway presents novel virologic, parasitic and
bacteriological issues. Since the start of production of farmed lumpfish, the bacteria most
commonly associated with disease in this fish species have been *Vibrio anguillarum* \[1\],
‘atypical’ *Aeromonas salmonicida* \[2\], *Moritella viscosa*, *Tenacibaculum* spp., and *Pasteurella* sp. \[3, 4\].
Following its first isolation in 2013, *Pseudomonas anguilliseptica* has however emerged as an
additional and serious threat to lumpfish production in Norway, and the number of outbreaks
has since increased annually \[5\]. *P. anguilliseptica* associated mortalities have also been
reported in lumpfish farmed in Scotland \[6\], Ireland, and the Faroe Islands (Scholz; Christian-
sen; unpublished data).

*P. anguilliseptica* is recognized as an opportunistic pathogen primarily affecting fish in
marine and brackish environments. It was originally described in 1971 as the causative agent
of “red spot disease” (sekiten-byo) in pond cultured Japanese eel (*Anguilla japonica*). Isolated
in 1981 from European eel (*Anguilla anguilla*) in Scotland \[7\], the bacterium became a fre-
cently occurring pathogen associated with eel farming throughout Europe. Initially, *P. angui-
lliseptica* was considered to be exclusively associated with disease in eel culture \[8\]. However,
the bacterium has since been recognized to display little or no host specificity as it has been
shown to be pathogenic in a range of cultured and wild fish species in different geographic
areas (Table 1). With the exception of a small number of isolates from farmed wolffish
(*Anarchichadidae*) and a single isolation from seawater farmed rainbow trout (*Oncorhynchus mykiss*)
in 2018 \[9\], *P. anguilliseptica* infection appears to be particularly associated with disease in
lumpfish in Norway.

Little is known of the population structure of this bacterium despite its association with dis-
ease in numerous fish species over a wide geographic range. While phenotypic studies \[12, 13,
21\], identified few differences, Random Amplification of Polymorphic DNA (RAPD) finger-
printing separated the population into two genetic clades, with one clade almost exclusively
represented by eel isolates.

A subsequent serological study \[22\] identified two serologically distinct groups, termed sero-
types O1 and O2, consistent with the two RAPD groups identified previously. While serotype
O1 were isolated from a diverse range of fish species, serotype O2 isolates were almost exclu-
sively associated with eels. The concordance observed between the genetic and serologic charac-
teristics culminated in the proposal of two separate clonal lineages for the bacterium \[23\].

Given the genetic and serological diversity demonstrated within the species and existence
of both species-specific variants and broad host range variants, the aim of the present study
was to elucidate the phylogenetic relationships amongst Norwegian isolates of *P. anguillisept-
tica* isolated from farmed lumpfish. A multilocus sequence analysis (MLSA) scheme compris-
ing nine housekeeping (HK) genes was therefore designed to phylogenetically reconstruct the
population structure of a variety of host derived, geographically disparate and historically dis-
tinct isolates of *P. anguilliseptica*. The knowledge generated may shed light on infection
dynamics, allow development of specific diagnostics and identify strains relevant for future
vaccine development.

**Materials and methods**

**Confirmation of isolate identity**

A total of 53 isolates were included in the analysis. These were sampled from a range of differ-
ent spatiotemporal origins and fish species (Table 2). All isolates originated from clinically
infected fish and were cultivated on blood agar w/2% NaCl at 15˚C for 48 hours. Matrix Assis-
ted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) was per-
formed on all isolates, while 16S rRNA gene sequencing and/or phenotypic characterization
was used in some cases to positively confirm species identity prior to the MLSA.
MALDI-TOF. MALDI-TOF analysis was performed on a Biotyper Microflex LT (Bruker Daltonics, Bremen, Germany). In-house main spectra (MSP) were generated for *P. anguilliseptica* isolate NVIB 50–2084 and type strain NCIMB 1949. For MSP generation, proteins from each reference strain were extracted according to the manufacturer’s protocol (S1 Protocol).

