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ORIGINAL ARTICLE

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Detection and characterization of a rhabdovirus causing mortality in black bullhead catfish, *Ameiurus melas*

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Abstract

This study fully describes a severe disease outbreak occurred in 2016 in black bullhead catfish farmed in Italy. Affected fish showed nervous clinical signs as well as emaciations and haemorrhagic petechiae on the skin at the fin bases, abdomen and gills. Viral isolation in cell culture allowed the subsequent identification of a rhabdovirus, tentatively named ictalurid rhabdovirus (IcRV), through electron microscopy, immunofluorescence and whole genome sequencing (WGS). The newly isolated virus, together with 14 additional viral strains stored in our repository and detected during similar mortality episodes in the period 1993–2016, was phylogenetically analysed on the basis of the nucleoprotein and the glycoprotein nucleotide and amino acid sequences. The genetic distances among Italian IcRV strains were also estimated. Our results show that all the IcRV strains belong to the genus *Sprivivirus* and are closely related to the tench rhabdovirus (TenRV). Italian catfish production is constantly decreasing, mainly due to viral infections, which include the newly characterized IcRV. Data presented in this work will assist to investigate the molecular epidemiology and the diffusive dynamics of this virus and to develop adequate surveillance activities.

KEYWORDS
Ameiurus melas, phylogenetic analysis, rhabdovirus, virus characterization, whole genome sequencing

1 | INTRODUCTION

The family *Rhabdoviridae* comprises viruses with a wide range of hosts, including fresh and marine water fish species, which are widespread in North America, Europe and Asia (Purcell, Laing, & Winton, 2012). According to the International Committee on Taxonomy of Viruses (ICTV) (2016), the *Rhabdoviridae* family encompasses 18 genera, four of which can infect finfish, namely *Vesiculovirus*, *Sprivivirus*, *Perhabdovirus*, *Novirhabdovirus*.

Rhabdoviruses are bullet-shaped, enveloped RNA viruses. The structure of their negative sense ssRNA genome is conserved along genera and includes five viral proteins in the following order: 3′nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L)5′. An additional gene coding for a non-virion protein (NV) has been described in *Novirhabdoviruses*, and it is considered distinctive of this genus (Kurath, Higman, & Björklund, 1997; Talbi et al., 2011).

From a pathogenic point of view, rhabdoviruses generally cause systemic haemorrhagic syndrome, exophthalmos and melanosis ending up with moderate-to-severe mortality. Affected finfish show flashing and impaired swimming, often revealing neurological signs. It must be underlined that clinical signs and mortality only appear when the

conditions are conducive to the clinical expression of the disease (i.e., water temperature, physiological stage, host, ...) (Kurath & Winton, 2008; Vo et al., 2015). Furthermore, rhabdoviruses are easily grown on cell monolayers, and this, together with typical lesions, generally makes its first diagnosis smooth. Survivors can recover and develop long-lasting immunity. However, a certain percentage of fish become carriers for their entire lifetime (Purcell et al., 2012).

Catfish farming is common in Hungary, Poland, Czech Republic, the Netherlands and Italy, but while Eastern European countries have a preference for African catfish, *Clarias gariepinus;* and wels catfish, *Silurus glanis;* the black bullhead catfish, *Ameiurus melas*, is almost exclusively farmed in Italy. The Italian production area is located in the northern part of the country, along the Po River. Black bullhead catfish naturally breed in hearth ponds in Eastern Europe and are transported to Italy via truck. Once in Italy, fish are grown in concrete or mud tanks with well/river water for 1–2 years until they reach the commercial size of 100–200 g. Finally, catfish are released in put and take ponds or sold to retail fish markets in the summer. The Italian production totalled more than 600 t in 2013–2014; however, the profitability of this industry has rapidly decreased to less than 200 t mainly because of recurrent outbreaks of viral diseases (FEAP 2016). Viral agents threatening black bullhead species include the following: the European catfish virus (ECV), a Ranavirus causing severe mortalities mainly during summer, the ictalurid herpesvirus (IcHV-2) and, sporadically, the spring viraemia of carps virus (SVCV) (Doszpoly et al., 2008; Gobbo, Cappellozza, Pastore, & Bovo, 2010).

In 2016, an Italian farm reported a severe mortality episode (90%) in a recently imported batch of black bullhead catfish. The aim of this study was to investigate the cause of the outbreak and characterize the aetiological agent. Additionally, we characterized 14 viral strains isolated from diseased black bullhead catfish between 1993 and 2016 during similar mortality episodes and stored in our repository.

2 | MATERIALS AND METHODS

2.1 | Case history and sampling

At the end of January 2016, an Italian fish farmer reported an epidemic outbreak which was causing an increased mortality in a black bullhead catfish (*Ameiurus melas*) batch imported from Hungary a few weeks before (December 2015). Approximately 1 t of juveniles catfish (around 50 g each) were farmed in a concrete tank (240 m^3) supplied by well water at a constant temperature (18°C in winter and 20°C in summer) and fed with commercial feed. Other fish species (sturgeon, eel, carp) reared in separated tanks of the same farm never showed any signs of disease nor mortality.

In accordance with the fish farmer, clinical inspections and samplings were performed on-site monthly, from January to June 2016. Pools of 10 healthy and 10 diseased fish were collected during each sampling and subjected to bacteriological, histological and virological examination. Diseased fish were always kept separate from the non-symptomatic ones. Blood samples for serological analyses were also taken from the fish collected between March and June 2016.

2.2 | Gross pathology, microscopic lesions and microbiological investigation

Upon arrival at the laboratory, fish were killed with an overdose of MS222 (Tricaine Pharmaq). Necropsy was immediately performed. Parasitological analysis was performed on gills and skin by fresh mounted glasses and microscopic observation according to standard procedures. The bacteriological analysis was conducted on kidney, liver and spleen using blood agar plates incubated at 22°C for 24–48 hr. Kidney, spleen, heart, gills and brain samples were collected to carry out histological analyses. Tissues were fixed in 4% phosphate-buffered formaldehyde and embedded in Paraplast applying standard histological protocols. Tissue sections (3 μm) were stained with haematoxylin–eosin (HE) and observed under Leitz Diaplan light microscope (Leica, UK). Digital images were obtained using an integrated Leica MC170HD (Leica, UK) camera and LAS 4.5.0 (Leica, UK) software.

