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Molecular investigations of outbreaks of *Perch perhabdovirus* infections in pike-perch

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ABSTRACT: In 2016, a total of 5 massive mortality episodes each affecting hundreds of thousands of pike-perch *Sander lucioperca* larvae occurred at 2 sites in 2 Western European countries. For each episode, perhabdoviruses related to the perch rhabdovirus (PRV) were detected in samples, using either PCR or cell culture combined with PCR. The sequences of the glycoprotein (*g*), phosphoprotein (*p*) and nucleoprotein (*n*) genes of these samples demonstrated that 2 different genotypes were present at 1 site, each associated with 1 of the 3 episodes. At the other site, a single genotype was associated with the 2 outbreaks. Furthermore, this genotype was strictly identical to 1 genotype involved in the outbreaks of the first site, strongly suggesting a common origin for these 2 viruses. The common origin was confirmed *a posteriori* because some larvae introduced to both sites had exactly the same geographic origin in Eastern Europe. Taken together, the molecular and epidemiological data suggest that both horizontal and vertical transmission of 2 distinct strains of perhabdoviruses were involved in the various outbreaks affecting pike-perch.

KEY WORDS: Pike-perch · *Sander lucioperca* · Perch rhabdovirus · Percidae · Virus · Epizooty

INTRODUCTION

The genus *Perhabdovirus* regroups 3 viral species pathogenic to fish: *Perch perhabdovirus* (e.g. perch rhabdovirus, PRV), *Sea trout perhabdovirus* (e.g. lake trout rhabdovirus, LTRV) and *Anguillid perhadovirus* (e.g. eel virus European X, EVEX) (www.talk.ictvonline.org). To date, *Anguillid perhabdovirus* has been found only in eels, whereas PRV and LTRV have been detected in various fish species during clinical episodes in farms or experimental facilities in Europe (Betts et al. 2003, Talbi et al. 2011, Gadd et al. 2013). In particular, in the last 3 decades, wild and farmed percids (European perch *Perca fluviatilis*, pike-perch *Sander lucioperca*) have been affected by PRV or LTRV in different countries, such as France, Norway, Ireland and Switzerland (Dorson et al. 1984, Nougayrède et al. 1992, Dannevig et al. 2001, Rodger & Girons 2008, Ruane et al. 2014, Wahli et al. 2015). The disease has been reported in fry and adults, with high mortality rates in some cases. Symptoms observed in adults are variable. Fish often exhibit abnormal swimming behavior and lethargy, as well as hemorrhaging at the base of the fins. Histopathology of affected juvenile perch reveals necrotic cells in the liver, spleen hematopoietic tissue and intestinal lamina propria as well as congestion in the central nervous tissue. PRV can be isolated by cell culture, starting from ground organs, e.g. anterior kidney, liver, spleen and brain of juveniles (Dorson et al. 1984).

The diagnostic of PRV is usually performed using cell culture confirmed by an immunofluorescence

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antibody test or using a generic RT-PCR starting from RNA extracted from the culture supernatant (Talbi et al. 2011). To our knowledge, to date, there is no PCR assay using total RNA from organs.

Perhabdoviruses have an ssRNA genome of about 11.5 kb encoding 5 genes (Stone et al. 2013). The glycoprotein (*g*) gene is commonly used for phylogenetic studies, although the partial sequences of the RNA-dependent RNA polymerase (*l*) and nucleoprotein (*n*) genes have been used as well, producing clusters similar to those obtained with the *g* gene (Ruane et al. 2014). Based on the partial *n* gene and the complete *g* gene, various genogroups of PRV (A–D) have been defined (Talbi et al. 2011). The phosphoprotein (*p*) gene has not been used to date for comparing PRV isolates, although it has been shown for EVEX that it may vary more than *g* and *n*, and therefore be more informative to compare isolates (Bellec et al. 2014).

Most percid perhabdoviruses reported to date originate from wild animals introduced into experimental facilities and farms (Dorson et al. 1984, Dannevig et al. 2001, Ruane et al. 2014). However, the interregional trade of contaminated fish either for farming or for studying their biology may play an increasing role in the spread of viruses, either horizontally or vertically. When performed correctly, the disinfection of eggs may prevent transmission, suggesting that the virus does not penetrate the eggs and that transmission is pseudo-vertical. Pike-perch is a fish species increasingly farmed in Europe (Dalsgaard et al. 2013, Kestemont et al. 2015) and is also sensitive to PRV as shown by an episode of high mortality caused by a virus in 1990 in France (Nougayrède et al. 1992). Since this episode, no mortality of this fish species has been published, although viruses have occasionally been isolated by the European Union Reference Laboratory for Fish Diseases (N. J. Olesen unpubl.). Here, we report a series of 5 outbreaks associated with variants of PRV during the same period at 2 distant sites. Based on our molecular data and other epidemiological information, we offer hypotheses on the origin of the virus involved in each outbreak.

