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Mortality outbreak by perch rhabdovirus in European perch (*Perca fluviatilis*) farmed in Italy: Clinical presentation and phylogenetic analysis

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Mortality outbreak by perch rhabdovirus in European perch (*Perca fluviatilis*) farmed in Italy: Clinical presentation and phylogenetic analysis / Caruso, Claudio; Gustinelli, Andrea; Pastorino, Paolo; Acutis, Pier Luigi; Prato, Riccardo; Masoero, Loretta; Peletto, Simone; Fioravanti, Maria Letizia; Prearo, Marino. - In: JOURNAL OF FISH DISEASES. - ISSN 0140-7775. - STAMPA. - 42:5(2019), pp. 773-776. [10.1111/jfd.12975]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/685962> since: 2019-08-28

*Published:*

DOI: <http://doi.org/10.1111/jfd.12975>

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(Article begins on next page)

This is the peer reviewed version of the following article:

Caruso, C, Gustinelli, A, Pastorino, P, et al. Mortality outbreak by perch rhabdovirus in European perch (*Perca fluviatilis*) farmed in Italy: Clinical presentation and phylogenetic analysis. *J Fish Dis.* 2019; 42: 773– 776.

which has been published in final form at <https://doi.org/10.1111/jfd.12975>

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1 **MORTALITY OUTBREAK BY PERCH RHABDOVIRUS (PRV) IN EUROPEAN PERCH**  
2 **(*PERCA FLUVIATILIS*) FARMED IN ITALY: CLINICAL PRESENTATION AND**  
3 **PHYLOGENETIC ANALYSIS**

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13 Running title: PrV in European perch

14 Abstract word count: **130**

15 Text word count: **1438** (including Acknowledgements, authors' contributions and conflict of  
16 interest statement).

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35 **ABSTRACT**

36 This work reports a mortality outbreak, occurred in 2015 and affecting juveniles of European perch  
37 (*Perca fluviatilis* L.) farmed in Italy. Perch Rhabdovirus (PRV) was detected by viral isolation and  
38 biomolecular investigations. Phylogenetic analysis clustered our isolate into genogroup B, which  
39 also includes PRV isolates from *Perca fluviatilis* identified in France (2004-2009); diagnostic  
40 investigations also revealed opportunistic bacteria (*Aeromonas hydrophila*) and parasites  
41 (*Chilodonella piscicola*). Since, occasionally, PRV has been reported in the natural environment,  
42 which is often a source of eggs and broodstock for farms, it could be possible that both similar  
43 France and Italian isolate were imported from a same place elsewhere and have a common origin.  
44 Improving biosecurity measures (batch control) and disinfection of egg strings with an iodine  
45 based solution helps prevent apparent vertical transmission of PRV.

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50 **KEYWORDS:** Perch Rhabdovirus; *Perca fluviatilis*; Rhabdoviridae; Phylogenetic analysis

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69 Among all known viral diseases in aquaculture, Rhabdoviruses have the most important impact,  
70 based on their pathogenicity, wide host-spectrum and worldwide diffusion. *Rhabdoviruses* (family  
71 *Rhabdoviridae*) contain a single molecule of linear, negative-sense RNA (Talbi et al., 2011). The  
72 International Committee on Taxonomy of Viruses (ICTV) divided the family *Rhabdoviridae* in  
73 eighteen different genera; among these, the genera *Novirhabdovirus*, *Vesiculovirus*, *Sprivirus*  
74 and *Perhabdovirus* contain viruses infecting both marine and freshwater fish. *Perhabdovirus*, a new  
75 genus of fish rhabdoviruses, includes three important species: *Perch Perhabdovirus* (PRV),  
76 *Anguillid perhabdovirus* (AngRV) and *Sea trout perhabdovirus* (STRV). PRV infecting European  
77 perch (*Perca fluviatilis*) is of particular interest since its impact is increasing with the farm  
78 intensification in Europe and North America.

