Vol. 112: 121–130, 2014
doi: 10.3354/dao02801

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 2

Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland

N. M. Ruane[1], H. D. Rodger[2], L. J. McCarthy[1], D. Swords[1], M. Dodge[3], R. C. Kerr[3], K. Henshilwood[1], D. M. Stone[3,*]

[1]Fish Health Unit, Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
[2]Vet Aqua International, Unit 7B, Oranmore Business Park, Oranmore, Co. Galway, Ireland
[3]Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, Weymouth, Dorset DT4 8UB, UK

ABSTRACT: Rhabdovirus infections are an emerging problem for both wild and farmed freshwater fish in Northern Europe. In October 2005, a clinical outbreak with an approximate mortality rate of 40% occurred in a single batch of juvenile perch on a farm in the Republic of Ireland. Clinical signs developed slowly and were consistent with a perch rhabdovirus infection: signs included haemorrhages at the base of the fins and apparent impairment of the central nervous system (manifested as loss of equilibrium and erratic swimming behaviour). Studies suggest that the infected fish originated from a hatchery within the country which relied on wild fish broodstock to supplement the production of perch juveniles. A related rhabdovirus was subsequently isolated from this hatchery. Virus isolation studies have shown that rhabdoviruses were often isolated from wild fish in the vicinity of the hatchery between 1993 and 2005. All isolates were analysed using a generic primer set specific for the L gene of fish vesiculotype viruses. Phylogenetic analysis revealed that all isolates recovered from perch clustered together with the European lake trout rhabdovirus (903/87) of the genus *Perhabdovirus*. In addition to this, anguillid rhabdovirus was isolated from eel, and the partial L-gene sequence of a previously reported isolate from tench clustered with the pike fry rhabdoviruses, in the genus *Sprivivirus*.

KEY WORDS: European lake trout rhabdovirus · Rhabdovirus · *Perhabdovirus*

INTRODUCTION

The European perch *Perca fluviatilis* is a common native freshwater species found throughout Europe and has also been introduced into Australia, New Zealand and South Africa. European perch aquaculture was historically confined to the Czech Republic, Hungary, Poland and France, with annual production varying between 100 and 300 t according to the Food & Agriculture Organisation (FAO; www.fao.org/fishery/species/2298/en). In Ireland, the European perch was identified as a possible species for the future diversification of aquaculture (Watson & Stokes 2003), and over the last decade, a number of hatcheries and grow-out facilities were established with the result that Ireland now has the potential to produce up to 170 t of perch per year.

As with any developing industry, perch aquaculture faces a number of potential bottlenecks, most notably the reliable production of perch fry (Toner & Rougeot 2008), and due to the lack of sufficient broodstock, wild broodfish have been used to establish production. As with all other farmed fish species, perch are susceptible to a range of diseases (Rodger & Girons 2008) and therefore, the use of wild fish as broodstock has increased the risk of introducing

*Corresponding author: david.stone@cefas.co.uk

pathogens to perch farms in Ireland. Parasites, bacteria and fungi have been the most frequently encountered causes of mortality in intensively reared perch (Grignard et al. 1996). Among the main bacterial pathogens of perch reported recently were mortalities associated with *Aeromonas sobria* in Switzerland (Wahli et al. 2005) and *Flavobacterium psychrophilum* in Finland (Lönnström et al. 2008). The main viral diseases are those caused by perch rhabdovirus, a member of the family *Rhabdoviridae* (Rodger & Girons 2008).

Within the family *Rhabdoviridae*, 3 genera are now recognized whose members infect fish: *Novirhabdovirus*, *Perhabdovirus* and *Sprivivirus*. Currently, the genus *Perhabdovirus* contains 3 species, *Perch rhabdovirus*, *Anguillid rhabdovirus* and *Sea trout rhabdovirus*. The genus *Sprivivirus* contains 2 species, *Spring viraemia of carp virus* and *Pike fry rhabdovirus* (Stone et al 2013, http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/vertebrate-official/4491.aspx). Rhabdovirus infections have long been recognized as an emerging issue for freshwater aquaculture in Europe (Betts et al. 2003). These have included viruses isolated from a wide range of fish species in a number of different countries, such as European perch, zander *Sander lucioperca*, pike fry *Esox lucius* and largemouth bass *Micropterus salmoides* in France (de Kinkelin et al. 1973, Dorson et al. 1984), roach *Rutilus rutilus* in the Netherlands (Haenan & Davidse 1989), pike in Denmark (Jørgensen et al. 1993), sea trout *Salmo trutta* in Sweden (Johansson et al. 2002), brown trout and grayling *Thymallus thymallus* in Finland (Koski et al. 1992, Björklund et al. 1994, Gadd et al. 2013), perch in Norway (Dannevig et al. 2001), bream *Abramis brama*, tench *Tinca tinca*, roach and crucian carp *Carassius carassius* in England (Way et al. 2003) and barbel *Barbus barbus* in the Czech Republic (Vicenova et al. 2011). Infections have also been reported in wild and farmed fish in Northern Ireland (Adair & McLoughlin 1986, Rowley et al. 2001).

The conserved amino acid motifs identified in alignments of the RNA-dependent RNA polymerase (L gene) have been used in examining phylogenetic relationships among the *Rhabdoviridae*. The sequence conservation displayed by this region is a useful target for determining evolutionary relationships among rhabdoviruses, both known and unclassified (Bourhy et al. 2005). This work builds on a previous report (Betts et al. 2003) which examined the relationship of a range of vesiculoviruses using the L gene. In this study, a simple assay using degenerate primer sets based on the polymerase (L) gene was used to identify the virus involved in disease outbreaks in perch farms and to determine the genetic relationship between these virus isolates and related viruses isolated from wild perch, bream, eel *Anguilla anguilla* and brown trout collected in Ireland between 1993 and 2013.

MATERIALS AND METHODS

Wild fish samples

Between 1993 and 2005, rhabdovirus was isolated from wild perch fry on 9 occasions from 7 different lakes (Table 1, Fig. 1). All isolations from perch were associated with reports of mortalities in the wild, and fish were submitted by staff of the Regional Fisheries Boards (now Inland Fisheries Ireland) to the Fish Health Unit, Marine Institute, for diagnostic testing. The numbers submitted varied between 5 and 30 fish sample^{-1}. All submissions occurred between July and September of the respective years when water temperatures were at their highest, ranging from 16 to 18°C. In September 1998, 5 bream with an average weight of 800 g were received by the laboratory for diagnostic testing (sample F1350-98), and all 5 had external signs of clinical disease. In July 1999, 30 clinically healthy eel elvers were submitted to the laboratory for diagnostic testing prior to export (sample F1737-99). In July 2000, 60 brown trout from a restocking facility were received (sample F1935-00). Mortality levels of up to 20% were reported on site in July when water temperatures were 18.5°C.