2.3 | Viral isolation, IFAT and TEM

Pools of organs (kidney, spleen, heart, gills and brain) from a maximum of five fish were homogenized with sterile quartz sand and diluted 1:10 with Eagle's minimum essential medium (EMEM) containing 1% antibiotics/antimycotic and 10% foetal calf serum (FCS). After clarification (800–1000 *g* for 15 min), samples were subjected to virological examination on bluegill fry (BF-2) and epithelioma papulosum cyprini (EPC) (Fijan et al., 1983; Wolf & Quimby, 1962) monolayers according to standard procedures (Dec 2015/1554/CE). Plates were incubated at 20°C and monitored for appearance of cytopathic effect (CPE).

All samples producing CPE were tested by indirect fluorescent antibodies test (IFAT) using VHSV (viral haemorrhagic septicaemia virus), IHNV (infectious hematopoietic necrosis virus), SVCV (spring viraemia of carp virus), ECV (European catfish virus) and IcHV (ictalurid herpesvirus) hyperimmune rabbit antisera. Polyclonal antisera were previously produced in-house and tested for sensitivity and specificity (December 2015/1554/CE). Briefly, positive supernatants (diluted 1:1,000–1:10,000) were added to EPC cells grown on glass supports, incubated for 24 hr at 20°C and fixed with 80% acetone. Samples were washed three times with phosphate buffer solution (PBS), air-dried and incubated at 37°C for 30 min with 50 μl of rabbit antiserum diluted 1:500 in PBS. After PBS washing, samples were incubated for 30 min at 37°C with 50 μl of anti-rabbit IgG conjugated with FITC (Sigma) and diluted 1:100 in PBS. Finally, samples were washed three times with PBS, air-dried, mounted on glass slides with a drop of glycerol and observed using an epifluorescence microscope (Zeiss Axioskop equipped with Axiocam MRC 5) at 20×–40× magnification.

Additionally, cell culture supernatants were put onto Formvar/carbon-coated grids, ultracentrifuged for 15 min in a Beckman Airfuge at 20 psi (125,000 *g*) using an A-100 rotor, stained with 1% phosphotungstic acid solution and subjected to direct transmission electron microscopy (TEM) (Philips 208S operating at 80 kV, at a magnification of 19,000–45,000).

2.4 | Serological methods

Blood samples collected monthly from the caudal vein were refrigerated overnight at 4°C to allow clot formation. Sera were harvested and inactivated at 45°C for 30 min. Each serum sample was tested in duplicate.

For the serum neutralization assay (SN), 50 μl of twofold serial dilutions of heat-treated catfish sera was mixed with an equal volume of titrated virus (2×10^3 $TCID_{50}$/ml) in 96-well plates and incubated overnight at 4°C. The serum–virus mixture (50 μl) was then seeded on semiconfluent EPC monolayer, incubated at 20°C for 6 days and monitored for appearance of CPE. Titre was calculated as the last serum dilution preventing the appearance of CPE (Hattenberger-Baudouy, Danton, Merle, & de Kinkelin, 1995).

To perform 50% plaques' neutralization test (PNT), 100 μl of twofold serial dilutions of heat-treated sera was mixed with an equal volume of titrated virus (10^3 PFU/mL) in 96-well plates and incubated overnight at 4°C. The serum–virus mixture (10 μl) was then seeded on semiconfluent EPC monolayer. Before inoculum, cell culture medium was discharged, and the samples left in adsorption for 1 hr at 20°C under gentle shaking; 150 μl solution of 1.6% carboxymethyl cellulose (MTC) (Sigma) was added to each well, and samples were incubated at 20°C for 24 hr. The supernatant was removed, and cells were fixed with formalin for 1 hr at room temperature. After three steps of PBS washing, plates were air-dried and stained for 30 min with 1% crystal violet (Sigma). The positive titre was calculated as the highest dilution at which the mean of the plaques number was less than the 50% of the mean of the plaques number in the control wells.

For both methods, serum samples were tested against the homologous IcRV viral strain isolated from diseased catfish (IcRV/A.melas/I/75/2016) and SVCV virus isolate 56/70 (Fijan, Petrinec, Sulimanović, & Zwillenberg, 1971). Sera were considered positive when titre was higher than 1:40.

The geometric mean titre (GMT) of the log2 last positive dilution of each serum collected at the same time point was then calculated (Brugh, 1978). Log2-transformed dilutions were subsequently analysed by Welch's ANOVA test.

2.5 | RNA extraction, RT-PCR and Sanger sequencing

Total RNA was purified from 100 μl of positive cell culture supernatant (sample IcRV/A.melas/I/75/2016) using the NucleoSpin RNA kit (Macherey-Nagel) following the manufacturer's protocol. RT-PCR was performed using a set of degenerated primers targeting the partial N gene sequence (amplicon size 670 bp) of fish rhabdoviruses, as described by Talbi et al. (2011), and the One-step RT-PCR kit (Qiagen). The reaction mix was constituted by 1X PCR Buffer, 0.2 mM dNTPs, 1 μM of each primer, 8U of RNase inhibitor, 0.5 μl RT-PCR Enzyme Mix and 5 μl of RNA in a total volume of 25 μl.

The RT-PCR was performed in a DNA Engine Thermal Cycler (Bio-Rad) by applying the following thermal profile: an initial step at 50°C for 30 min, 94°C for 15 min, 40 cycles at 94°C for 30 s, 47°C for 1 min, 68°C for 1 min and finally 68°C for 7 min. The purity and size of the amplicon were verified with 7% acrylamide gel electrophoresis. The PCR product was treated with the A'SAP PCR Clean-up kit (ArcticZymes) and sequenced in both directions using the BrightDye Terminator cycle sequencing kit (Nimagen). Sequence reaction was subsequently purified using Centri-Sep 96-well plates (Princeton Separation) and analysed on a 16 capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The nucleotide (nt) sequences were assembled and edited using the SeqScape v3.1 software (Applied Biosystems).

In addition to strain IcRV/A.melas/I/75/2016 isolated during the disease outbreak herein reported, 14 additional viral isolates, collected between 1993 and 2016 during similar disease outbreaks in black bullhead catfish and stored in our repository, were also analysed as described above (Table 1).

2.6 | Whole genome sequencing (WGS)

The total RNA purified as described above from sample IcRV/A.melas/I/75/2016 was subjected to retrotranscription with SuperScript III Reverse Transcriptase (Invitrogen). Double-stranded cDNA was then synthesized using NEBNext mRNA Second Strand Synthesis Module (Euroclone), purified with Agencourt AMPure XP (Beckman Coulter) and quantified with Qubit dsDNA HS assay kit (Thermo Fisher). The cDNA library was prepared using Illumina Nextera XT DNA Sample Preparation kit (Illumina), and fragments were selected with Agencourt AMPure XP (Beckman Coulter). Library was checked for quality and size with Agilent 2100 Bioanalyzer (Agilent High Sensitivity DNA kit, Agilent Technologies), diluted at a concentration of 2 nM and finally sequenced with Miseq v3 Reagent Kit (300PE) using Illumina MiSeq platform.