Following incorporation of constructed MSPs into the reference library, single colonies of putative *P. anguilliseptica* were subjected to MALDI-TOF analysis by the Direct Transfer Method according to the manufacturer’s instructions. Briefly, single colonies were smeared with a toothpick as a thin film onto two successive spots on a MALDI target plate. After drying at room temperature, the spots were overlaid with HCCA matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile 2.5% trifluoracetic acid) and air-dried. Identification was then performed using the standard Biotyper Database supplemented with the in house generated MSPs. Similarity scores ≥ 2.0 in relation to any particular MSP were considered to represent good identification to the species/genomovar level.

**Phenotypic characterization.** Phenotypic characterization was conducted on a representative selection of six isolates derived from various fish host species and geographic-/temporal origins (NVIO 10973, NVIO 8905, NVIO 11214, NVIB 50–2255, NVIB 50–1910 and NVIO 9976; Table 2). The characteristics examined were: colony morphology, gram staining, motility, anaerobic respiration and hemolysis on blood agar. Biochemical properties such as oxidative/fermentative (O/F) production of acid from glucose, as well as the ability to produce decarboxylases from the amino acids arginine, lysine and ornithine (ALO), were also tested. Production of oxidase was tested using the DrySlide Oxidase (BD, Franklin Lakes, USA).

**DNA extraction, PCR and sequencing**

Template DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer’s protocol. Concentration and purity of nucleic acid in the final eluate was assessed with the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, USA). All samples were diluted to 2 ng/μl before being stored at -20°C.

The following housekeeping (HK) genes were included in the MLSA assay: *rpoB* (RNA polymerase beta subunit), *atpD* (ATP synthase F1 beta subunit), *gyrB* (DNA gyrase beta subunit).
# Table 2. Isolates of *Pseudomonas anguilliseptica* included in this study.

| Isolate number | Year of isolation | Strain | Serotype | Fish species          | Origin of isolate | Source |
|----------------|-------------------|--------|----------|-----------------------|-------------------|--------|
| NCIMB 1949$^{T}$ | 1998              | NCIMB 1949 | O2       | European eel          | Japan             | NCIMB  |
| NVIO 11299      | 2018              | Lumpfish |          | Ireland               | FVGI              |        |
| NVIO 11300      | 2018              | Lumpfish |          | Ireland               | FVGI              |        |
| NVIO 11301      | 2018              | Lumpfish |          | Ireland               | FVGI              |        |
| NVIO 11302      | 2018              | Lumpfish |          | Ireland               | FVGI              |        |
| NVIO 11303      | 2018              | Lumpfish |          | Ireland               | FVGI              |        |
| NVIO 9942       | 2015              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 10973      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 11158      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 11159      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 11160      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 11161      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 11162      | 2017              | Lumpfish |          | Faroe Islands         | FFVA              |        |
| NVIO 8180       | 2011              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 8227       | 2012              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 9976       | 2015              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 10039      | 2015              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 10341      | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 10449      | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 10550      | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 10726      | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–927     | 2015              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1353    | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1579    | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1705    | 2016              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1763    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1825    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1846    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1895    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1910    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1914    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–1952    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–2015    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–2095    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIB 50–2040    | 2017              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 11313      | 2018              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 11370      | 2019              | Lumpfish |          | Norway                | NVI               |        |
| NVIO 8905       | 2013              | Wolfish (*Anarhichas lupus*) | | Norway                | NVI               |        |
| NVIB 50–2084    | 2017              | Ballan wrasse (*Labrus bergylta*) | | Norway                | NVI               |        |
| NVIB 50–2255    | 1992              | STR-6   |          | Baltic herring        | Baltic sea        | ÅAU    |
| NVIB 50–2260    | 1992              | STR2-1  |          | Baltic herring        | Baltic sea        | ÅAU    |
| NVIB 50–2256    | 1991              | P57B/91 |          | Rainbow trout         | Finland           | ÅAU    |
| NVIB 50–2257    | 1993              | 0506-F05|          | Brown trout (*Salmo trutta*) | Finland       | ÅAU    |
| NVIB 50–2258    | 2000              | P19/00  | O1       | Atlantic salmon       | Finland           | ÅAU    |
| NVIB 50–2259    | 2008              | P33-6/08|          | Whitefish             | Finland           | ÅAU    |
| NVIB 50–2262    | 2015              | P17-3/15|          | Whitefish             | Finland           | ÅAU    |
| NVIB 50–2261    | 2009              | P30-5/09|          | Rainbow trout         | Finland           | ÅAU    |