MATERIALS AND METHODS

From a total of 5 distinct mortality episodes (M1–5) in 2016, pike-perch (zander) larvae were collected from 2 sites in 2 European countries to elucidate the etiology (Fig. 1). One site, a farm in Belgium, was affected by 3 successive episodes of mortalities (M1–M3) within a few months, each episode affecting at least 99% of the population. The larvae were kept in a recirculating water system at 17°C with appropriate levels of O_2, NH_4 and NO_2. The second site, an experimental facility in France, experienced 2 episodes (M4–5) almost simultaneously on 2 bat-

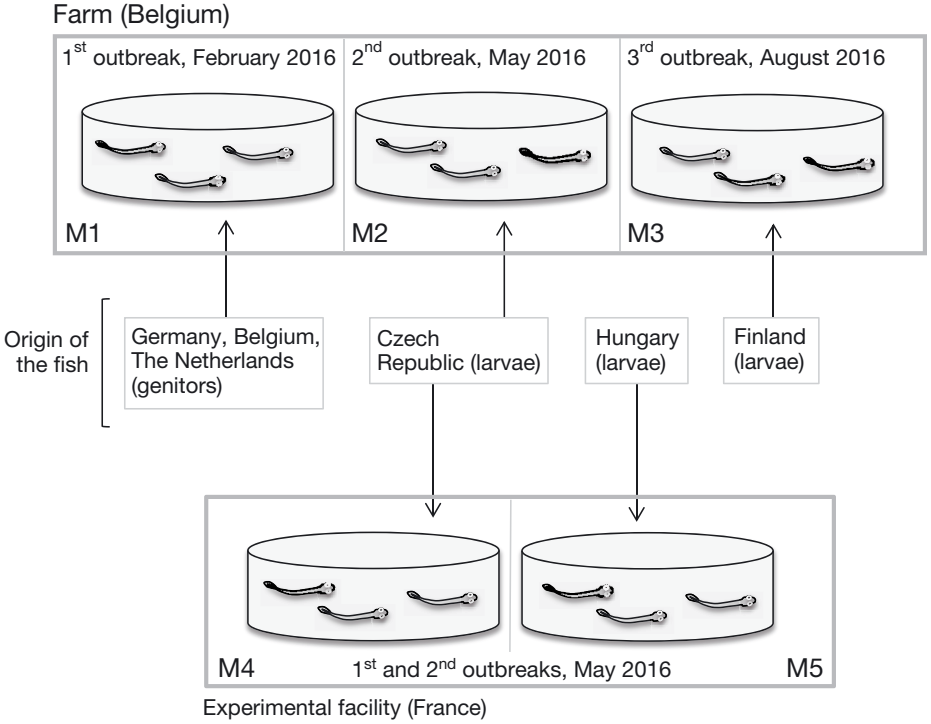


Fig. 1. Features of 5 mortality episodes (M1–5) affecting pike-perch larvae at 2 distant sites in 2016. M1, M2 and M3 occurred on a farm in Belgium; M4 and M5 at an experimental facility in France. M4 and M5 affected larvae of distinct geographic origins that were distributed in 8 tanks (4 and 4) at the French site. For simplification, only 1 tank is represented for each episode

ches of fry freshly imported from the Czech Republic and Hungary (240 000 and 200 000 fry, respectively). Both batches were distributed into 4 tanks each, with common recirculating water maintained at 21.5°C.

Histology

In the Belgian farm, the first batch of larvae affected by mortality was analyzed by histology. Larvae were fixed in 10% formalin and embedded in paraffin. Sections of 4–5 µm thick were stained with hematoxylin-eosin-saffron.