Farmed fish samples

In September 2005, a clinical outbreak of disease with an approximate mortality rate of 40% occurred in 1 batch of juvenile perch in a grow-out farm (Farm 1, F1) in the Republic of Ireland. Two sample pools of perch fry (5 fish pool^{-1}) were received by the laboratory for virological testing (F3003-05). A follow-up sample at the same farm took place in October, when 30 perch fry were tested during a routine surveillance visit by the authorities (F3032-05).

In July 2007, 38 perch fry were sampled from Hatchery 1 (H1) during a routine surveillance visit (F3382-07). Another positive sample from this site occurred in February 2009, when 3 brood fish obtained from the wild were sampled (F009-09). Fungus had been observed on the skin and gills along

Table 1. Rhabdovirus isolates used in the construction of the phylogenetic tree in this study

Virus (abbreviation)	Virus strain	Host	Source	Reference
Lake trout rhabdovirus (LTRV)	903/87	*Salmo trutta lucustris*	Finland	Koski et al. (1992)
American eel rhabdovirus (EVA)	No strain designation	*Anguilla rostrata*		
European eel rhabdovirus (EVEX)	No strain designation	*Anguilla anguilla*		Sano (1976)
Pike rhabdovirus	DK5533	*Esox lucius*	Denmark	Jørgensen et al. (1993)
Grayling virus isolate	47-90	*Thymallus thymallus*	France	Betts et al. (2003)
Pike-perch rhabdovirus	48-90	*Sander lucioperca*	France	Nougayrède et al. (1992)
Perch rhabdovirus (PRV)	No strain designation	*Perca fluviatilis*	France	Dorson et al. (1984)
Perch virus isolate	02-95	*Perca fluviatilis*	France	Betts et al. (2003)
Largemouth bass virus isolate	03-95	*Micropterus salmoides*	France	Betts et al. (2003)
Pike fry rhabdovirus (PFRV)	PFRV F4	*Exos lucius*	France	De Kinkelin et al. (1973)
Tench rhabdovirus (TenRV)	80560	*Rutilus rutilus*	Netherlands	Haenen & Davidse (1989)
Grass carp rhabdovirus (GrCRV)	V76	*Ctenopharyngodon idella*	Germany	Ahne (1975)
Spring viraemia of carp virus (SVCV)	S30	*Cyprinus carpio*	Yugoslavia	Fijan et al. (1971)
Present study				
Perch virus isolate	F593-93	*Perca fluviatilis*	Lough Gowna	
Perch virus isolate	F609-93	*Perca fluviatilis*	Lough Avaghan	
Perch virus isolate	F1075-96	*Perca fluviatilis*[a]	Lough Conn	
Perch virus isolate	F1082-96	*Perca fluviatilis*[a]	Lough Conn	
Perch virus isolate	F1318-97	*Perca fluviatilis*[a]	Lough Lavey	
Bream virus isolate	F1350-97	*Abramis brama*	Lough Oughter	
Perch virus isolate	F1564-98	*Perca fluviatilis*[a]	Lough Drumlaheen	
Perch virus isolate	F1575-98	*Perca fluviatilis*	Lough Lavey	
Eel virus isolate	F1737-99	*Anguilla anguilla*	River Shannon	
Trout virus isolate	F1935-00	*Salmo trutta*	Cullion	
Perch virus isolate	F2764-04	*Perca fluviatilis*[a]	Poulaphouca	
Perch virus isolate	F2973-05	*Perca fluviatilis*[a]	Lough Cloone	
Perch virus isolate	F3003-05	*Perca fluviatilis*	Farmed (Farm 1, F1)	
Perch virus isolate	F3032-05	*Perca fluviatilis*	Farmed (F1)	
Perch virus isolate	F3382-07	*Perca fluviatilis*[a]	Farmed (Hatchery 1, H1)	
Perch virus isolate	F3446-07	*Perca fluviatilis*[b]	Farmed (Farm 2, F2)	
Perch virus isolate	F100-08	*Perca fluviatilis*	Farmed (F2)	
Perch virus isolate	F147-08	*Perca fluviatilis*	Farmed (F2)	
Perch virus isolate	F009-09	*Perca fluviatilis*[b]	Farmed (H1)	
Perch virus isolate	F044-09	*Perca fluviatilis*	Farmed (F2)	
Perch virus isolate	F108-09	*Perca fluviatilis*	Farmed (F2)	
Perch virus isolate	F004-13	*Perca fluviatilis*[a]	Farmed (H1)	

[a]Virus isolated from fry
[b]Virus isolated from wild-caught broodstock

with necrotic patches in the gills. A final positive sample was received from this site in January 2013, consisting of 2 sample pools of perch fry (5 fish pool^{-1}; F004-13).

In November 2007, 1 brood fish and 26 perch fry were sampled during a routine surveillance visit (F3446-07) to Farm 2 (F2). The brood fish had fungus on the skin but otherwise, all fish appeared healthy. In July 2008, 2 sample pools of perch fry (5 fish pool^{-1}) were received for virological testing (F100-08). In October 2008, 10 fry were sampled during a routine surveillance visit (F147-08). Fish were observed swimming at the top of the water column. In May 2009, 1 sample pool of perch fry (5 fish pool^{-1}) was received for virological testing (F044-09) and again in August 2009, 4 perch fry were received for virological testing (F108-09).

Tissue sampling and virus culture

At the aquaculture facilities, fish were removed from the tanks using hand nets and euthanized with an overdose of MS-222 (10% w/v). Wild fish were sampled from lakes using gill nets and similarly euthanized. All fish sampling was performed on site, i.e.