79 The first case of PRV pathogenic for perch was reported in the early 1980 in France, in wild *Perca*  
80 *fluviatilis* (Dorson et al., 1984). Outbreaks of PRV infection were also reported in Denmark,  
81 Ireland, Germany, Norway, and Switzerland (Jørgensen et al. 1993; Dorson et al., 1984; Dannevig  
82 et al., 2001; Bigarrè et al., 2017; Wahli et al., 2015). More recently, PRV was detected for the first  
83 time in Finland in grayling fry (*Thymallus thymallus* L.) (Gadd et al., 2013).

84 In 2015, a batch of juveniles of European perch (*Perca fluviatilis* L.) from a broodstock group  
85 composed of internal production and one bought abroad in another farm, were stocked in tanks in a  
86 commercial perch farm located in Italy. Juveniles showed abnormal swimming behavior 3 days  
87 after stocking and cumulative mortality reached 95% in a week. Clinical signs were rather aspecific,  
88 with respiratory distress, swimming on the water surface and looping and they gave rise to concerns  
89 over the possibility of a transmissible disease. A sample of 30 fish were submitted to  
90 parasitological, bacteriological and virological examinations, according to standard laboratory  
91 procedures. For virological analysis monolayer cell cultures of bluegill fry (BF-2) and epithelioma  
92 papulosum cyprinid (EPC) cell lines were used. The 24-well plates were incubated at  $15\pm 2^{\circ}\text{C}$ .  
93 Cultures were daily inspected for the occurrence of cytopathic effect (CPE). For PRV biomolecular  
94 detection, total RNA was extracted both from pooled juvenile and from 350  $\mu\text{l}$  of cell culture  
95 supernatant as previously described by Caruso et al. (2014). Reverse transcription (RT) was carried  
96 out using High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) in a total  
97 volume of 20  $\mu\text{l}$  per reaction. To detect PRV in samples, the primers from Gadd et al. (2013) were  
98 used to amplify the L gene. PCR was performed using Platinum<sup>TM</sup> Taq DNA Polymerase (Invitro-  
99 gen) in a final volume of 25  $\mu\text{l}$  containing 2.5  $\mu\text{l}$  of 10 $\times$  PCR Reaction Mix, 500 nM of forward and  
100 reverse primer, 200  $\mu\text{M}$  of dNTP, 500  $\mu\text{M}$  of  $\text{MgCl}_2$ , and 1U/reaction of Taq DNA polymerase and  
101 2.5  $\mu\text{l}$  of cDNA. The PCR conditions were as follows: initial step of 10 min at  $95^{\circ}\text{C}$ , 30 cycles of  
102  $94^{\circ}\text{C}$  for 1 min,  $50^{\circ}\text{C}$  for 2 min and  $72^{\circ}\text{C}$  for 2 min, followed by a final extension step of 15 min at

103 72°C. PCR products were visualized by gel electrophoresis and sequenced with both the forward  
104 and reverse PCR primers using ABI - PRISM Big Dye Terminator v.3.1 cycle sequencing kit.  
105 Subsequently, for phylogenetic analysis, primers pairs oPVP116 and oPVP118 (Talbi et al., 2011),  
106 and oPVP126 (Talbi et al., 2011) and Rha-G-seqR2 (Gadd et al., 2013) were used to obtain the  
107 complete viral G gene sequence in two overlapping fragments. The expected amplicon size for  
108 primers oPVP116&oPVP118 was approximately 1400 bp, while for primers oPVP126&Rha-G-  
109 seqR2 was 676 bp. Purified DNA was used for sequencing both strands with an ABIPRISM Big  
110 Dye Terminator v.3.1 cycle sequencing kit. The consensus sequence was determined by the  
111 alignment of forward and reverse strand sequences of each genetic region in a single alignment  
112 using SeqMan and sequence similarities were calculated using MegAlign (Lasergene package,  
113 DNASTAR Inc.). Multiple sequence alignment was performed with BioEdit version 7.0.5.2  
114 software using CLUSTAL W. MEGA version 7 was used for p-distance matrices calculation and  
115 phylogeny inference according to the Neighbor-Joining method. The nucleotide substitution model  
116 was Kimura two-parameters. The robustness of the hypothesis was tested in 1.000 parametric  
117 bootstrap analyses.

118 Differential diagnosis with other common viral pathogens was also carried out. Parasitological  
119 analyses allowed to detect *Chilodonella piscicola* in the gills with mild to severe infection intensity.  
120 Bacteriological analysis allowed the isolation of *Aeromonas hydrophila*.