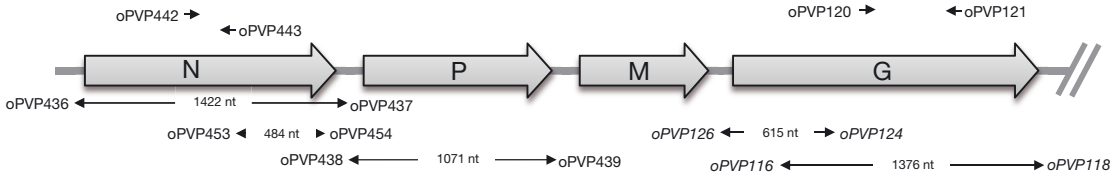
Cell culture

Larvae from 3 batches affected by massive mortalities (M1–3) in Belgium were processed in the CER laboratory for virus isolation (samples 16/65, 16/121 and 16/122 on 31 March, 27 May and 9 June, respectively). For these samples, hundreds of dead, moribund or alive larvae were euthanized (overdose of benzocaine) and immediately collected by filtration, divided in 3 batches and ground in mortar and pestle with sterile sand. The ground larvae were suspended in 10% antibiotic culture medium (v/v, penicillin at 5000 units ml^{-1} and streptomycin at 5000 µg ml^{-1}) and centrifuged at 4000 rpm (3750 × *g*) during at least 15 min. Three cell lines were inoculated with the supernatant of the processed tissue samples, pure and diluted at 10^{-1}, 10^{-2} and 10^{-3}, with 2 wells of a 24-well plate per dilution: epithelioma papulosum cyprini (EPC), blue gill fry (BF-2) and fathead minnow (FHM; Gravell & Malsberger 1965). The 3 cell lines were grown in BHK21 medium (Gibco 21710) supplemented with 10% fetal bovine serum, 5% antibiotics (v/v of penicillin and streptomycin), glutamax and tryptose phosphate broth. After inoculation, the 3 cell line plates were incubated at 14 and 21°C in 5% CO_2 incubators.

Two batches of dead larvae from France were assigned the references 16/179 (origin of production, Czech Republic) and 16/183 (origin Hungary). They were processed in the LDA39 laboratory after a 1 d transportation at 4°C. For these 2 batches, weights of 1.5 and 0.57 g of 13 d old larvae were ground in MEM BHK21 medium (1/10, w/v) and inoculated in duplicates on EPC cells at 20°C and rainbow trout gonad (RTG-2) cells at 14°C at 2 dilutions (10^{-1} and 10^{-2}) in 24-well plates. All plates were regularly inspected under a microscope for cytopathic effects (CPE); if no CPE were noted upon the first observation at 1 wk, a second passage was carried out. For the Belgian samples, aliquots of supernatants from cell cultures developing strong CPE were tested for the presence of various fish viruses, namely viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), spring viremia carp virus (SVCV) and infectious pancreatic necrosis virus (IPNV) using commercial ELISA kits (BioX) according to the manufacturer's instructions.

PCR

Total nucleic acids were isolated either from 200 µl of cell culture supernatant or 200 µl of larvae ground in phosphate-buffered saline (PBS) buffer (10% w/v) using a Nucleospin virus kit (Macherey-Nagel) according to the provided protocol, except that the incubation with Proteinase K was 15 min at 70°C rather than 3 min at room temperature. Elution was performed in 50 µl of RNase-free water. For the 3 genes *g*, *p* and *n*, the Superscript III kit (Invitrogen) was used starting from 2–10 µl or 500 ng of total RNA extracted from cell culture or ground larvae, respectively. The amplification of the complete *g* gene was performed by targeting 2 overlapping fragments as already described, except that the reverse transcription and PCR were performed in a single reaction using the Superscript III kit (Talbi et al. 2011). For amplifying the complete *n* gene, primers oPVP436 and oPVP437 (600 nM each) were designed from PRV sequences, upstream and downstream from the gene (Fig. 2). The RT-PCR started with a step at 48°C for 30 min, followed by a step at 94°C for 2 min and 35 cycles of 94°C for 30 s, 48°C for 30 s and 68°C for 80 s. For the complete *p* gene, primers pPVP438 and oPVP439 (800 nM each) were designed based on the complete PRV sequence. The RT-PCR started with a step at 48°C for 30 min, followed by a step at 94°C for 2 min, and 40 cycles of 94°C for 30 s, 48°C for 30 s and 68°C for 60 s. For viral amplification starting from total RNA from whole larvae, specific primers oPVP453 and oPVP454 were visually designed from the newly obtained *n* sequences from the viruses isolated in Belgium. They match perfectly with known PRV isolates belonging to the genogroups A, B and C previously described (Talbi et al. 2011). However, they have a number of mismatches (4 and 5) with isolate 4925 from the genogroup D. Moreover, these 2 primers have respectively 8 and 4–5 mismatches with 2 members of another *Perhabdovirus* species, sea trout rhabdovirus (STRV) (GenBank AF434992) and LTRV (GenBank AF434991). Both primers were



oPVP120*	GTC AGA CAA GAG TGG AAT C
oPVP121*	GGA TTG AAT CCT TGT GTC G
oPVP124	CAC ATC CCT TGA TGG AAT AC
oPVP442*	GAG CAA AGA GGC TGA ATG TG
oPVP443*	ATC ATT CTG GAC ACC TCT TG
oPVP453	ARG AGG TGT CCA GAA TGA TG
oPVP454	RGG TAA GCT CCA ATG GTG TC
oPVP436	GAA AAC ACT AAC AGA AAT CT
oPVP437	CTG TTA AAG TTT TTT CAT ATC
oPVP438	GAT ATG AAA AAA CTT TAA CAG
oPVP439	CAT TTC ATC ACT TTA AAT CAT C

Fig. 2. Position and sequences of the PCR primers placed on the partial genome of the reference perch rhabdovirus (PRV; JX679246). The 5′–>3′ sequences of the newly designed primers are indicated. Others indicated on the map in *italics* have been previously published (Talbi et al. 2011). The exact sizes of the PCR products expected for PRV (JX679246) are indicated. Primers with * were used for sequencing

used at 400 nM; 500 ng of total RNA were added to the reaction. The RT-PCR started with a step of 58°C for 30 min, followed by a step at 94°C for 2 min and 40 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 30 s. All RT-PCR reactions ended with a 7 min elongation step. For sequencing, all PCR products were cloned in a TA vector (PCR4 TOPO, Invitrogen), and 3 clones were sequenced using universal M13 R and F primers and the Sanger method with a 3130 Genetic Analyzer (Applied Biosystems). In addition to the universal oligonucleotides, specific internal primers were used for sequencing long fragments of the *n* and *g* genes (Fig. 2).