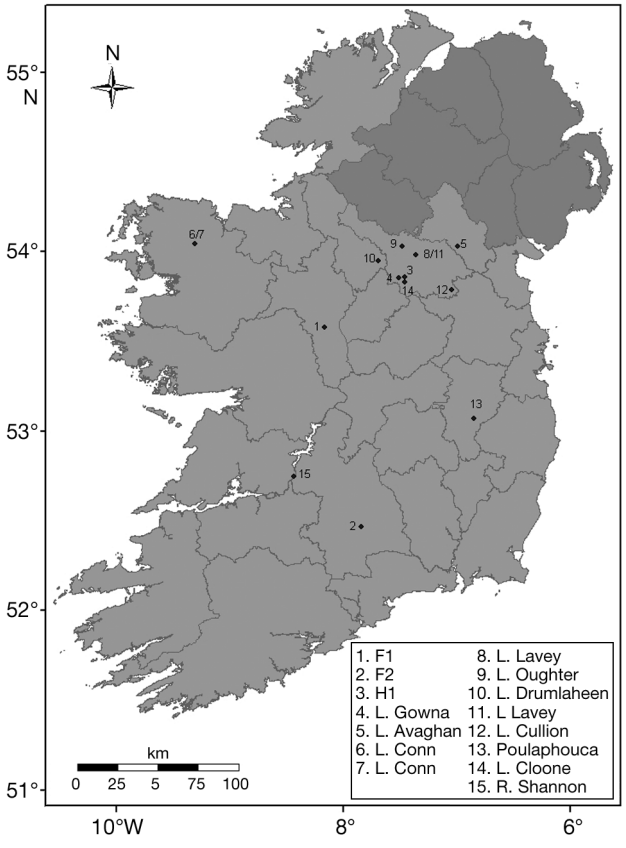

Fig. 1. Sites in the Republic of Ireland where vesiculovirus has been isolated, 1993–2013. F1, F2: Farms 1 and 2; H1: Hatchery 1

at the farm or lake (in the case of wild fish). For virological testing, selected tissues (heart, kidney and spleen or brain) were aseptically removed and stored in 4 ml of cooled Eagle's minimum essential medium (GIBCO, Invitrogen) containing 12.5% (v/v) 0.1% Tris-HCl, 10% (v/v) foetal bovine serum, 1200 U penicillin and 1200 µg streptomycin (GIBCO, Invitrogen) at pH 7.6 for transport to the laboratory. Kidney swabs were plated onto tryptone soya agar and Colombia blood agar plates and incubated at 22°C. Bacterial colonies were identified by standard primary (including vibriostatic disc 0129, Oxoid) and secondary biochemical tests (API 20E, API NE, API ZYM; Biomerieux). Portions of gill, heart, kidney, liver, spleen, brain, gastrointestinal tract, pancreas, skin and muscle from diseased fish were prepared for light microscopy by fixing tissues in neutral buffered saline with 10% formaldehyde, paraffin wax embedding, sectioning at 5 µm and staining with haematoxylin-eosin (H&E).

The tissue sample was adjusted to a final ratio of 1:10, tissue material to media volume. The samples were homogenized using a stomacher 80® (Seward), the homogenate was clarified by centrifugation at 3000 × *g* (15 min at 4°C), and the supernatant was incubated at 15°C for 4 h with 2.5% gentamicin (v/v 50 mg ml^{-1}; GIBCO). Virus isolation was carried out on epithelioma papulosum cyprini (EPC) and bluegill fry *Lepomis macrochirus* (BF2) cell lines. Monolayers grown on 24-well cell culture plates at 20°C were inoculated with an aliquot of sterile filtered (0.22 µm) supernatant at final dilutions of 1:10, 1:100 and 1:1000 and incubated at 15°C. Plates were monitored daily for the development of viral cytopathic effect (CPE), and after 7 d, media were collected from the wells and inoculated onto fresh monolayers of EPC and BF2 cells. If no CPE was observed after a period of 14 d (following passage on Day 7), the sample was recorded as negative. When CPE was observed, a range of immunofluorescence antibody tests (IFAT; Bio-X Diagnostics) and enzyme-linked immunosorbent assays (ELISA; Test Line) were performed to rule out infectious haematopoietic necrosis (IHN), viral haemorrhagic septicaemia (VHS), infectious pancreatic necrosis (IPN) and spring viraemia of carp (SVC) viruses.

RNA extraction

All molecular analyses in this study were carried out on cell-cultured virus which was passaged no more than 3 times. Once CPE was well established in a well, total nucleic acid was extracted from 140 µl of the cell culture medium using the QIAamp® Viral RNA mini kit (Qiagen) according to the manufacturer's recommendations and eluted in 60 µl diethyl-pyrocalborate-treated dH_2O and stored at −80°C until required. Control wells inoculated with media only were also extracted. The concentration and purity was estimated by measuring absorbance at 260 and 280 nm on a Nanodrop 1000 (Thermo Scientific).

RT-PCR and sequence analysis

Generic primers were designed to conserved sequence motifs identified using Clustal W alignment of the vesiculo-type virus sequences generated by Betts et al. (2003). An outer primer set was designed to generate a 350 bp partial RNA polymerase (L) gene product (VesGen1: 5′-GGR MGD TTY TTY TCH YTR ATG TC-3′; VesGen2: 5′-ATC AGR TCN GGY CTN CCR TTR TAR TA-3′) and a second nega-

tive sense primer was designed to generate a 260 bp product in a semi-nested PCR assay using the 350 bp product as a template (VesGen4: 5′-ATN ACK CKR AAN ACN GGN CCR TT-3′). The semi-nested step was introduced to ensure that sufficient product was obtained for direct sequencing of the PCR amplicons. The 350 bp product corresponds to nucleotides 1579 to 1934 of the pike fry rhabdovirus L gene (ACP-28002), and the 260 bp product corresponds to nucleotides 1579 to 1841 of the pike fry rhabdovirus L gene (ACP28002). Reverse transcription (RT) PCR was performed in triplicate for each virus isolate.

RT was performed at 37°C for 1 h in a 20 µl volume consisting of 1× M-MLV RT reaction buffer (50 mM Tris pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol random primers, 20 U M-MLV reverse transcriptase (Promega) and 4 µl of the nucleic acid extracted above. The PCR was performed in a 50 µl reaction volume consisting of 1× GoTaq flexi buffer (Promega), 2.5 mM $MgCl_2$, 1 mM dNTP mix, 50 pmol VesGen1 and VesGen2, 1.25 U of GoTaq® DNA Polymerase (Promega) and 2.5 µl of RT reaction from above. In each case, the reaction mix was subjected to 35 temperature cycles (1 min at 95°C, 1 min at 55°C and 1 min at 72°C) after an initial denaturing step (5 min at 95°C) followed by a final extension step of 10 min at 72°C, in a DNA Engine® Peltier Thermal Cycler (Bio-Rad). The second round of PCR using VesGen1 and VesGen4 was performed using the same cycling conditions as those used in the first round of amplification. PCR products were visualised on 1.5% agarose gels stained with ethidium bromide. Products of interest were excised from the gels and frozen at −20°C until required. DNA was extracted and purified by ethanol precipitation and sequenced using the ABI PRISM Big Dye Terminator v3.1 cycle sequencing kit using the same primers as in the amplification.