(Continued)
subunit), rpoD (RNA polymerase, sigma factor), ileS (isoleucyl-tRNA synthetase), aroE (shikimate dehydrogenase), carA (carbamoyl-phosphate synthase small chain), glnS (glutamyl-tRNA synthetase), and recA (recombinase A). PCR primers were designed for each of the nine housekeeping genes based on homologous sequences from *Pseudomonas aeruginosa*, *P. orzhizhabitans* and *P. anguilliseptica* Type strain DSM 12111T, retrieved from public databases (S1 Table). Alignment and primer design was performed in Geneious (Biomatters, Auckland, New Zealand). Primers are listed in Table 3. Forward and reverse M13 primer sequences (Thermo Fisher Scientific) were added to each primer set for downstream sequencing of PCR amplicons. M13 Forward: 5’d[GTAAAACGACGGCCAG]3’, M13 Reverse: 5’d[CAGGAAACAGCTATGAC]3’.

Amplification of HK genes by PCR was based on a standard reaction mixture containing (per reaction) 4 μl 5xGreen GoTaq Flexi buffer, 1.5 μl MgCl₂ Solution (25 mM), 0.4 μl dNTP (10 mM), 1 μl of forward and reverse primers (10 μM), 0.1 μl Go Taq G2 Flex DNA

**Table 2. (Continued)**

| Isolate number | Year of isolation | Strain  | Serotype | Fish species | Origin of isolate | Source |
|----------------|-------------------|---------|----------|--------------|-------------------|--------|
| NVIO 11214     | 2018              | AZ/210-1| O1       | Turbot       | Spain             | CIBUS  |
| NVIO 11215     | 2018              | AZ/210-2| O1       | Turbot       | Spain             | CIBUS  |
| NVIO 11216     | 2018              | AZ/211-1| O1       | Turbot       | Spain             | CIBUS  |
| NVIO 11217     | 2018              | AZ/211-2| O1       | Turbot       | Spain             | CIBUS  |
| NVIO 11219     | 2018              | TW47/L1 | O1       | Gilthead Sea Bream | Spain | CIBUS  |
| NVIO 11220     | 2018              | TW75/L3 | O1       | Gilthead Sea Bream | Spain | CIBUS  |

Source annotation: NCIMB (National Collection of Industrial Food and Marine Bacteria), FVGI (Fish Vet Group Ireland), FFVA (Faroes Food and Veterinary Agency), NVI-B/O (Norwegian Veterinary Institute, Bergen/Oslo), ÅAU (Åbo Akademi University), CIBUS (Centro de Investigaciones Biológicas, Universidade de Santiago de Compostela). Type strain NCIMB 1949.