All obtained sequences were assembled and edited using VNTI11.5 (Thermofisher Scientific). For cloned PCR products, a consensus sequence of 3 clones was created (without the primer sequences) and used for phylogenetic analysis. New sequences were deposited in GenBank and compared with others already available (Table 1). The alignments and the phylogenetic analyses were performed using ClustalW and the maximum likelihood method (1000 bootstraps) implemented in MEGA7.0 (Kumar et al. 2016).

RESULTS

History of the outbreaks

In 2016, a total of 5 batches pike-perch larvae of various origins experienced massive mortality events in 2 Western European countries (Fig. 1): Belgium and France. The first batch of eggs was produced in February 2016 in a farm in Belgium. Because this farm used genitors from 7 different origins (wild and farmed pike-perch from 3 European countries) for egg production, it was not possible to identify the exact origin of the affected larvae. One day after hatching, the batch of larvae exhibited the first mortalities and virtually all larvae died. The tanks were disinfected and a second batch originating from the Czech Republic was introduced in May. It also succumbed massively at 10–11 d post hatching. A third batch from a different origin (Finland) was then introduced in June, but it was also affected by massive mortality at 12–14 d post hatching. In the same period of 2016, in France, 2 batches of larvae imported from the Czech Republic and Hungary were introduced into 2 different tanks in an experimental facility. Three days after feeding with *Artemia* nauplii, the batch originating from the Czech Republic was affected by mortality, reaching

Table 1. GenBank accession numbers of glycoprotein (*g*), phosphoprotein (*p*) and nucleoprotein (*n*) genes sequenced in this study. The reference of each sample and its corresponding mortality episode (M1–5) are indicated in the first 2 columns. Samples 4890 and 9574 are from France, isolated from pike-perch and perch in 1990 and 2009, respectively. Accession numbers starting with MF are from the present study; na: not applicable, nd: not determined

Sample	Mortality episode	*g*	*p*	*n*
16/065	M1	MF101756	MF112218	MF112225
16/121	M2	MF124890	MF112219	MF112226
16/122	M3	MF124891	MF112220	MF112227
16/179	M4	nd	MF112221	nd
16/183	M5	nd	MF112222	nd
4890	na	JF502607	MF112223	MF170027
9574	na	JF502613	MF112224	MF170028