Multiple sequence alignments and phylogenetic analysis were performed using a 217 bp partial L-gene sequence obtained for the 22 virus isolates from Ireland and another 13 vesiculo-type viruses isolated from different fish species (Table 1). A partial L-gene sequence from SVC virus (AJ318079) was used as an outgroup. Multiple alignments were performed using Clustal W (Thompson et al. 1997) with the following Clustal parameters: a gap opening penalty of 15 and gap extension penalty of 6.66. Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al. 2007). The neighbour-joining tree was constructed using a maximum composite likelihood model, and the robustness of the tree was tested using 1000 bootstrap replicates.

RESULTS

Wild fish: clinical signs and pathology

In each case, the wild perch sent to the laboratory for diagnostic testing were found to be infected with parasites such as *Scyphidia* sp., *Ichthyobodo* sp., *Chilodonella* sp. and *Tricodina* sp. Fungal and bacterial infections were also noted on occasion, with mixed growths of *Aeromonas hydrophila* and *A. sobria* often found during bacteriological analysis. The 5 bream submitted in 1997 all showed haemorrhagic skin lesions and a heavy infestation of *Trichodina* sp. Bacteriological analysis showed a mixed growth of *A. hydrophila* and *A. sobria*. The brown trout from the restocking facility showed a severe *Ichthyophthirius multifiliis* infestation on the skin and gills along with moderate levels of *Chilodonella* sp. and *Scyphidia* sp. Fifteen fish had signs of exophthalmia, and 10 fish had an extended abdomen. Internally, the fish showed signs of proliferative kidney disease.

Farmed fish: clinical signs and pathology

Mortality of 40% was reported in 1 batch of 100 000 juvenile perch from a grow-out farm (F1) in September 2005 (F3003-05). The juveniles averaged 6 g in weight, and all 36 examined individuals presented with similar findings. The clinical signs observed in moribund fish included lethargy, loss of balance (presented as fish temporarily losing their position in the water column), erratic swimming behaviour, some darkening of the skin at the caudal region and haemorrhages at the bases of the fins. Scales projected from the fins (Fig. 2A) and internally, fish had clear ascitic fluid in the abdominal cavity but no other abnormalities, other than not having fed. Histopathology was undertaken on 8 fish and revealed scattered necrotic hepatocytes in the liver, multifocal necrosis in the haematopoietic tissue of the spleen (Fig. 2B) and kidney as well as necrotic cells in the lamina propria of the intestine. The central nervous tissue (brain) presented with congestion in the meninges and in 1 fish, large areas of haemorrhage. Another fish had marked endocardial cell proliferation in the cardiac atrium (Fig. 2C). A follow-up sample at the same farm in October 2005 (F3032-05) resulted in the isolation of perch rhabdovirus; however, no pathology associated with a viral infection was found after histological analysis.

The perch fry from hatchery H1 in July 2007 (F3382-07) showed no obvious clinical signs of a viral

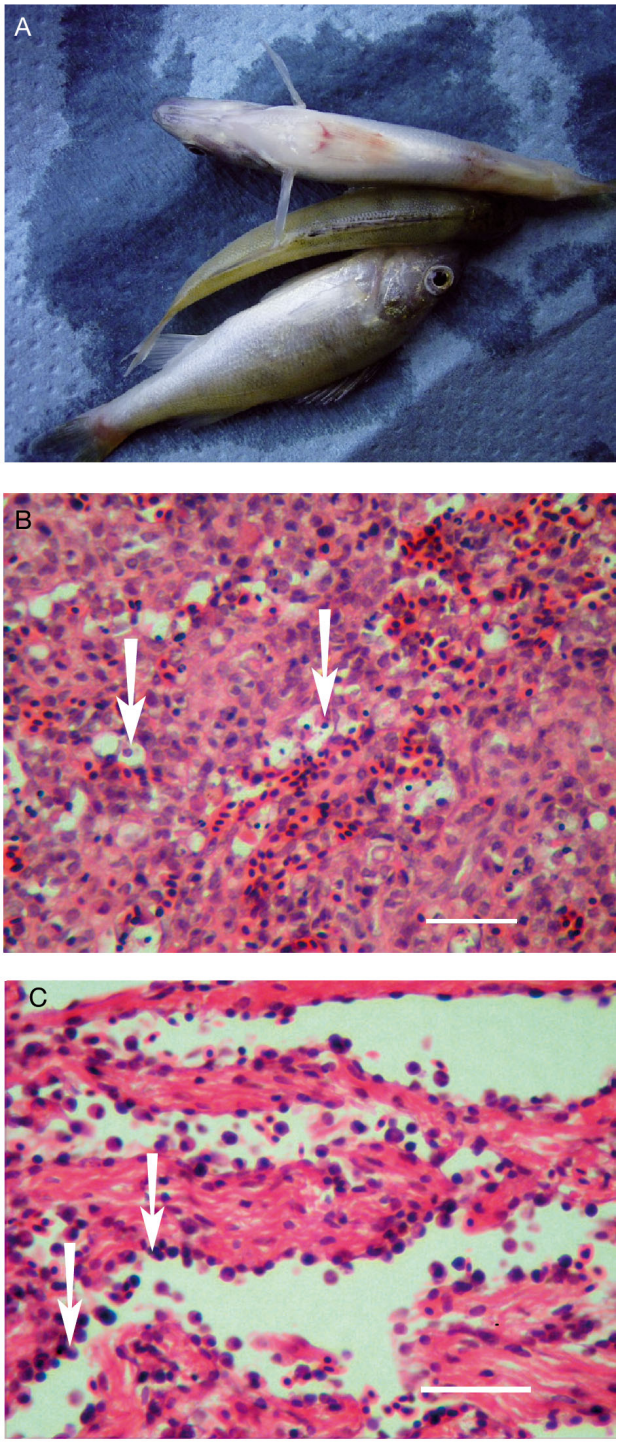

Fig. 2. (A) Juvenile European perch *Perca fluviatilis* exhibiting congestion at the base of the pelvic, anal and tail fins and projection of dermal scales following infection with perch rhabdovirus. (B) Histopathological section of perch spleen presenting with focal necrosis (arrows) in the haematopoietic tissue. H & E stain. Scale bar 60 µm(C) Histopathological section of perch cardiac atrium presenting with endocardial cell proliferation (arrows). H&E stain. Scale bar = 60 µm

infection. *Ichthyobodo* sp. were present on the gills of all fish, and 1 fish had coccidian spores in the intestinal wall (results not shown). In February 2009 (F009-09), the 3 wild-caught brood fish were observed to have focal hyperplasia, inflammation and haemorrhages in the gills. Two of the fish had granulomas present in the liver along with areas of liquefactive necrosis.