Reads quality was assessed using FastQC v0.11.2 (Andrews & Fast, 2014). Raw data were filtered by removing: (i) reads with more than 10% of undetermined ("N") bases; (ii) reads with more than 100 bases with Q score below 7; (iii) duplicated paired-end reads. The remaining reads were clipped from Illumina adaptors Nextera with scythe v0.991 (https://github.com/vsbuffalo/scythe) and trimmed with sickle v1.33 (https://github.com/najoshi/sickle). Reads shorter than 80 bases or unpaired after previous filters were discarded. The taxonomic assignment of high-quality reads was carried out using BLASTN 2.3.0+ alignment (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the integrated NT database (version 26 January 2016) and diamond v0.8.16 alignment (Buchfink, Xie, & Huson, 2015) against the integrated NR database (version 26, January 2016). Alignment hits with e-values higher than 1×10^{-3} were discarded. The taxonomical level of each read was determined by the lowest common ancestor (LCA)-based algorithm implemented in MEGAN v6.5.5 (Huson et al., 2016). For the reconstruction of the consensus sequence, reads taxonomically classified as belonging to the *Rhabdoviridae* family were selected and *de novo* assembled using IDBA-UD v1.1.1 (Peng, Leung, Yiu, & Chin, 2012). Multikmer approach was applied, using a minimum value of 114, a maximum value of

TABLE 1 List of black bullhead catfish isolates characterized in this study. The year of isolation and the GenBank accession numbers for both N and G genes sequences are reported

Strain	Year	Genbank Acc. No. Partial N Sequence	Genbank Acc. No. Complete G Sequence
IcRV/A.melas/I/305/1993	1993	MF960893	MF960883
IcRV/A.melas/I/330/1997	1997	MF960894	MF960878
IcRV/A.melas/I/6/1997	1997	MF960887	MF960884
IcRV/A.melas/I/2600/1997	1997	MF960897	MF960872
IcRV/A.melas/I/555/1999	1999	MF960896	MF960880
IcRV/A.melas/I/533/2000	2000	MF960895	MF960879
IcRV/A.melas/I/2905/2000	2000	MF960898	MF960873
IcRV/A.melas/I/138/2001	2001	MF960890	MF960877
IcRV/A.melas/I/3314/2001	2001	MF960899	MF960874
IcRV/A.melas/I/9185/2002	2002	MF960900	MF960875
IcRV/A.melas/I/228/2003	2003	MF960892	MF960881
IcRV/A.melas/I/20983/2004	2004	MF960886	MF960876
IcRV/A.melas/I/135/2009	2009	MF960889	MF960882
IcRV/A.melas/I/75/2016[a]	2016	MF960888	MF960885
IcRV/A.melas/I/149/2016	2016	MF960881	MF960871

[a]Subjected to WGS.

124 and an inner increment of 10. The longest assembled sequence with a genome length comparable to that of *Rhabdoviridae* (10–15 Kb) was selected. To assure that the selected sequence was truly representative of IcRV genome, we aligned all the reads classified as belonging to the *Rhabdoviridae* family against the newly assembled consensus sequence with BWA v0.7.12 (Li & Durbin, 2010). We performed a visual inspection of the alignment with tablet v1.14.10.21 (Milne et al., 2010) and manually edited the consensus sequence. For IcRV genome annotation, we performed an online BLASTn search (https://www.ncbi.nlm.nih.gov/BLAST/) and transferred to the consensus sequence produced the annotation of the most similar *Rhabdoviridae* genome available in GenBank using MEGA v5.2.2 (Tamura et al., 2011).

2.7 | Phylogenetic analysis

To classify the newly identified catfish viruses within the *Rhabdoviridae* family, sample IcRV/A.melas/I/75/2016 as well as viruses isolated in the period 1993–2016 (Table 1) underwent molecular characterization. For this purpose, a comprehensive phylogenetic tree based on the deduced amino acid (aa) sequence of the partial N gene of the known *Rhabdoviridae* genera (Hoffmann, Beer, Schütze, & Mettenleiter, 2005; Stone, Kerr, Hughes, Radford, & Darby, 2013; Talbi et al., 2011) was first developed. The N gene partial sequences of catfish rhabdoviruses were obtained as described above. Furthermore, to better characterize our samples, a more confined data set including nt and aa sequences related to the glycoprotein of fish rhabdoviruses only was used for additional phylogenetic analyses (Table 2).

To this aim, a new primer set targeting the complete IcRV glycoprotein gene (total amplicon size 1630 bp) was developed based on the genome sequence obtained through the WGS of strain IcRV/A.melas/I/75/2016 (Table 3).

The reaction mix was prepared with 1× PCR Buffer, 0.2 mM dNTPs, 1 μM of each primer, 8U RNase Inhibitor, 0.5 μl RT-PCR Enzyme Mix and 5 μl of RNA in a total volume of 25 μl (One-step RT-PCR kit, Qiagen). PCR was performed in a DNA Engine Thermal Cycler (Bio-Rad) with an initial step at 50°C for 30 min followed by 94°C for 15 min, 40 cycles at 94°C for 30 s, 54°C for 1 min, 68°C for 2 min and a final elongation at 68°C for 10 min. Amplicons purity and size were verified with 7% acrylamide gel electrophoresis. Amplification products were sequenced with the Sanger method as previously described. Sequences alignments were conducted with MAFFT 7 (https://mafft.cbrc.jp/alignment/server) by adopting the G-INS-i iterative refinement method with a gap penalty of 5.0 (Katoh, Kuma, Toh, & Miyata, 2005; Kuraku, Zmasek, Nishimura, & Katoh, 2013).