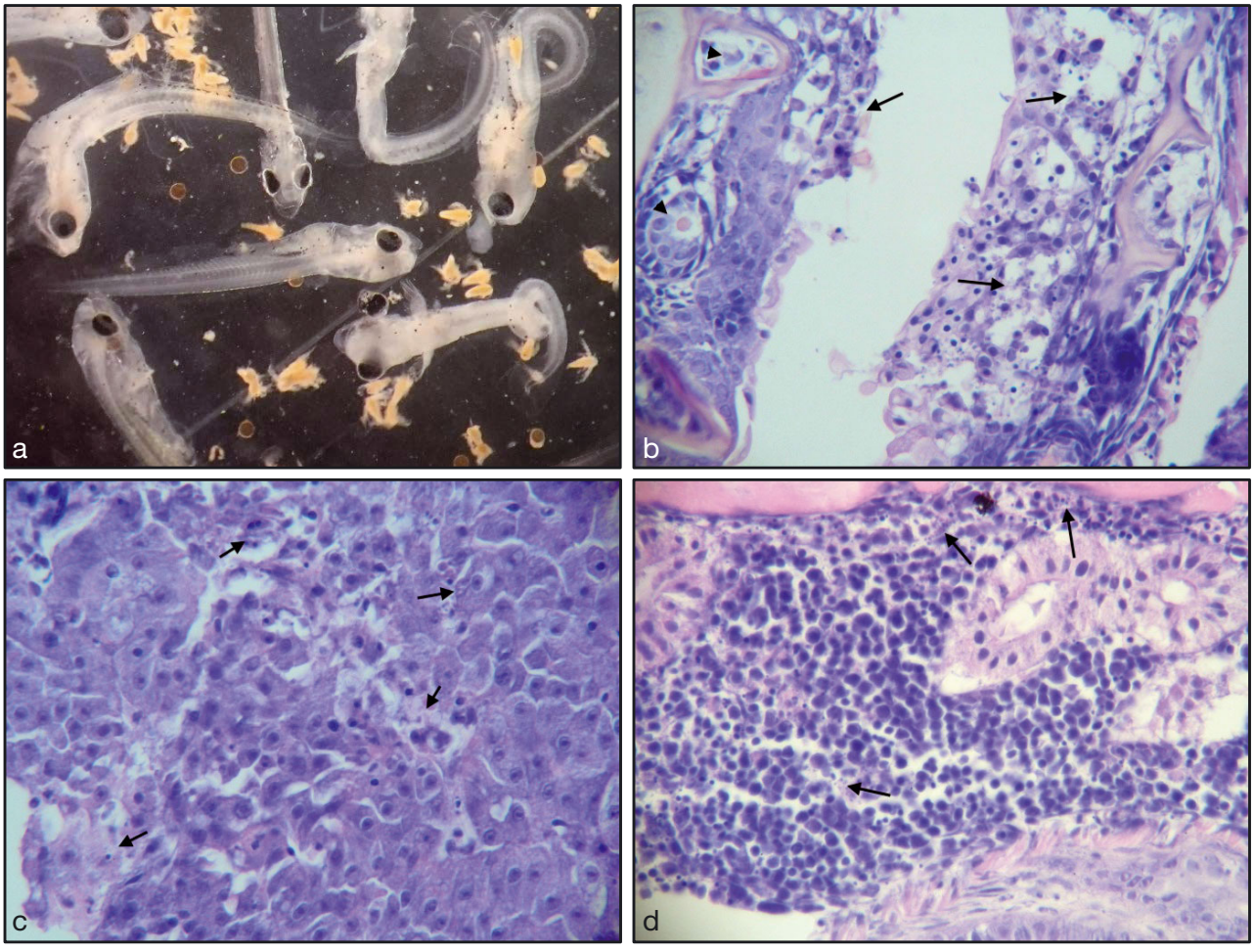


Fig. 3. Histopathology of dead pike-perch larvae from a mortality episode. (a) Typical stage of mortality (episode M4 in France). Also shown are semi-thin sections of various organs of dead larvae (episode M1 in Belgium) (×400): (b) necrosis of mouth epithelial cells with formation of small vesicles (black arrows); dental embryonic structures (black arrowheads); (c) liver parenchyma scattered with tiny necrotic foci (black arrows); (d) intertubular hematopoietic tissue is scattered with pycnotic cells (black arrows)

100% after 24 h. Four days later, the second batch (Hungary) exhibited a similar severe mortality event, which reached almost 100% after 1 d. No macroscopic lesions or parasites were observed on/in the moribund larvae, using a stereomicroscope and light microscope.

Histology

Histological observations were performed on a few larvae from the first outbreak (M1). One animal presented scattered lesions in the liver, kidney and oral mucosa (Fig. 3). In the liver, they consisted of small necrotic foci. Similar foci were also observed in the malpighian epithelia in the mouth. These (hydropic-type) lesions were associated with some vesiculation of the epithelium. In the kidney, the lesions were limited to the hematopoietic tissue. They were characterized by isolated or small foci of grouped necrotic cells. The brain, eyes and heart, when observed, remained free of any abnormalities.

Cell culture

Dead larvae from the 5 mortality episodes were assayed in cell culture in distinct laboratories. After incubation for 4–5 d, obvious CPE appeared for 3 samples (outbreaks M1, M2 and M3) tested at the CER laboratory in Belgium on 3 different cell lines (FHM, EPC and BF2) and at 14 and 21°C. The CPE were characterized by scattered foci of rounded and refringent cells (not shown). The foci became larger

and centrifuge lysis ensued, leading to the total destruction of the cell layers after 3–4 d. These effects suggested the putative presence of a rhabdovirus according to CPE features. The 2 samples from episodes M4 and M5, tested at LDA39 in France, did not produce any CPE after 3 passages of 7 d on EPC cells at 20°C and on RTG cells at 14°C and were therefore initially diagnosed as negative.

PCR detection and virus identification

The 3 CPE-positive cell culture supernatants were tested with a series of PCR targeting 2 portions of the *g* as well as the complete *n* and *p* genes of PRV, a common percid pathogen. All PCRs were positive and gave products at the expected sizes demonstrating the presence of viruses related to PRV in the 3 batches of larvae from Belgium (not shown). The sequences of the full length *g*, *p* and *n* genes were obtained for these 3 isolates and were genetically highly related to the type species PRV (GenBank no. JX679246), with similarities of 95, 94 and 92–93% (nucleic acids) for the *g, n* and *p* genes, respectively, between each sample and PRV. However, the highest identity levels (98–99%) were observed for the *g* gene of a variant of PRV isolated from pike-perch from France in 1990 (reference 4890). The phylogenetic trees for *n*, *p* and *g* of the 3 viruses were consistent and grouped them in the genogroup A which includes the type species PRV (see Fig. 5) (Talbi et al. 2011).