**Table 3. Primers (without M13) for each of the nine housekeeping genes included in the analysis.**

| Primer        | Sequence (5’-3’) | Gene product                  | Size of target sequence (bp) | PCR product post trim (bp) | Annealing temp (˚C) | Reference |
|---------------|------------------|-------------------------------|-------------------------------|----------------------------|---------------------|-----------|
| aroE-42 F     | CAAGTGCAGGCCCTGATCTCATC | Shikimate dehydrogenase | 653                           | 504                        | 56˚C                | This study |
| aroE-761 R    | GTTCAGACACGATGCCCAG  |                               |                               |                            |                     |           |
| atpD-114 F    | ACCCTGAAGTTGTCAGCAGA | ATP synthase F1, beta subunit | 808                           | 645                        | 56˚C                | This study |
| atpD-965 R    | TACAAAGGTGCGCTGCCAGA |                               |                               |                            |                     |           |
| carA-143 F    | CGGATTCTCTCTAATGCCAGC | Carbamoyl-phosphate synthase  | 762                           | 525                        | 56˚C                | This study |
| carA-956 R    | GTTCTGCTCGGATCTACAC  |                               |                               |                            |                     |           |
| glnS-238 F    | GCCCAAGAGACCCAGGAG   | Glutamyl-tRNA synthetase      | 587                           | 528                        | 56˚C                | [24]      |
| glnS-863 R    | CTTGCCTTGCTGTAATCG   |                               |                               |                            |                     |           |
| ileS-43 F     | TTTCCGATGAAAGCCCGGC  | Isoleucyl-tRNA synthetase     | 708                           | 645                        | 56˚C                | [24]      |
| ileS-788 R    | GGTAAACTCGGCGTGAAAGCT |                               |                               |                            |                     |           |
| rpoB-3,307 F  | TGGGTTCTCGGTGATCTACGT | RNA polymerase, beta subunit  | 529                           | 504                        | 56˚C                | [24]      |
| rpoB-3,878 R  | GAACCTGACCCATTACACCC  |                               |                               |                            |                     |           |
| rpoD-294 F    | GACCCAGTCGGCATGTACAT  | RNA polymerase sigma factor   | 766                           | 732                        | 56˚C                | This study |
| rpoD-1,204 R  | ATGCGACGGTGATCTCCCTT  |                               |                               |                            |                     |           |
| recA-136 F    | CTCGGCTTGAGTCCACGATCG | Recombinase A           | 740                           | 636                        | 56˚C                | This study |
| recA-959 R    | CTCGGCTTGAGTCCACGATCG |                               |                               |                            |                     |           |
| gyrB-329 F    | ACAGTCTAACAGTGGTTTCCGC | DNA gyrase, beta subunit B    | 703                           | 645                        | 56˚C                | This study |
| gyrB-1,089 R  | CTGGCCATTTTCCGCTGGA   |                               |                               |                            |                     |           |

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polymerase, 8 μl nuclease-free water, and 4 μl DNA template, amounting to a total reaction volume of 20 μl. All reagents were supplied by Promega (Madison, USA).

PCR was performed in an Agilent SureCycler 8800 Thermal Cycler (Santa Clara, USA) and involved 95˚C for 3 min, 30 cycles of denaturation (95˚C for 1 min), annealing (56˚C for 1 min) and elongation (72˚C for 1 min), followed by 72˚C for 5 min and cooling to 4˚C indefinitely. Amplification of PCR-products of the desired size was confirmed by gel electrophoresis using the GelPilot® 100bp Plus Ladder (QIAGEN, Venlo, Netherlands) as reference. PCR products were purified with AMPureXP® (Beckman Coulter, Pasadena, USA) using the Biomek4000 pipetting robot (Beckman Coulter, Pasadena, USA).

Cycle sequencing of purified PCR products was based on a reaction mixture containing (per reaction) 0.5 μl BigDye v3.1, 2.0 μl 5X Sequencing Buffer, 2.0 μl M13 primer (2.5 μM), 2.0 μl purified PCR product and 3.5 μl MilliQ-water, and involved 96˚C for 1 min, 25 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 4 min, followed by cooling to 4˚C indefinitely. This was performed using an Applied Biosystems® Veriti® 96-Well Thermo Cycler (ThermoFisher Scientific, Waltham, USA). Sequence products were purified using the BigDye® XTerminator Purification Kit (ThermoFisher Scientific, Waltham, USA) in accordance with its protocol. Capillary electrophoresis of purified sequence products was performed using the 3500XL Genetic Analyzer (ThermoFisher Scientific, Waltham, USA) according to the manufactures protocol.

Data analysis
Forward and reverse strands for each amplicon were sequenced for all isolates and imported to Geneious 11.1.5 (Biomatters, Auckland, New Zealand) for contig assembly and further processing. Contigs were manually checked and edited and re-sequencing performed when necessary. Contigs were then trimmed at the 5’ and 3’-ends to exclude primer sequences and areas of poorer quality towards the amplicon ends. All sequences were trimmed to preserve the reading frame. Final processed sequences were imported and concatenated in Microsoft Excel. Concatenated sequences were then re-imported to Geneious to construct the final alignment. Complete record of partial gene sequences for all isolates was submitted to GenBank (accession nos. MW684870—MW685347).

Multi-Locus sequence analysis
Based on the concatenated alignment, a maximum likelihood phylogenetic reconstruction was run in MEGA X using the TN93 substitution model [25] (identified as optimal by the model test module). Number of bootstraps was set to 1000, while all other inputs were set to default. The phylogeny was similarly reconstructed for individual genes and for a concatenated amino acid alignment (LG substitution model [26] used for the latter).