For both targets, maximum-likelihood (ML) phylogenetic trees were estimated using PhyML 3.1 (Guindon et al., 2010) incorporating the GTR (nt) and the WAG (aa) models. Nodes robustness was assessed with 100 bootstrap resamplings. Phylogenetic trees were visualized using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Pairwise nt and aa distances were estimated using MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

3 | RESULTS

3.1 | Clinical signs of the disease

Clinical signs started in January 2016 and lasted until mid-April 2016. Diseased catfish showed anorexia and a severe lethargic behaviour, with the majority of fish lying at the bottom or at the side of

TABLE 2 List of reference strains of the *Rhabdoviridae* family used for the phylogenetic analyses based on the aa N sequences and the aa/nt G sequences. GenBank accession numbers are reported

		Genbank accession numbers		
Genus	**Abbreviation**	**Whole genome (nt)**	**N (aa)**	**G (aa)**
Lyssavirus	RABV	NC001542	NP056793	
	ABLV	NC003243	NP478339	
	EBLV-1	–	AA62892	
	DUVV	EU293120	ABZ81217	
	LBV	–	ABV01112	
	MOKV	NC006429	YP142350	
	EBLV-2	EU293114	ABZ81187	
Ephemerovirus	ARV	–	AAC54627	
	BEFV	NC002526	NP065398	
Hapavirus	WONV	NC011639	YP002333271	
	FLAV	–	AAN73283	
Tupavirus	TUPV	NC007020	YP238528	
Perhabdovirus	SCRV	NC008514	YP802937	
	EPRV	KR612230	ALJ30355	
	EVEX	JX827265		AFX58971
	EVEX	–	AIY29139	–
	EVEX	NC022581	YP008686602	YP008686606
	PRV	JX679246	AF72893	AFX72891
	LTRV	AF434991	AAL38515	AAL38518
	STRV	AF434992	AAL38520	AAL38523
Vesciculovirus	CHPV	GU212858	ADO63665	
	ISFV	AJ810084	CAH17544	
	VSNJV	JX121111	AFO67842	
	VSAV	EU373658	ACB47439	
	COCV	EU373657	ACB47434	
	VSIV	NC001560	NP041712	
Sprivivirus	SVCV	DQ491000	ABF06660	ABF06663
	SVCV	DQ097384	AAZ20270	AAZ20272
	SVCV	EU177782	ABW24033	ABW24036
	SVCV	AJ318079	CAC51333	CAC51336
	SVCV	NC002803	NP116744	NP116747
	GRCRV	NC025376	YP009094263	
	GRCRV	KC113518	AGE10374	AGE10377
	PFRV	FJ872827	ACP27998	ACP28001
	TenRV	KC113517	AGE10369	AGE10372
Sigmavirus	SIGMAV	GQ410979	ACU65439	
Novirhabdovirus	SHRV	NC000903	NP050580	
	VHSV	EU481506	ACA34520	
	VHSV	AB672616	BAM29132	
	HIRRV	NC005093	NP919030	
	IHNV	X89213	CAA61495	
	IHNV	L40883	AC42150	
	IHNV	NC001652		NP042679

(Continues)

TABLE 2 (Continued)

		Genbank accession numbers		
Genus	**Abbreviation**	**Whole genome (nt)**	**N (aa)**	**G (aa)**
Cytorhabdovirus	LNYV	NC007642	YP425087	
	NCMV	NC002251	NP057954	
Nucleorhabdovirus	PYDV	NC016136	YP004927965	
	SYNV	NC001615	NP042281	

TABLE 3 Primers used for N and G genes amplification

Target	Primer name	Sequence	Amplicon size	Reference
N gene	PVP 141	5′ GAWWTCTGTTAAAGTTTTTTTC 3′	670 bp	Talbi et al., 2011
	PVP 143	5′ TGGATATGTTCTACTTCCACTTCA 3′		
G gene	IcRV F1	5′ CAATGTAGTCCCAAGAGTCCC 3′	907 bp	This work
	IcrRV R1	5′ CTGAGCAATCTGGTACGTCGTC 3′		
	IcrRV F2	5′ CACAGGGTTTACAAATTCAAAGGGGC 3′	833 bp	
	IcrRV R2	5′ CATCTCATGGGATTCCACGGAC 3′		
	IcRV F3	5′ GGAGGAGAGTGTGACGC 3′	575 bp	
	IcRV R3	5′ GTTCTGGCACTATTGGACCTT 3′		
	IcrRV F4	5′ CCAGTGGATGCAGTAGTTGAG 3′	379 bp	
	IcrRV R4	5′ GGAGATGAGTGGTGAATTCAGG 3′		

the tank. Some fish showed breathing difficulties with gasping at the water surface. Clear neurological signs (whirling, flashing), especially after stimulation, were present. The clinical pattern is available as supplemental material (Video S1).

Mortality reached its peak in mid-January and lasted until mid-February, then started to decrease until mid-April, 2016. In May 2016, no clinical signs nor more mortality was detected. In June, the fish farm owner decided to empty the tank and only 150 kg of catfish of 1,500 kg introduced in the tank in January were collected. Although a precise measurement of daily mortality was not available, the cumulative percentage reached 90%.

3.2 | Gross pathology, microscopic lesions and microbiological analysis

Diseased fish presented enlarged abdomen, haemorrhagic suffusion and congested vent. Some specimens appeared darker than normal and emaciated (Figure 1a, b). Necroscopy revealed haemorrhagic petechiae scattered on the skin, at the fin bases, on the abdomen and on the gills (Figure 1c, d). Air bubbles inside the stomach were often detected and accounted for the distended abdomen (Figure 2a). Internally, general congestion of the viscera was observed in the majority of specimens. Enlarged and discoloured liver, splenic hypoplasia and pale kidney were other common findings. Small petechial haemorrhages were sporadically observed also in internal organs (mainly kidney and spleen) (Figure 2b).

Histology confirmed the presence of haemorrhages and severe fusion of the gill lamellae (Figure 3a), with hypertrophy and hyperplasia of gill epithelium. Reduction and necrosis of the renal interstitial lymphoid tissue were also observed (Figure 3c, d). The liver revealed congestion and turbid degeneration of hepatocytes (Figure 3b). Despite the neurological clinical signs, no significant microscopic or macroscopic lesions were found in the brain of the diseased fish, except for a mild congestion.

Bacteriological analysis showed a low number of colonies referred to *Aeromonas sp.* only in few specimens.

Microscopic examination of gills and skin revealed the absence of parasites.

3.3 | Virological investigations

Samples collected from the diseased fish (in January, February and April) showed clear CPE in both EPC and BF-2 cells. Typical CPE was characterized by early appearance (12–24 hr post-infection) of foci of refractive round cells rapidly evolving in the total destruction of cell monolayer (24–48 hr post-infection) (Figure 4b).

Recovered catfish collected in May and June, when the clinical signs ceased, tested negative for virus isolation.

Sera against VHSV, IHNV, ECV and IcHV yielded negative results by IFAT. Interestingly, the presence of fluorescent foci was evident with the use of rabbit antiserum raised against SVCV (Figure 4c). Electron microscopy of positive cell supernatants revealed the massive presence of bullet-shaped viral particles, clearly indicating the presence of a virus belonging to the *Rhabdoviridae* family (Figure 4d).