Surprisingly, the virus identified in the first outbreak M1 was slightly different (98% identity for *g*, *n* and *p*) from the viruses from the 2 subsequent outbreaks at the same site (M2 and M3), these last two being identical (*p*) or nearly identical one to another (only 2 substitutions each for *g* and *n*). Considering that the 2 substitutions between the sequences of *g* and *n* of M2 and M3 may have originated from the quasi-species nature of the sample or errors during the PCR, despite the use of an error-proof *Taq* polymerase, viruses from M2 and M3 were likely the same. There were 9 (or 10) amino acid changes between the predicted G proteins of the M1 virus and the M2 (or M3). These results strongly suggested that the first outbreak had a different viral origin from the other two, while the second and third outbreaks were associated with a unique genotype.

Despite the negative results obtained in cell culture, samples 16/179 and 16/183 from the 2 mortality episodes in France (M4 and M5) were analyzed by RT-PCR in search of viruses similar to those previously found. The generic primers used for RT-PCR (highly degenerated and low melting temperature [Tm]) work well with concentrated viruses produced in cell culture, but are not adapted to viral RNA diluted in total nucleic acids from tissues. Thus, we designed new sets of primers (avoiding degeneracy and having high Tm), specific to the *n* gene for a range of genotypes related to those recently found. Using these primers, both samples from France appeared unambiguously positive, producing a unique band at the expected size (Fig. 4). As a positive control, an identical PCR product was obtained when testing isolate 16/121 from Belgium. The identities of the PCR products were confirmed by sequencing directly with the amplifying primers. Both PCR products had exactly the same sequences over 444 bp. Moreover, they were also strictly identical with the sequences obtained from isolates 16/121 and 16/122 (M2 and M3). There was therefore a strong evidence for the spread of the same virus between M2, M3, M4 and M5. This strong genetic link was further confirmed by the amplification and sequencing of the full-length *p* genes of the viruses identified in M2, M3, M4 and M5, which were strictly identical (Fig. 5).

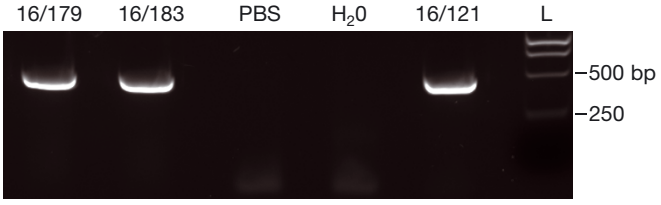


Fig. 4. Detection of the partial nucleoprotein (*n*) gene from samples of pike-perch from outbreaks M4 and M5. Two negative controls (PBS buffer extraction and no template PCR) and a positive control (isolate 16/121) are shown in Lanes 3 to 5. L: 1 kb ladder (Promega)

DISCUSSION

In spring and summer 2016, 5 mortality episodes affecting pike-perch larvae occurred in 2 very distant sites without any obvious relationship. Semi-thin sections of larvae from the first outbreak did not reveal the presence of an observable pathogen, but showed lesions that were compatible with a viral infection. For instance, multifocal necrosis was observed in the kidney and the liver, as already described in tissues infected by perch perhabdoviruses (Henshilwood et al. 2009, Gadd et al. 2013). To our knowledge, the oral lesions observed in our study have never been