121 Virus isolation revealed CPE at the second passage both on BF-2 and EPC cells. CPE consisted in  
122 large area of monolayer destruction in BF-2 cell line, and multifocal syncytia in EPC. Scientific  
123 literature describes the possibility to evaluate the CPE of Rhabdovirus in many cell cultures  
124 (Lorenzen et al., 1999) but in this case, EPC and BF-2 cell lines have been successfully used for  
125 viral isolation resulting particularly suitable for Rhabdoviruses isolation according to Bigarrè et al.  
126 (2017). Afterwards, biomolecular methods carried both on pooled juvenile fry (screening) and the  
127 supernatant of EPC and BF-2 cells allowed the identification of PRV showing a 399 bp band  
128 corresponding to the expected length, even if weaker in pooled fry. Differential diagnosis with other  
129 common viral pathogens was negative. Phylogenetic analysis (Figure 1) based on the complete  
130 sequence of the glycoprotein G gene (1.560 nts) revealed that our isolate clusters into genogroup B,  
131 which includes other PRV isolates from *Perca fluviatilis* identified in France (2004-2009) that share  
132 98.6-99.2% similarity with our sequence at the glycoprotein G gene. Genogroup B also includes  
133 one isolate from the Baltic Sea and PRV sequences 196 identified in other host species, like *Salmo*  
134 *trutta* L. (Baltic Sea, 2009), *Thymallus thymallus* (Finland, 2002-2010) (Gadd et al., 2013).  
135 However, all these sequences have relatively lower similarities (95.3-96.9%). Interestingly, the only  
136 other Italian isolate (27V10) clusters within genogroup A.

137 Molecular data showed high identity of the Italian PRV isolate with a France strain; in a previous  
138 work, Talbi et al., (2011) also showed a striking genetic similarities between perch rhabdoviruses  
139 and isolates from other European countries and various ecological niches, most likely reflecting the  
140 circulation of viruses through fish trade as well as putative transfers from marine to freshwater fish;  
141 indeed, PRV is not a problem only connected to fish farming since, occasionally, it has been  
142 reported in the natural environment, which is often a source of eggs and broodstock for farms. It  
143 could be possible that both similar France and Italian isolate were imported from a same place  
144 elsewhere in Europe and have a common origin. Diagnostic investigations also revealed the  
145 presence of *Aeromonas hydrophila*, an opportunistic pathogen widely distributed in freshwater  
146 environment (Austin & Adams, 1996; Burr et al., 2012), and the parasites *Chilodonella piscicola* in  
147 the gills; these microorganism may have had a minor role in the onset of symptoms with a  
148 secondary pathogenic role to the PRV infection. No quarantine or biosecurity measures (such as  
149 isolation of new stock) were applied in the Italian farm; since most percid Perhabdoviruses  
150 reported to date originate from wild animals introduced into farms (Dorson et al., 1984; Dannevig et  
151 al., 2001; Ruane et al., 2014), careful disinfection of egg strings with an iodine based solution helps  
152 prevent apparent vertical transmission of PRV.

153 Following observed results, it could be useful to insert the research of PRV in monitoring plans,  
154 above all for trading purpose. Despite the increasing interest on the impact of rhabdoviruses in  
155 percid farming, few data are still available in literature and the diversity of the viral populations is  
156 still poorly investigated (Talbi et al., 2011). Therefore, it is important to acquire epidemiological  
157 data, through reporting PRV outbreaks, which allow to add and share more clinical and molecular  
158 information. Moreover, additional information on genetic diversity, transmission routes and  
159 alternative hosts for perch rhabdoviruses are needed to understand their evolution; improving  
160 knowledge on PRV epidemiology will lead to better prevent and control the disease both in farmed  
161 and in wild perch populations.

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#### 164 **ACKNOWLEDGEMENTS**

165 We warmly would like to thank Dr. Riikka Holopainen Finnish Food Safety Authority (Evira) to  
166 provide the positive PRV PCR control. We also thank to Luisa De Marco, Elena Gobbi, Erika  
167 Messana, Sabrina Nodari and Paola Arsieni of IZS PLV (Turin) for excellent technical assistance.