The adult perch and fry collected from farm F2 in November 2007 (F3446-07) showed no clinical signs of a viral infection. The gills had necrotic epithelia, and fungal hyphae and cellular debris were observed in the interlamellar spaces. The fry sampled in October 2008 showed pathology consistent with a chronic bacterial infection (F147-08). Kidney tissue showed a loss of normal architecture, with areas of coagulative necrosis and fibrosis, and *Aeromonas sobria* was isolated from kidney swabs of these fish.

Virus culture

All samples tested produced CPE in both the EPC and BF-2 cell lines within 7 d. The only exceptions to these were isolates F044-09, F108-09 and F004-13, which resulted in CPE on BF-2 cells only. All samples tested negative for IHN, VHS, IPN and SVC viruses by IFAT and ELISA.

Sequence analysis

PCR amplifications using the generic primer sets produced products of the expected size for all of the viruses tested. Sequence analysis based on a 217 nucleotide region of the product obtained for the perch virus isolates revealed 3 novel sequences that shared 98.2 to 100% nucleotide identity (0 to 4 nucleotide substitutions) when compared to each other. Samples F108-09, F147-08, F3382-07, F100-08, F004-13 and F044-09 shared 100% nucleotide identity over the 217 nucleotides, and all were isolated from farmed perch. The partial L-gene sequence was identical for all of the 9 wild perch isolates, and these shared 100% nucleotide identity with a virus isolated from farm site F1 (F3032-05), a single virus isolate from farm site F2 (F3446-07) and a single virus isolate from the hatchery site H1 (F009-09). Another sample from farm site F1 (F3003-05) differed from the latter by a single nucleotide (Fig. 3). The nucleotide variations observed between the perch virus isolates were synonymous and did not lead to an amino acid substitution.

```
903/87      GTGGGAATTG AGAGAGTACT TCGTTATCAC AGAATATCTG ATAAAAACAC ACTATGTGCC TCTATTCAAA GGATTGACAA TGGCAGACGA CATGACGGAA GTTGTGAAGA  [110]
F3003-05    C........A .......... ....C..... .........A .......... .T.....A.. ......T... ..G....... .......... ......A..G ..........  [110]
F3446-07    A........A .......... ....C..... .........A .......... .T.....A.. ......T... ..G....... .......... ......A..G ..........  [110]
F108-09     A........A .....A.... ....C..... .........A .......... .T.....A.. ......T... ..G....... .......... ......A..G ..........  [110]
F1737-99    C......... ..G.....T. .T..A..A.. T.....C... ..C.....T. .T.....C.. ...C..T... ........T. .......T.. ......A..G ..C.......  [110]
DK5533      A......C.. .....A..T. .T..A..... ......C..C .....G.... .T........ CT........ ..G..A..C. .......T.. T........G .....A..A.  [110]
EVA         T........A ........T. .T..G..A.. C..G..C... ..C.....C. .T..C..C.. ...C..T... ...C....T. ....C..... ......A... ..C..C..A.  [110]
EVEX        C......... ..G.....T. .T..A..A.. T.....C... ..C.....T. .T..C..C.. ...C..T... ........C. .......T.. ......A..G ..C..C....  [110]
PRV         A......... .....A..T. .T..A..A.. ......CT.A ..C..G.... .......... CT........ ..G..A..C. .......T.. T......... .....A....  [110]
PRV 47-90   A......... .....A..T. .-..A..A.. .......T.A ..C..G.... .......... CT........ ..G.....C. .......... T.....A..G .....A....  [110]
PRV 48-90   A......... .....A..T. .T..A..AC. ...G...T.A ..C..G..C. .......... CT......C. ..G.....C. ....G..T.. T...T....G ..........  [110]
02-95       A......... .....A..T. ....A..A.. ......CT.. ..C..G.... .......... CT........ ..G..A..C. .......T.. T......... .....A....  [110]
80560       T...C.G... .......... .T..G..A.. G......... ..T..G..C. .T.....T.. A.....T... ........T. .......T.. .C.C..ATC. ..C.......  [110]
F1350-97    T...C.G... .......... .T..G..A.. G......... ..T..G..C. .T.....T.. A.....T... ........C. .......T.. .C.C..ATC. ..........  [110]
PFRV        C...C..C.. .....A..T. .T..C..... C..G..CT.A .....G..T. .T.....A.. AT........ ...C.C.... ....G..T.. TC....ATC. .....A....  [110]
V76         A...C.G..A ..G....... ....C..... ...G..CT.. ..C....... .......C.. GT.G.....G ...C....G. .......T.. .C.T..ATCC ..........  [110]
S30         A...C..... ..G....... .T..A..T.. ...G...... ..C..G.... .T.....T.. G..C.....G .....A..G. ....C..... TC.C..CTCT ..G..A....  [110]

903/87      AAATGTTAGA GAGAAGCCAG GGACAGGGAG AAGAAGATTA CGAACATATC AGTATCGCCA ACCACATAGA CTACGAAAAA TGGAACAATC ACCAGAGGAA AGCTTCT     [217]
F3003-05    .......... A..G...... .....A.... .......... ......C..T ..C..T.... .......... .......... ........C. .......... .......     [217]
F3446-07    .......... A..G...... .....A.... .......... ......C..T ..C..T.... .......... .......... ........C. .......... .......     [217]
F108-09     .......G.. A..G...... .....A.... .G........ ......C..T ..C..T.... .......... .......... ........C. .......... .......     [217]
F1737-99    .G........ A.....T..A .....A.... .G..T..... ...G...G.. ..C..T.... .......T.. T..T...... ........C. .T..A..... ..AG...     [217]
DK5533      .G...C.G.. A.....T... ..G..A.... ....T..C.. T..G..C..T ..C....... ....T..T.. ...T.....G .......... .T.....A.. G.AA..A     [217]
EVA         .G.....G.. A.....T..A .......... .G..T..... T..G...G.. ..C..T.... .T..T..T.. T......... ........C. .T..A..... ..AG..C     [217]
EVEX        .G........ A.....T..A .....A.... .G..T..... ...G...G.. ..C..T.... .......T.. T..T...... ........C. .T..A..... ..AG...     [217]
PRV         .....C.... A..G..T..A ..G....... ....T..C.. ...G..C... ..C..T.... .......T.. ...T..G..G .....T.... .T.....A.. G.AA..A     [217]
PRV 47-90   .....C.... A.....T..A .......... ....T..C.. ...G..C... ..C..T.... .......C.. ...T..G..G .....T.... .T..A..A.. G.AA..A     [217]
PRV 48-90   .....C.G.. A.....T..A .......... ....T..C.. ...G..C... ..C..T.... .......T.. ...T..G..G .....T.... .T.....A.. G.AA..A     [217]
PRV 02-95   .....C.G.. A..G..T..A ..G....... ....T..C.. ...G..C... ..C..T.... .......T.. ...T.....G .....T.... .T.....A.. G.AA..A     [217]
80560       .G.....G.. C.AC.CAA.C .....A...T T...C..C.. TTC.TCG..T T....A..G. .T.....T.. T......... .......... .T........ ..AG..A     [217]
F1350-97    .G.....G.. C.AC.CAA.C .....A...C T...C..C.. TTC.TCG..T T....A..G. .T.....T.. T......... .......... .T........ ..AG..A     [217]
PFRV        .G.....G.. C.AC.CGA.T .....A...C TG..T..... TTC.TCG..T T.C....... .T.....C.. ...T..G..G .....T.... .T.....A.. ..AA..C     [217]
V76         .G.....G.. C.AC.CAA.T .....A...T TG..T..C.. .TCCTC.... T....T.... .......T.. .......... .....T.... .T........ ..AG...     [217]
S30         .G........ T.AC.CTA.T ..G..A..CT TG..C..C.. .TC.TC...A T.C..T..G. .......T.. T..T...... .......... .T..AC.... ..AA..C     [217]
```