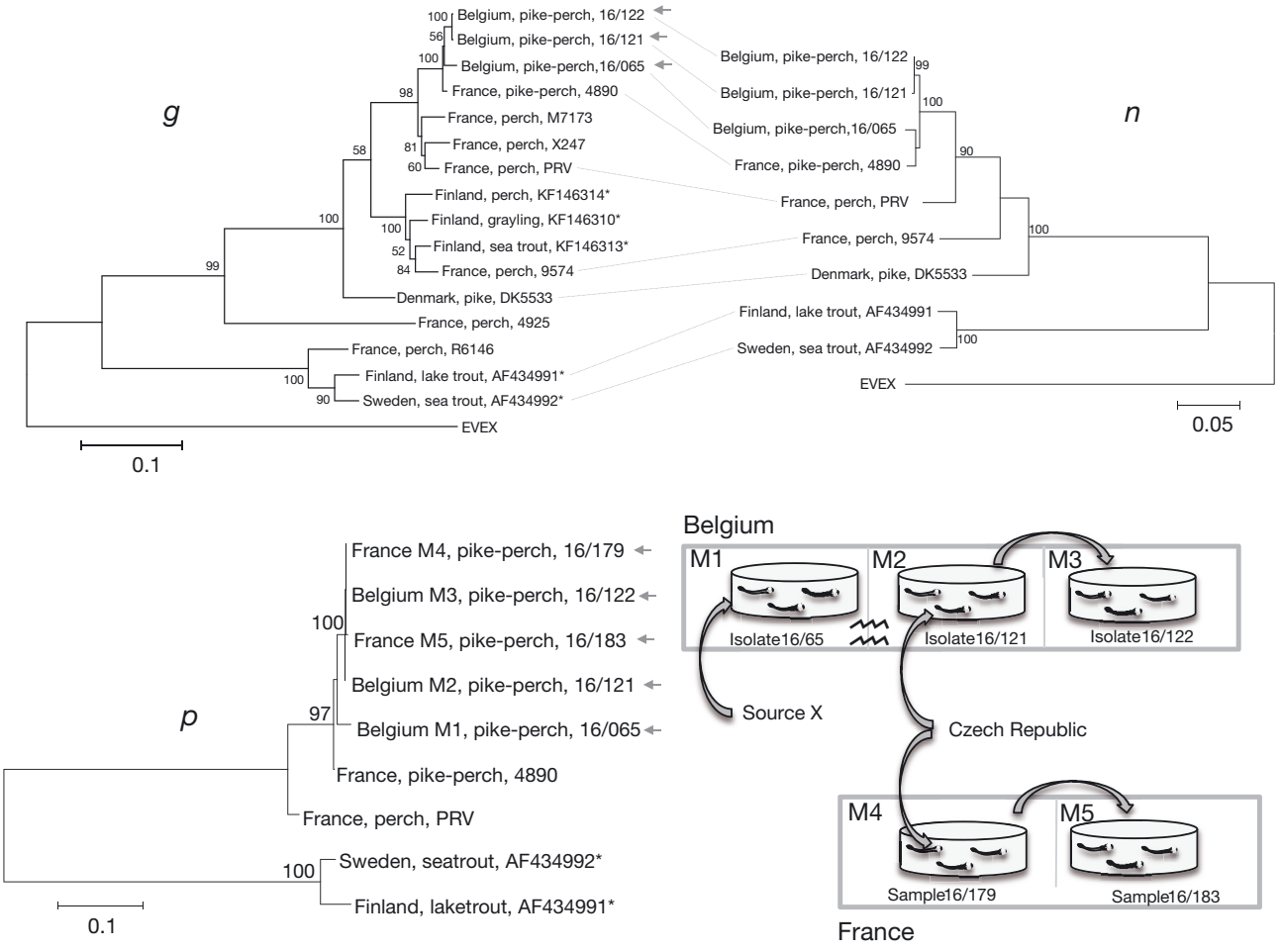


Fig. 5. Phylogenetic and epidemiological relations between perhabdoviruses. Small grey arrows indicate viruses from our study. The number of substitutions per site is indicated below each tree. Some viruses are identified by their GenBank numbers (*). When not indicated in the trees, GenBank numbers are available in Talbi et al. (2011) or in Table 1. For the phosphoprotein (*p*) gene tree, the episode of mortality (Mx) is indicated with the name of the associated sample. For each mortality episode, a source of contamination (curved arrow) is proposed on the drawing. Source X was not identified (genitors from 3 countries) while a second putative source was from Czech Republic. The symbol ≈ indicates culling of fish and disinfection of the facilities. *g*: glycoprotein gene, *n*: nucleoprotein gene. EVEX: eel virus European X; PRV: perch rabdovirus

reported before in PRV infections. More reports of lesions observed in this organ are needed to evaluate the pathognomonic potential. Conversely, no lesions were observed in the brain despite the likely presence of the virus in this tissue and previous reports of congestion in the meninges (Dorson et al. 1984, Henshilwood et al. 2009). Unfortunately, we did not examine the pancreas despite the frequent lesions reported in other cases. Until the best target organ for detecting PRV is identified, either by histopathology or PCR, it is recommended to sample various organs in any future putative cases.

For each outbreak, rhabdoviruses related to PRV were found either by cell culture and PCR (M1, M2 and M3), or only by PCR with new assays developed here (M4 and M5). To our knowledge, this is the first report of the presence of PRV in Belgium. For outbreaks M4 and M5, the virus could not be cultured for unknown reasons, possibly due to poor conservation of the samples or a toxic effect. However, PRV was unambiguously detected by PCR in these 2 samples. Finally, 2 related, yet distinct, variants of PRV were found among the 5 episodes. Despite the apparent absence of connections between the 2 sites, the complete and partial sequences of key viral genes and other epidemiological data suggest the involvement of 2 viral genotypes in the various outbreaks. The first episode M1 occurred in larvae originating from 7 batches of genitors from farmed and wild fish from 3 European countries. Therefore, 1 genotype was likely transmitted to the larvae from 1 or several of these genitors. After culling, the farmer thoroughly