Results
Confirmation of isolate identity
MALDI-TOF analysis of samples submitted for the study positively identified all 53 isolates as *P. anguilliseptica* with similarity scores ≥2.0. 16S rRNA gene sequencing of 39 isolates also demonstrated concordance with MALDI-TOF results.

With the exception of production of arginine dihydrolase (isolate NVIO 8905 negative), the studied isolates were phenotypically similar. The results of phenotypical testing are listed in Table 4.
Multi-Locus sequence analysis

The concatenated alignment of nine housekeeping genes (5,364bp) displayed average sequence identities of 99.9% between all isolates. Phylogenies inferred from MLSA based on nucleotide sequences revealed seven genotypes distributed amongst three major lineages (Fig 1). Inspection of the tree shows that lumpfish isolates are dispersed throughout depending on country of origin (i.e. Norwegian isolates in lineages 1 and 2, Faroese in lineage 1 and Irish in lineages 1 and 3), while isolates from other fish species (irrespective of geography) belong almost exclusively to lineage 1. The type strain NCIMB 1949 from Japanese eel in Japan constitutes one exception to this, as it forms a singleton genotype within lineage 3. Phylogenetic reconstruction based on concatenated amino acid sequences displayed compatible topologies (Fig 2). Maximum likelihood trees generated from single gene alignments displaying genetic heterogeneity (\textit{rpoD}, \textit{rpoB}, \textit{carA}, and \textit{atpD}) are attached as (S1–S4 Figs). For \textit{aroE}, \textit{glnS}, \textit{ileS}, \textit{recA} and \textit{gyrB}, complete genetic homogeneity was observed across all 53 isolates.

Discussion

The present work represents the most comprehensive phylogenetic study to date of the fish pathogenic bacterium \textit{P. anguilliseptica} originating from a range of different geographic localities, fish species and time of isolation.

MLSA revealed a high degree of genetic similarity irrespective of geographic origin, fish host species or time of isolation. The significant degree of genetic conservation identified amongst isolates retrieved from a wide geographic area over a period of 21 years suggest ‘purifying’ selection pressure towards a restricted fish parasitic/pathogenic niche. Evolution of virulent host associated lineages from ancestral environmental strains has previously been described from \textit{Francisella tularensis} [27]. As in previous studies relating to \textit{P. anguilliseptica}, all isolates studied here originated from clinically infected fish. No environmental isolates were available. Had such isolates been available they may have revealed a greater intraspecific diversity. While the possibility that fish pathogenic \textit{P. anguilliseptica} represent expansion of a fish pathogenic clone against a background of environmental, possibly non-virulent strains cannot be ruled out, there is, however, no literature published to date suggesting the existence of environmental strains of this bacterium.
A degree of phylogenetic heterogeneity was, however, identified. The phylogenetic patterns identified appear to be more related to geographic origin than chronology or host fish species. The Norwegian isolates, originating with the exception of two isolates from wolffish and ballan wrasse, from farmed lumpfish, were distributed between two of the three major lineages (lineage 1 and 2, Fig 1). Lineage 1 is the most numerous of the three, with isolates originating from most geographic localities and fish species. Approximately a third of all Norwegian isolates are situated within this lineage, while the rest are situated in the exclusively Norwegian lineage 2. Although the Norwegian isolates were distributed amongst two phylogenetic lineages, no distinct differences were registered between these lineages in terms of domestic geography or time of isolation. MLSA based on concatenated amino acid sequences displayed a pattern consistent with nucleotide sequence analysis in the formation of three major lineages.

Following identification of the various lineages by MLSA, MALDI-TOF MSP’s were generated from NVIB 50–2084 and NCIMB 1949, representing MLSA lineages 1 and 3 respectively (Fig 1). Subsequent MALDI-TOF analysis of the entire study collection utilizing these MSP’s revealed that MALDI-TOF may be capable, with further refinement, of distinguishing between the different lineages observed, and may therefore prove a valuable future diagnostic tool for discrimination of these lineages.
The results of phenotypic characterization were in compliance with previously published descriptions [12, 13, 21] with the exception of a single isolate which was negative for production of arginine dihydrolase (Table 4). A complementary analysis of all isolates included in the present study showed three isolates (NVIO 8827, NVIO 8905, and NVIB 50–1579), all situated within lineage 2, negative for arginine dihydrolase production. This appears to be a variable trait only within this lineage. The existence of phenotypic variation beyond the phylogenetic resolution achieved through MLSA can thus not be ruled out.