Fig. 3. Alignment of partial (217 bp) L-gene sequences of the lake trout rhabdovirus 903/87 and a range of fish rhabdoviruses with the 3 unique sequences generated for the viruses isolated from European perch *Perca fluviatilis* from Ireland (F3003-05, F3446-07 and F108-09), and the isolates from bream (F1350-97), trout (F1935-00) and eel (F1737-99). The origins of the viruses are provided in Table 1

The partial L-gene sequences obtained for the Irish perch isolates shared 89 to 92% nucleotide identity with the partial L-gene sequence determined for lake trout rhabdovirus 903/87. They shared 83 to 84% nucleotide identity with the equivalent region from eel virus European X (EVEX; JN639009), and only 78 to 81% nucleotide identity with the original perch rhabdovirus isolate (PRV, JX679246) from France. Phylogenetic analysis assigned the perch isolates to a single clade that was most closely related to the lake trout rhabdovirus isolate 903/87. The isolate from trout (F1935-00) was also assigned to the same lineage. The isolates from eel (F1737-99) and bream (F1350-97) were assigned to lineages with EVEX and Tench rhabdovirus 80560, respectively (Fig. 4).

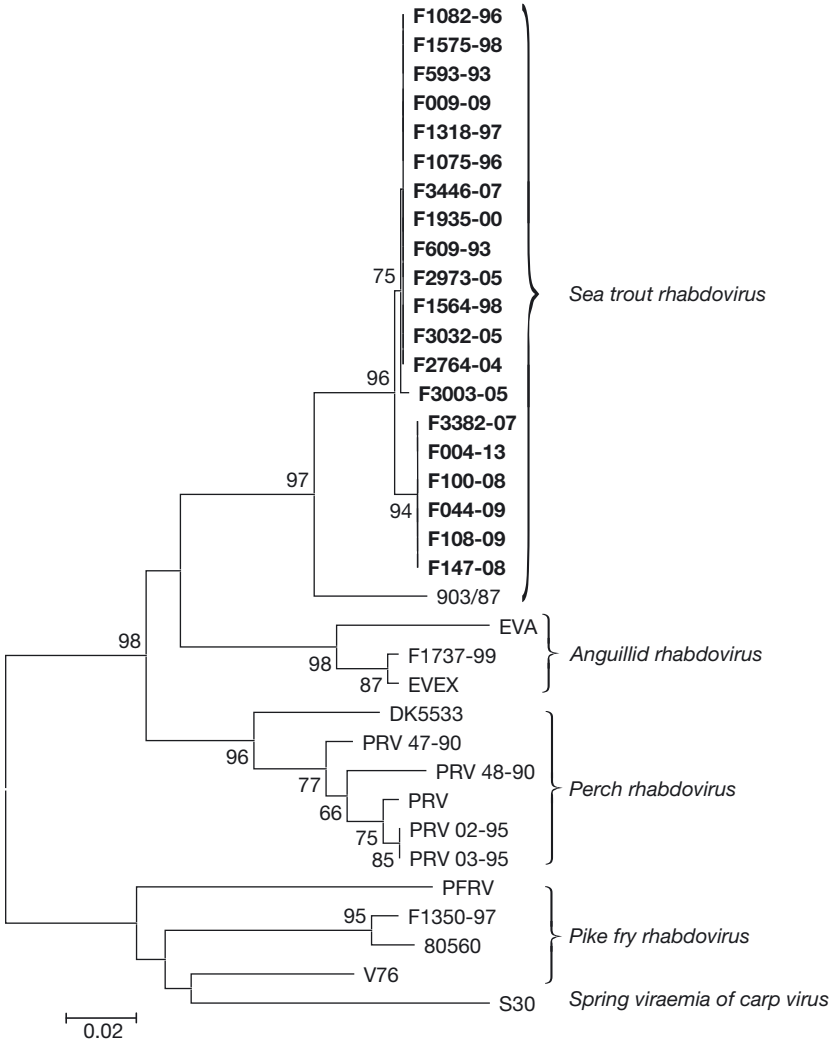

Fig. 4. Phylogenetic tree showing relationships between Irish vesiculovirus isolates (in **bold**) from European perch *Perca fluviatilis* and the main vesiculovirus groups (as listed in Table 1) based on partial (217 bp) RNA polymerase gene sequences. The tree was constructed using the neighbour-joining method, and 1000 bootstrap replicates were performed for each analysis to assess the likelihood of the tree construction. Only values greater than 60 are indicated. The Irish perch isolates form 2 related but distinct lineages which are more closely related to the lake trout rhabdovirus isolate 903/87 and represent new isolates of the species *Sea trout rhabdovirus* in the genus *Perhabdo-virus* rather than an isolate of the species *Perch rhabdovirus*

DISCUSSION

This paper describes the molecular characterisation of the partial RNA polymerase gene sequence of perch rhabdovirus isolates from wild and farmed fish in Ireland between 1993 and 2013. Perch farming was identified as a potential growth area in the diversification of the Irish aquaculture industry (Watson & Stokes 2003), and this was followed by the setting up of a small number of perch hatcheries and grow-out farms. A steady reliable source of juveniles has proved a bottleneck to the establishment of the industry (Toner & Rougeot 2008) and has resulted in some sites using wild-caught broodstock to supplement the production of juveniles. The practice of using wild-caught broodstock was linked to a clinical outbreak of disease in a batch of farmed perch (F3003-05) on a grow-out farm (F1) in Ireland in 2005. Fish exhibited pathology consistent with a disease of viral aetiology which was confirmed with the isolation of a virus and by the sequencing performed in this study. A second isolate (F3032-05) from the same farm was detected later the same year during a routine surveillance visit, even though the fish were not showing any clinical signs of disease at that time. Both virus isolates from this farm shared 99.5% nucleotide identity in the partial RNA polymerase gene, indicating that they were derived from a genetically related source.