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171 **AUTHORS' CONTRIBUTIONS**

172 MP and MLF coordinated this study. CC and AG wrote the paper; CC, RP and PP performed the  
173 molecular and virological analysis. SP performed typing PCRs, sequencing and phylogenetic  
174 analyses. AG and MLF carried out the sampling, bacteriological and parasitological examination.  
175 PLA, LM, MLF and MP critically read the manuscript. All Authors read and approved the final  
176 manuscript.

177

178 **CONFLICT OF INTEREST STATEMENT**

179 None of the authors of this paper has a financial or personal relationship with other people or  
180 organizations that could inappropriately influence or bias the content of the paper.

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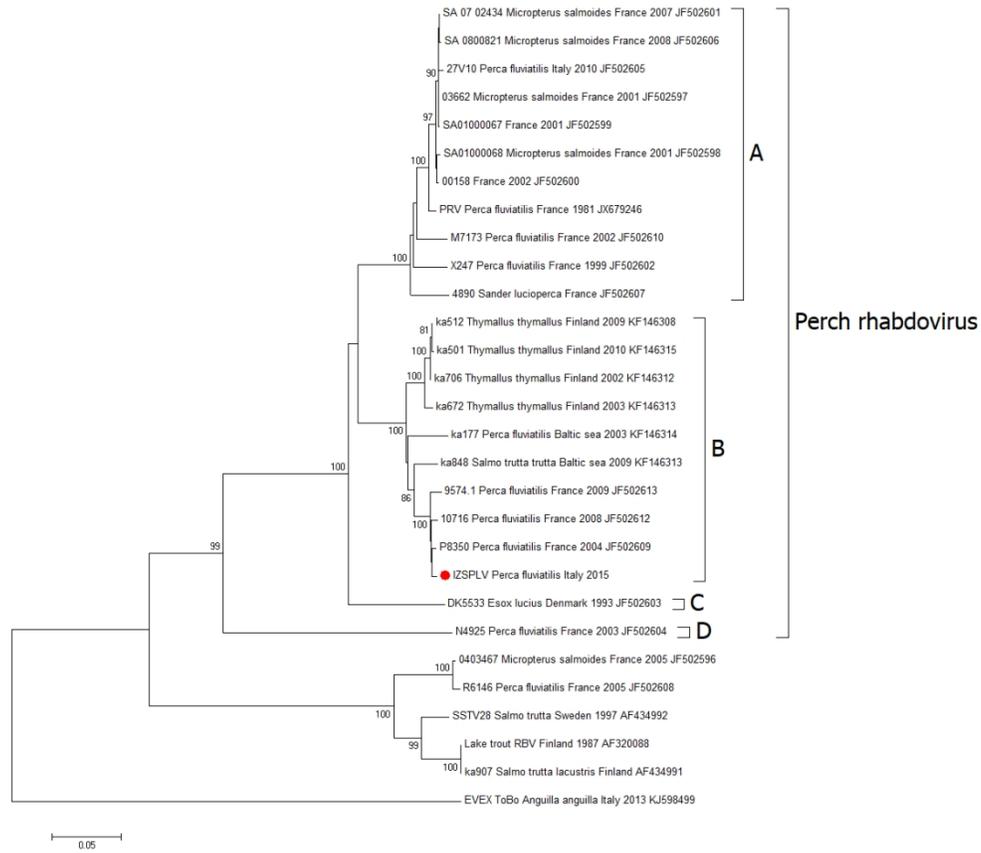


Figure 1 Phylogeny inferred by Neighbor-Joining analysis obtained by an alignment of 1,560 nucleotides, covering the glycoprotein (G) gene region of PRV. The phylogenetic tree includes the PRV isolate of this study (marked by the red circle; GenBank acc. No. MK360920) and sequences available in GenBank. Host species, country of origin, collection year and accession number are indicated for each sequence. Eel Virus European X (EVEX) was used as outgroup. Bootstraps (1,000 replicates) values > 80 are shown at the internal nodes. The length of each pair of branches represents the distance between sequence pairs. The scale bar represents the percentage of nucleotide differences.