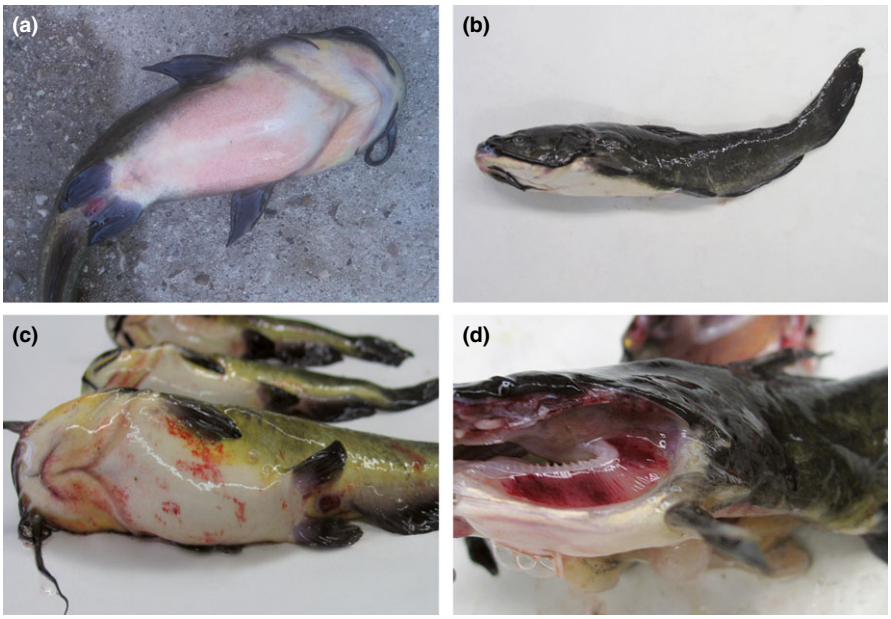

FIGURE 1 External lesions. (a) Live catfish showing distended abdomen with haemorrhagic suffusions and extruding vent; (b) darker and emaciated black bullhead catfish; (c) post-mortem aspect of diseased catfish; (d) haemorrhages of the gills

3.4 | Serological analysis

All catfish sera tested with the homologous virus by SN assay yielded negative results, while with PNT the presence of antibodies in sera of survivors was detected. In details, PNT showed a growing serological titre in surviving catfish over time, and the estimated GMT was 1.33 ± 2.29; 4.10 ± 1.77; 4.34 ± 1.74; 4.80 ± 1.72 from March to June 2016, corresponding to mean plaque neutralization titre of 1:25, 1:170; 1:202 and 1:277, respectively (Figure 5).

Despite it was observed that the antibodies titres increased over time, the Welch's ANOVA showed no significant difference among the monthly GTMs estimated (F = 2.9235, num df = 3.000, denom df = 25.022, *p*-value = .05352).

Sera tested with heterologous antigens (SVCV) showed no reactivity in SN assay, while low neutralization titres attributable to cross-reactivity between SVCV and IcRV in PTN assay were detected (data not shown).

Sera tested with heterologous antigens (SVCV) confirmed previous results: all sera tested negative in SN assay, and low titres attributable to cross-reactivity in PTN assay were detected (data not shown).

3.5 | Whole genome sequencing

The *de novo* assembly of strain IcRV/A.melas/I/75/2016 generated a consensus sequence of 11.086 nucleotides. The BLASTn search identified tench rhabdovirus (TenRV) strain s64 isolated from *Tinca tinca* as being the most similar to IcRV available in GenBank (accession number KC113517). Genome annotation revealed that genome organization of IcRV is consistent with that of other fish rhabdoviruses, especially with those belonging to the *Sprivivirus* family (Amarasinghe et al., 2017; Stone et al., 2013). As a matter of facts, IcRV genome consists of five open reading frames (ORFs) encoding five different proteins in the order: 3′nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), RNA polymerase (L)5′ (Figure 6). ORFs are flanked by the transcriptional start signal 3′-UUGUC-5′, the termination/polyadenylation signal 3′-AUAC(U)$_7$-5′ and interspersed by short 3′-GA-5′ intergenic regions with the exception of the G-L junction which harbours the 3′-GAUA-5′ motif.

3.6 | Phylogenetic analyses

The IcRV subjected to RT-PCR yielded partial N sequences and complete G sequences of 603 bp and 1,533 bp, respectively (length of GenBank submitted sequences). The phylogenetic analyses based on the deduced aa sequence of the N protein, the G gene and the deduced aa sequence of the glycoprotein consistently placed all the strains isolated from catfish within the *Sprivivirus* genus (Figure 7a, b, c). These strains showed the highest aa (97.9%–98.4% for the partial N protein; 97%–99% for the G protein) and nt (96.7%–98.6% for the G gene) identity with TenRV (GenBank accession number KC113517), thus confirming the results previously obtained

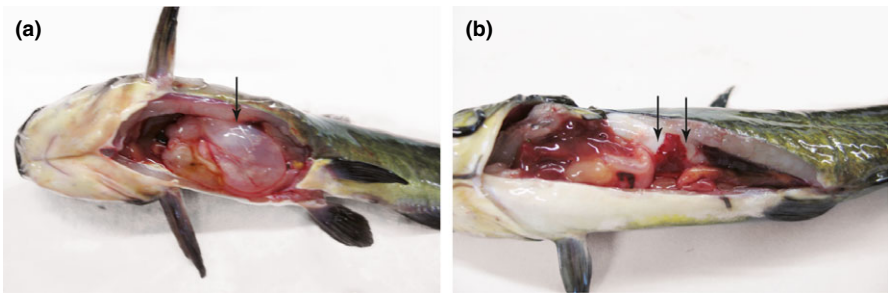

FIGURE 2 Internal lesions. (a) Abundance of gas bubbles in the stomach (arrow) and discoloured liver; (b) haemorrhages on the kidney's edges (arrow)