To better understand the origins of the virus, archived material from wild fish collected between 1993 and 2005 was screened for the perch rhabdovirus by a combination of cell culture and RT-PCR. The majority of these samples had been previously confirmed as perch rhabdovirus by the EU Reference Laboratory for fish diseases using immunofluorescence and neutralisation tests as described by Dannevig et al. (2001). Phylogenetic analysis showed that the Irish perch isolates formed 2 related but distinct lineages which were more closely related to the lake trout rhabdovirus isolate 903/87 (Koski et al. 1992, Björklund et al. 1994, Johansson et al. 2001) and represent new isolates of the species *Sea trout rhabdovirus* in the genus *Perhabdovirus* (Stone et al. 2013) rather than an isolate of the species *Perch rhabdovirus.* Interestingly, all of the isolates from wild perch shared 100% nucleotide identity with the isolate from farm F1 (F3032-05), indicating that a virus similar to the disease-causing agent had been circulating in wild perch since 1993. The presence of the virus in wild fish in Ireland between 1993 and 2005 high-

lights the risk posed to perch aquaculture when using wild-caught broodstock and identifies the potential source of the initial outbreak in the grow-out farm (F1) in 2005. The same virus was isolated from farmed perch from a second grow-out farm (F2) in 2007 (F3446-07) and a perch hatchery (H1) in 2009 (F009-09). In both of these cases, the virus was isolated from wild-caught adult perch destined for use as broodstock.

The second cluster consisted of 6 isolates, all originating from farm sites. Four of the viruses (F100-08, F147-08, F044-09 and F108-09) were isolated in 2008 and 2009 in a grow-out farm (F2). The other 2 isolates within this group came from fry in the hatchery (H1), one isolated in 2007 (F3382-07) and the other in 2013 (F004-13). They share 98.2% nucleotide identity with the isolate from F1 in 2005, suggesting that they may have originated from a closely related but distinct source within Ireland. They also share 98.2% nucleotide identity with the virus isolated in 2009 from wild-caught adult perch on the same site H1 (F009-09).

The virus appears endemic in the wild perch population surrounding the hatchery having been isolated from wild-caught broodstock and juvenile perch. It is not surprising, then, that there appears to be a genetic link between the viruses associated with outbreaks of disease on these farm sites supplied by the hatchery and the virus discovered in the broodstock on the hatchery site in 2009. The reason for the sequence differences between the 2 virus clusters is unclear at present, and whether the sequence differences have any effect on the virulence of the isolates is not known. Surprisingly, although all of the wild samples of perch were associated with cases of mortality, no obvious pathology was observed which could be associated with viral infections. The majority of the fish submitted for examination showed significant gill pathology and had high numbers of ectoparasites, all of which are more indicative of poor environmental conditions. A similar situation was seen in the farmed perch, where, with the exception of the first case in 2005, all subsequent samples failed to indicate clinical viral infections. This would suggest that the environmental conditions may play a primary role in the health of these fish, with viral and bacterial pathogens acting as secondary, opportunistic pathogens.

The viruses isolated from perch in Ireland showed greatest nucleotide sequence identity (90.4 to 91.7%) to the lake trout rhabdovirus 903/87 isolated from lake trout *Salmo trutta lacustris* in Finland (Koski et al. 1992). Although the virus in our study was recovered primarily from perch, it was also isolated from brown trout fingerlings from a state hatchery used for restocking purposes in 2000 (F1935-00). Elevated mortalities were noted at the time and although clinical signs (which included loss of equilibrium and disorganised swimming behaviour) were consistent with a viral infection, the fish were also heavily infested with gill parasites and fungal infections, so it could not be demonstrated that the mortalities were due to the viral infection. Isolation of viruses from perch and black bass (*Micropterus* sp.) that share a close genetic relationship to the lake trout rhabdovirus were described previously (Talbi et al. 2011), but since the analysis was based on partial N- and complete G-gene sequences, it was not possible to determine their relationship to the viruses isolated from perch in Ireland.

Based on partial G-gene sequence data, the rhabdovirus isolated from wild bream in 1997 (F1350-97) was previously shown to be genetically similar to other rhabdovirus isolates from wild bream, roach and farmed brown trout and rainbow trout *Oncorhynchus mykiss* in Northern Ireland (Rowley et al. 2001; isolate E1350). It was also found to be genetically similar to rhabdovirus isolates from diseased bream, roach, tench and crucian carp in England (Way et al. 2003). The phylogenetic analysis based on the partial L-gene sequences generated in our study confirmed the initial findings that isolate F1350-97 is closely related to the tench rhabdovirus 80560, a virus of the species *Pike fry rhabdovirus* in the genus *Sprivivirus* and indicates that there is more than 1 type of rhabdovirus circulating within the wild fish population in that region of Ireland and their ability to transfer between hosts. In fact, pike fry rhabdovirus was reported from brown trout in Northern Ireland in the 1980s (Adair & McLoughlin 1986). The virus isolated from eels in the River Shannon in 1999 was identified as an isolate of the species *Anguillid rhabdovirus* in the genus *Perhabdovirus*, further demonstrating the variety of rhabdoviruses circulating in the environment.

The L gene is highly conserved and is usually well suited to determine the evolutionary relationships between more distantly related rhabdoviruses. In our study, a generic PCR assay targeting a short region of the L gene proved to be a useful diagnostic tool for the detection and discrimination of closely related rhabdovirus isolates from the genera *Sprivivirus* and *Perhabdovirus* without the need to screen other more divergent regions of the genome such as the G and N genes. Given the apparent widespread nature of sea trout rhabdoviruses in Ireland there is an urgent need for more data on the host susceptibility range

for the virus so that any potential threat posed to the expansion of the aquaculture industry into new species can be assessed. Moreover, it will be important to make a thorough assessment on the risk of the transmission from farm and hatchery to wild fish and the potential disease impact that sea trout rhabdovirus could have on wild fish populations.