Previous studies have suggested the existence of antigenic variation within *P. anguilliseptica*. Seven of the isolates studied in Lopez-Romalde et al. [15, 22] were used in the present study (see Table 1). All included isolates representing serotype O1 were situated within the same phylogenetic lineage (Fig 1) and it is reasonable to assume that all isolates within this lineage probably belong to serotype O1.
Notably, all but one of the Irish lumpfish isolates included in this study clustered closely with the type strain NCIMB 1949, isolated from Japanese eel in Japan, within lineage 3 (Fig 1). The serological differences identified by López Romalde et al. [22] between representatives of lineage 1 (O1) and NCIMB 1949 (O2) suggest that these Irish isolates might potentially also belong to serotype O2. Future antigenic characterization would be required to determine whether this is the case. Irish isolate NVIO 11302 deviates, however, from this pattern and is situated within the geographically spread lineage 1 (Fig 1). This situation could either be due to the existence of several genotypes associated with disease in Irish lumpfish or possibly indicate spread of infection via import of Norwegian lumpfish-roe to Ireland.

Of the Norwegian isolates, 8 of 26 were situated within lineage 1, likely representing serotype O1. The remaining, and thereby the majority of Norwegian isolates, were situated in lineage 2, which shows comparable phylogenetic separation from lineage 1 as are at least as phylogenetically distant, if not slightly more distant to lineage 1 than NCIMB 1949 (serotype O2) (Fig 1). This raises the question as to whether lineage 2 may represent a previously yet undescribed serotype. The fact that all Norwegian isolates are distributed between two of the major phylogenetic lineages (1 and 2), possibly representing different serotypes, complicates identification of a suitable candidate strain for vaccine development. Characterization of the serological properties of Norwegian isolates will therefore be necessary with respect to the potential development of a mono- or multi-genotype vaccine for future use in lumpfish.

Interestingly, isolates of identical genotype were identified from Norwegian lumpfish and diseased Atlantic salmon in Finland (Fig 1). The identical genotype and the pathogenic nature of the bacterium [12] suggests that *P. anguilliseptica* might potentially pose a threat to cohabiting salmonids in commercial net pens in Norway. However, considering the ever-increasing number of outbreaks among lumpfish and the lack of reported infections in cohabiting salmon, this indicates that Norwegian salmon are not highly susceptible to *P. anguilliseptica* infection. While future whole genome sequencing studies may reveal sub-MLSA genotype differences related to host specificity, establishment of a challenge model to test salmon susceptibility to the Norwegian lumpfish strain should be considered to evaluate the potential risk of cross-species transmission.

**Conclusion**

The nine-locus MLSA employed in this study identified a considerable degree of genetic similarity among isolates of *P. anguilliseptica*, separating the studied isolates amongst seven closely related genotypes. Several genotypes were identified amongst Norwegian lumpfish isolates, although the majority belonged a single overarching lineage. Antigenic study of representative isolates from each genotype should be evaluated as a basis for development of vaccines against this important lumpfish pathogen.

**Supporting information**

S1 Protocol. MALDI-TOF Main Spectra Profile (MSP) generation for *P. anguilliseptica*. (DOCX)

S1 Table. Locus accession numbers (NCBI) for *P. aeruginosa*, *P. oryssihabitans* and *P. anguilliseptica* for alignment and PCR primer design. (DOCX)

S1 Fig. Maximum likelihood tree based on *rpoD* sequence (766 bp). (DOCX)
S2 Fig. Maximum likelihood tree based on rpoB sequence (507 bp).
(DOCX)

S3 Fig. Maximum likelihood tree based on carA sequence (526 bp).
(DOCX)

S4 Fig. Maximum likelihood tree based on atpD sequence (645 bp).
(DOCX)

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