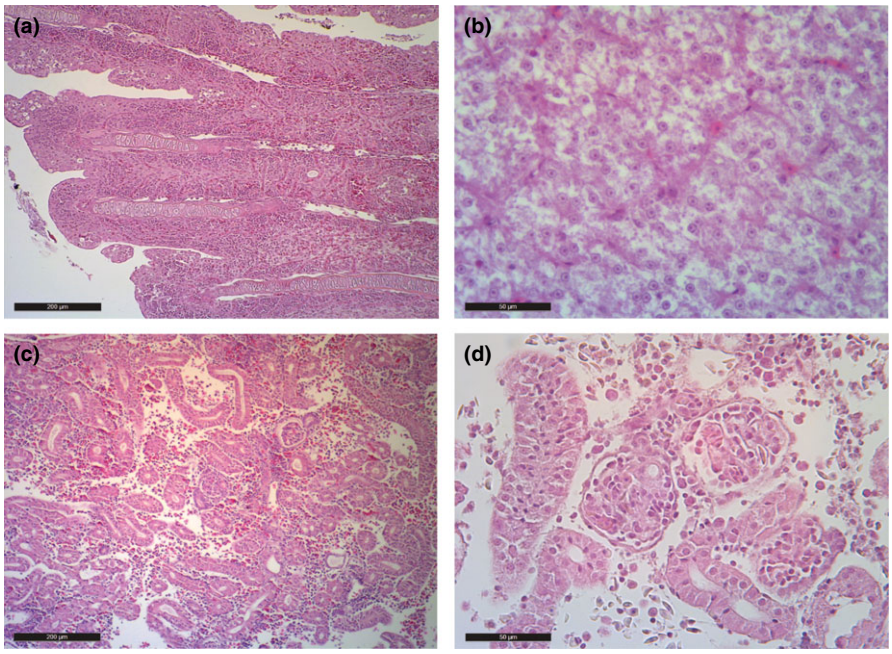

FIGURE 3 Histological lesions. (a) Severe fusions and haemorrhages of gills (10× magnification); (b) congestion and turbid degeneration of hepatocytes (40× magnification); (c) and (d) haemorrhages and reduction in the interstitial tissue of the kidney (10× and 40× magnifications)

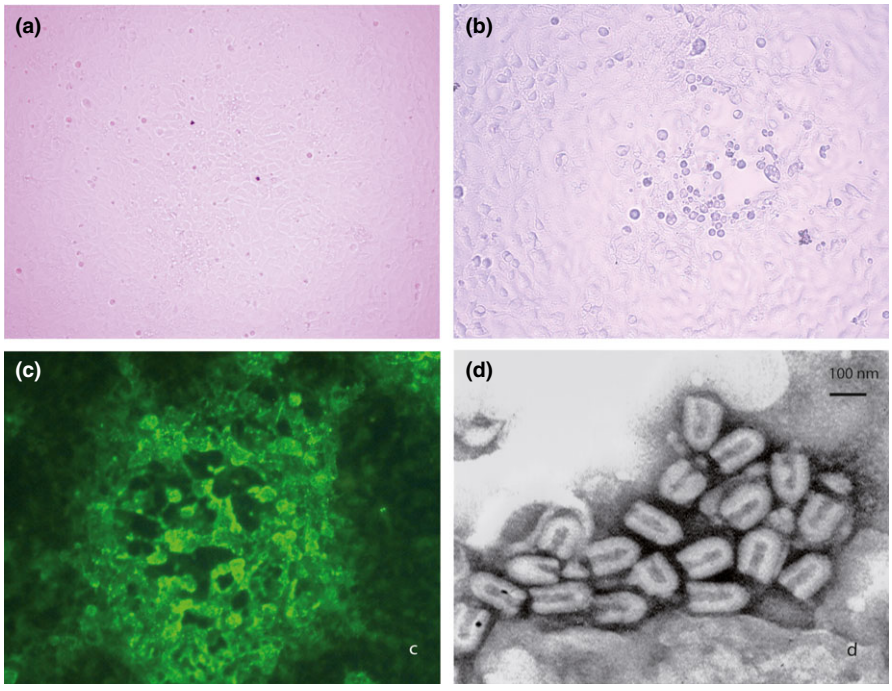

FIGURE 4 Cytopathic effect (CPE) of IcRV on EPC cells. (a) Non-infected EPC cells (20× magnification); (b) infected cells at 24 hr post-infection (20× magnification); (c) indirect immunofluorescence using SVCV antiserum on infected EPC cells at 12 hr post-infection (40× magnification); (d) transmission electron microscopy of cell culture supernatant showing typical bullet-shaped viral particles

with the WGS for strain IcRV/A.melas/I/75/2016. The overall identity values between IcRV and the members of the genus *Sprivivirus* are as follows: 88.5%–98.4% for the N protein, 69.98%–97% for the G protein and 65.7–96.7 for the G gene. The 15 IcRV herein characterized shared a high level of aa and nt identity with each other, that is 99.5%–100% for the nucleoprotein, 99%–100% for the glycoprotein and 98.6%–100% for the G gene.

4 | DISCUSSION

Viral infections are the cause of some of the most important diseases in aquaculture. As shown by the increasing number of outbreaks, a lack of preventive measures and therapies makes such infections the bottleneck for the development of the fish farming industry (Gomez-Casado, Estepa, & Coll, 2011; Wahli et al., 2015). On the other hand, while approaching an emerging viral disease, many problems are often encountered: the delay in developing diagnostic and prophylactic tools, poor knowledge on the host range and the geographic distribution of the pathogen, lack of information on disease epidemiology and insufficient knowledge on pathogens interplay (Walker & Winton, 2010). Therefore, the straight identification of the aetiological agent and its phenotypic and genetic characterization is of utmost importance for the correct management of the disease.

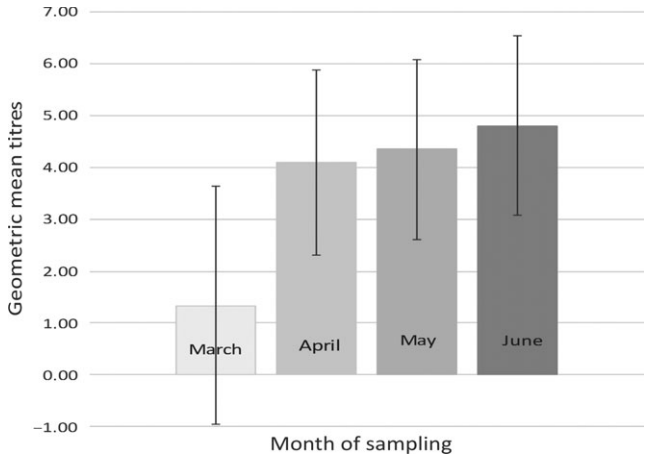

FIGURE 5 Histogram of the serological titre in surviving catfish over time, and the estimated GMT: 1.33; 4.10; 4.34; 4.80 (±*SD*) from March to June 2016

In this study, the isolation and the characterization of a new rhabdovirus detected during a severe mortality event in black bullhead catfish have been described. The virus was associated with marked neurological signs and heavy mortality. The suspicion of a rhabdovirus disease was initially based on clinical evidence and on results obtained by virus isolation and electron microscopy that displayed typical bullet-shaped viral particles. Interestingly, indirect immunofluorescence showed the presence of positive foci when using hyperimmune antiserum against SVSV while the partial N gene sequence obtained with a generic rhabdoviridae primer set was not consistent with this identification. This finding was confusing, but not surprising, as it is already known that polyclonal antibodies against SVCV cross-react at various degrees with other fish rhabdoviruses (OIE Manual of Diagnostic Tests for Aquatic Animals, Ch. 2.3.9, 2017). The partial N gene sequence obtained with a generic rhabdoviridae primer set (Talbi et al., 2011) was not consistent with SVCV. The identity of the newly isolated virus was finally resolved by means of WGS, which unequivocally showed how the black bullhead catfish virus clearly differs from SVCV, having the TenRV as its closest relative. Subsequent phylogenetic analyses based on the aa N and aa/nt G sequences were consistent with this finding and ultimately classified the catfish rhabdovirus within genus *Sprivivirus*.