Acknowledgements. This work was funded by the Marine Institute and under Defra contracts F1152 and F1191. We acknowledge Cathy Hickey and Frank McKiernan (Marine Institute) and the staff at the EU Reference Laboratory for Finfish Diseases for their work during the original isolations of the virus. We thank the following for kindly providing virus isolates: Dr N. J. Olesen, for TenRV 80560 and GrRV V76; and Dr. T. Sano for EVEX and EVA.

LITERATURE CITED

Adair BM, McLoughlin M (1986) Isolation of pike fry rhabdovirus from brown trout (*Salmo trutta*). Bull Eur Assoc Fish Pathol 6:85–86

Ahne W (1975) A rhabdovirus isolated from grass carp (*Ctenopharyngodon idella* Val.). Arch Virol 48:181–185

Betts AM, Stone DM, Way K, Torhy C, Chilmonczyk S, Benmansour A, de Kinkelin P (2003) Emerging vesiculo-type virus infections of freshwater fishes in Europe. Dis Aquat Org 57:201–212

Björklund HV, Olesen NJ, Jørgensen PEV (1994) Biophysical and serological characterization of rhabdovirus 903/87 isolated from European lake trout *Salmo trutta lacustris*. Dis Aquat Org 19:21–26

Bourhy H, Cowley JA, Larrous F, Holmes EC, Walker PJ (2005) Phylogenetic relationships among rhabdoviruses inferred using the L polymerase gene. J Gen Virol 86: 2849–2858

Dannevig BH, Olesen NJ, Jentoft S, Kvellestad A, Taksdal T, Håstein T (2001) The first isolation of a rhabdovirus from perch (*Perca fluviatilis*) in Norway. Bull Eur Assoc Fish Pathol 21:186–194

De Kinkelin P, Galimard B, Bootsma R (1973) Isolation and identification of the causative agent of 'red disease' of pike (*Esox lucius* L. 1766). Nature 241:465–467

Dorson M, Torchy C, Chilmonczyk S, de Kinkelin P, Michel C (1984) A rhabdovirus pathogenic for perch; *Perca fluviatilis* L.: isolation and preliminary study. J Fish Dis 7: 241–245

Fijan N, Petrinec Z, Sulimanovic D, Zwillenberg LO (1971) Isolation of the causative agent from the acute form of infectious dropsy of carp. Vet Arh 41:125–138

Gadd T, Viljamaa-Dirks S, Holopainen R, Koski P, Jakava-Viljanen M (2013) Characterization of perch rhabdovirus (PRV) in farmed grayling *Thymallus thymallus*. Dis Aquat Org 106:117–127

Grignard JC, Mélard C, Kestemont P (1996) A preliminary study of parasites and diseases of perch in an intensive culture system. J Appl Ichthyol 12:195–199

Haenen O, Davidse A (1989) Isolation of pike fry rhabdovirus from roach (*Rutilus rutilus*). Bull Eur Assoc Fish Pathol 9:116

Johansson T, Nylund S, Olesen NJ, Björklund H (2001) Molecular characterisation of the nucleocapsid protein gene, glycoprotein gene and gene junctions of rhabdovirus 903/87, a novel fish pathogenic rhabdovirus. Virus Res 80:11–22

Johansson T, Östman-Myllyoja L, Hellström A, Martelius S, Olesen NJ, Björklund H (2002) A novel fish rhabdovirus from Sweden is closely related to the Finnish rhabdovirus 903/87. Virus Genes 25:127–138

Jørgensen PEV, Olesen NJ, Ahne W, Wahli T, Meier W (1993) Isolation of a previously undescribed rhabdovirus from pike *Esox lucius*. Dis Aquat Org 16:171–179

Koski P, Hill BJ, Way K, Neuvonen E, Rintamäki P (1992) A rhabdovirus isolated from brown trout (*Salmo trutta* m. *lacustris* (L.)) with lesions in parenchymatous organs. Bull Eur Assoc Fish Pathol 12:177–178

Lönnström LG, Hoffrén ML, Wiklund T (2008) *Flavobacterium psychrophilum* associated with mortality of farmed perch, *Perca fluviatilis* L. J Fish Dis 31:793–797

Nougayrède P, de Kinkelin P, Chilmonczyk S, Vuillaume A (1992) Isolation of a rhabdovirus from the pike-perch *Stizostedium lucioperca* L 1758. Bull Eur Assoc Fish Pathol 12:5–7

Rodger HD, Girons A (2008) Diseases and health management in perch farming. Fish Vet J 10:9–18

Rowley H, Graham DA, Campbell S, Way K, Stone DM, Curran WL, Bryson DG (2001) Isolation and characterisation of rhabdovirus from wild common bream *Abramis brama*, roach *Rutilus rutilus*, farmed brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* in Northern Ireland. Dis Aquat Org 48:7–15

Sano T (1976) Viral diseases of cultured fishes in Japan. Fish Pathol 10:221–226

Stone DM, Kerr RC, Hughes M, Radford AD, Darby AC (2013) Characterisation of the genomes of four putative vesiculoviruses: tench rhabdovirus, grass carp rhabdovirus, perch rhabdovirus and eel rhabdovirus European X. Arch Virol 158:2371–2377

Talbi C, Cabon J, Baud M, Bourjaily M, de Boisséson C, Castric J, Bigarré L (2011) Genetic diversity of perch rhabdoviruses isolates based on the nucleoprotein and glycoprotein genes. Arch Virol 156:2133–2144

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882

Toner D, Rougeot C (2008) Farming of Eurasian perch, Vol 1. Juvenile production. Aquaculture Explained no. 24. Bord Iascaigh Mhara (Irish Sea Fisheries Board), Dublin

Vicenova M, Reschova S, Pokorova D, Hulova J, Vesely T (2011) First detection of pike fry-like rhabdovirus in barbel and spring viraemia of carp virus in sturgeon and pike in aquaculture in the Czech Republic. Dis Aquat Org 95:87–95

Wahli T, Burr SE, Pugovkin D, Mueller O, Frey J (2005) *Aeromonas sobria*, a causative agent of disease in farmed perch, *Perca fluviatilis* L. J Fish Dis 28:141–150

Watson L, Stokes A (2003) Seahorses to sea urchins: the next big splash in Irish aquaculture. Bord Iascaigh Mhara (Irish Sea Fisheries Board), Dublin

Way K, Bark SJ, Longshaw CB, Denham KL and others (2003) Isolation of a rhabdovirus during outbreaks of disease in cyprinid fish species at fishery sites in England. Dis Aquat Org 57:43–50

Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: September 17, 2013; Accepted: August 24, 2014
Proofs received from author(s): November 24, 2014