disinfected the tanks, a procedure that probably eradicated the first virus. The following outbreak M2 affected larvae from the Czech Republic, and the third outbreak affected larvae originating from Finland. Between outbreaks M2 and M3, the facilities were not disinfected. Therefore, for the site in Belgium, a likely scenario consistent with the molecular data is an initial contamination of the first batch of larvae by a virus carried by genitors of different origins, followed by its eradication during the disinfection performed just after the outbreak. After this first episode, a second distinct virus was likely introduced with the second batch of larvae from the Czech Republic, affecting this batch and also contaminating the third batch originating from Finland, due to the absence of disinfection between batches. Under this scenario, disinfection efficiently inactivated the first virus and prevented the infection of the subsequent batch. Other scenarios are possible, such as the introduction of 2 or more viruses with the first batch, and the subsequent isolation of only 1 virus during each episode. This hypothesis is plausible considering the limited sampling of the fish combined with a putative selective effect of cell culture and a limited sequencing of the clones obtained from the PCR products. With the new PCR tools described here, it will be possible in the future to amplify and sequence viral sequences directly from different batches of larvae without using cell culture and PCR cloning.

Regarding episodes M4 and M5 successively affecting 2 batches of different origins at the same site, the same virus was found as determined by the perfect identity between the compared full-length *p* sequences. Therefore, the same virus was likely transmitted between the 2 batches, probably via the water system. Surprisingly, this virus was also identical to those of episodes M2 and M3, despite the absence of any direct epidemiological connection. Upon inquiry, it was found that the batches from M2 and M4 had exactly the same origin, from a site in the Czech Republic, epidemiologically linking the farm in Belgium and the experimental site in France. This link explained both the identities between M2 and M4 viruses as well as between M2/M3 and M4/M5 through horizontal transmission.

The likely scenario of contamination of the present outbreaks implicated that both horizontal and pseudo-vertical transmission modes were active in spreading the viruses between countries and batches of fish. The control of the disease at the continental scale will therefore require the use of a large panel of methods to limit both modes of dissemination. Regarding vertical transmission, thorough disinfection of pike-perch eggs can prevent the infection of fry, although protocols of disinfection still need to be standardized and optimized, i.e. to avoid toxic effects (Rodger & Girons 2008, Lahnsteiner & Kletzl 2016). Regarding horizontal transmission, the proposed scenario of infection for the second Belgian batch highlights that disinfection of the tanks and the water circulation system is efficient and can prevent the transmission of the virus from a first batch to a second. Diagnostic tools and gene sequencing were useful in the present cases, both to detect the presence of PRV and to formulate hypotheses of spread.

Although cell culture allowed the diagnosis of the 3 outbreaks in Belgium, it was not efficient for isolating the virus in France, and it did not lead to PRV detection. Conversely, PCR led to rapid diagnosis and, combined with sequencing and phylogenetic analysis, allowed an accurate comparison of the sequences between outbreaks and with those already in databases. Among the new PCR protocols proposed in our work, the one amplifying a portion of the *n* gene is the first, to our knowledge, that can be widely used for detecting a range of PRV variants directly from tissue. The range of variants potentially detected encompasses the genogroups A, B and C previously described (Talbi et al. 2011). This method should be extensively validated and transferred to European laboratories receiving samples of percids for diagnosis. Other more rapid and specific methods, such as Taqman-based real-time PCR, should be developed as well. More globally, the control of perhabdoviruses dispersal in European percid farms requires the generation of genetic data and the development of PCR-based methods specific of viral species (LTRV, PRV, etc.).

The high similarities between the 2 distinct viruses presently identified and the pike-perch virus isolated in France in 1990 are puzzling since there is no known link between outbreaks that occurred 26 years apart. The simplest explanation is that the virus from 1990 has the same geographic origin as those from 2016, namely the Czech Republic. We could not find information on the origin of the fish in 1990, but transfers of pike-perch have been frequent these last years from the Czech Republic to France. Therefore, we can only speculate on such a transfer of contaminated fish, which implies that the virus from 1990 is an ancestor of the 2 recent ones and has slightly diverged into variants occasionally dispersed over almost 3 decades. Another hypothesis involves an unknown source of virus which spread in various European countries before 1990. More generally, this high genetic similarity underlies the existence of a

viral population which has been widely spread across Europe, most probably by trade of pike-perch or an alternative reservoir, possibly perch. Further accurate identification of perhabdoviruses from percids should be a priority to continuously improve probe-based diagnostic tools and trace the movements of viruses. This effort, combined with disinfection of eggs and the facilities, should help to reduce the present and future burdens of the disease on this fish species, and perch, which are both increasingly farmed. However, although some European farms have already successfully developed some control measures, many others still ignore the risks associated with this group of perhabdoviruses or fully depend on the introduction of fry, genitors or juveniles from sources that are not certified virus-free. Methods that can diagnose healthy carriers with very low viral loads or sick animals carrying unknown genotypes are needed to select and maintain virus-free genitors and protect inter-regional exchanges of farmed juveniles.

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