Serological results showed that only the PTN assay can be considered a suitable method for detecting antibodies in catfish sera, while the SN test was not able to identify any antibodies response. This finding was confirmed by two antigens, the homologous IcRV and the heterologous SVCV. On the other hand, a certain level of cross-reactivity of catfish antibodies was observed by PTN assay further upholding the antigenic similarities amongst fish rhabdoviruses. The antibodies titres increasing over time, observed in survivors, suggest that a specific humoral response could be developed by catfish. The in vivo protection conferred by such a seroconversion must be experimentally proved but could represent a promising evidence for the development of an efficient vaccine.

The retrospective analysis of additional 14 uncharacterized isolates stored in our repository and detected in diseased catfish in the period 1993–2016 revealed that these viruses were actually members of the same genus and were arbitrarily named Ictalurid rhabdoviruses (IcRV). It is noteworthy that catfish and tench can share the same environment in feral conditions. Therefore, it cannot be ruled out that the genetic relatedness of IcRV and TenRV might be a consequence of the adaptation of the same virus to different fish species (Bandín & Dopazo, 2011).

Rhabdoviruses appear to be the primary aetiological agents of many important diseases of freshwater and marine species (Rowley et al., 2001). Some of them, such as IHNV and VHSV, have been the object of an important work and are listed in Europe (Directive 2006/88/EC), while few of those infecting fish of secondary interest were fully sequenced, such as the anguillid rhabdovirus (NC022581, FN557213), the spring viraemia carp virus (NC00283), the perch rhabdovirus (NC0208031), the siniperca chuatsi virus (NC008514), the pike fry rhabdovirus (NC02356, FJ872827), the tench rhabdovirus (NC025371) and the grass carp rhabdovirus (NC025376) (Bourhy, Cowley, Larrous, Holmes, & Walker, 2005; Walker, Dietzgen, Joubert, & Blasdell, 2011). As their immunological characterization can be confounding due to cross-reactivity reactions, the OIE recommends using RT-PCR and sequencing to confirm diagnosis. Moreover, fish rhabdoviruses generally show broad host range (Kurath & Winton, 2008). As a matter of fact, it is already known that black bullhead catfish and other *Ictaluridae* can also be infected by SVCV, the causative agent of an OIE notifiable disease (OIE - Manual of Diagnostic Tests for Aquatic Animals, Ch. 2.3.9, 2017). This is why it is of utmost importance to accurately differentiate among

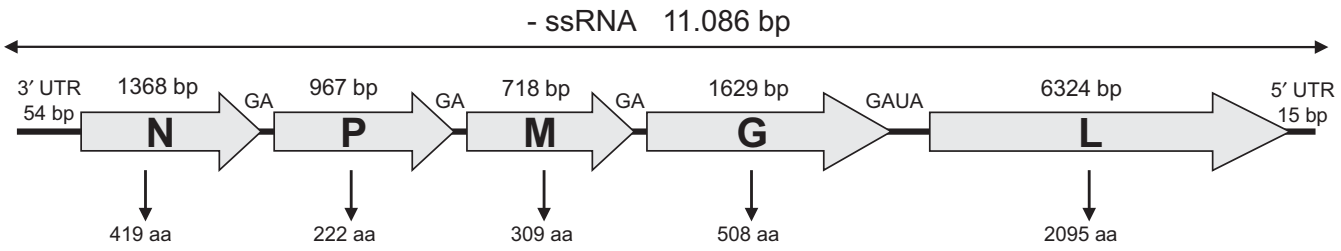

FIGURE 6 Structure of the IcRV genome. The five ORFs' coding for structural proteins (3′-N-P-M-G-L-5′) divided by short intergenic motives are represented

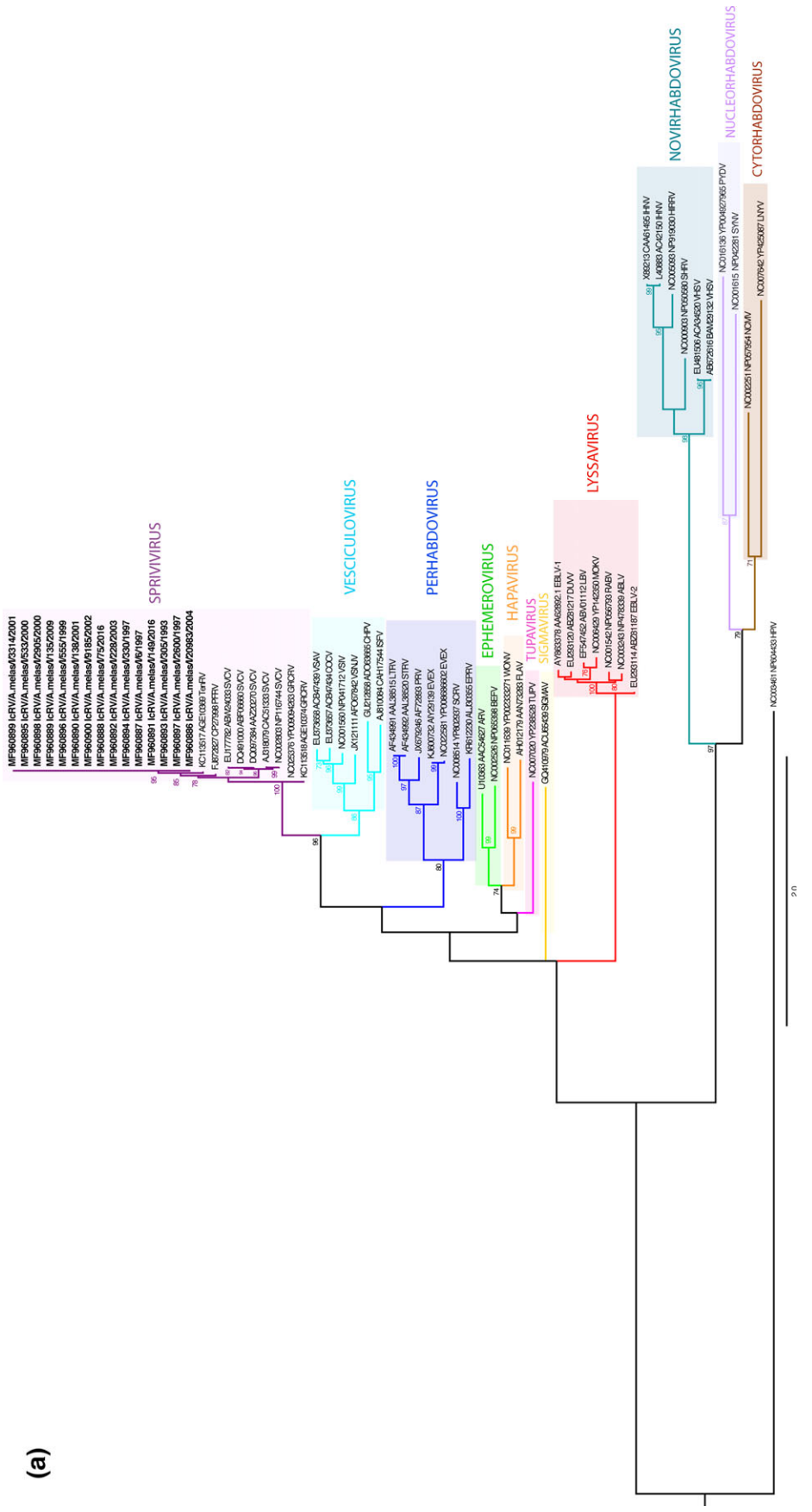

FIGURE 7 Continued

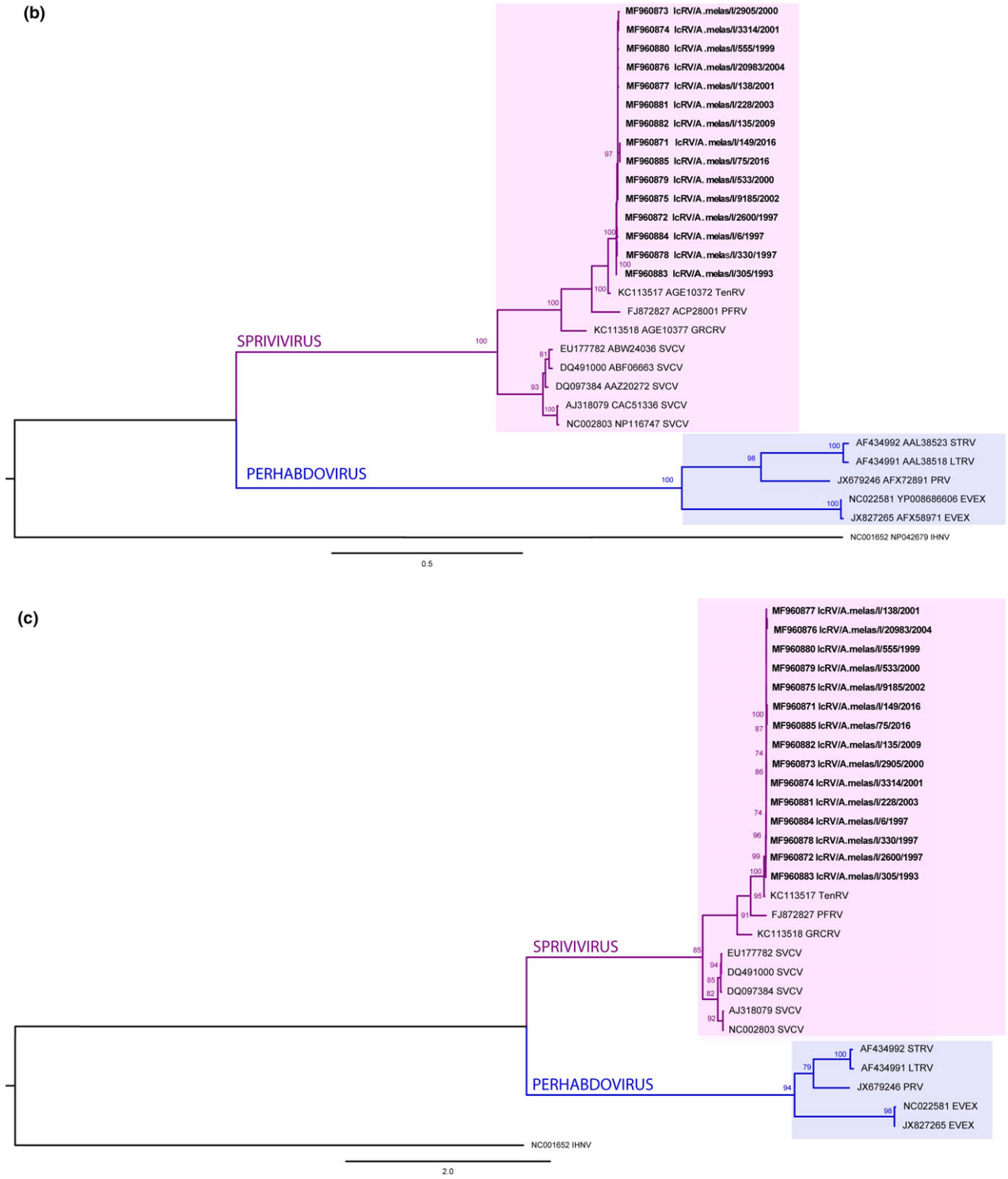

FIGURE 7 ML phylogenetic trees. (a) Partial deduced aa sequence of the N protein; (b) complete deduced aa sequence of the G protein; (c) complete G gene sequence. The Italian IcRV strains are highlighted in bold. Subdivision of *Rhabdoviridae* into genera is indicated. The numbers at nodes represent bootstrap values (only values >70% are reported). Branch lengths are scaled according to the number of aa/nt substitutions per site. The scale bar is reported. The tree is mid-point rooted for clarity only

rhabdoviruses and to implement their molecular diagnostics in order to make a correct diagnosis and undertake proper measures in case OIE notifiable diseases are detected. The development and validation of a specific molecular assay such as real-time RT-PCR could be an interesting future output of the present work. Such test could be used to implement surveillance of imported fish and to prevent the introduction of the disease in disease-free farms.

Italian catfish farming is severely threatened by recurrent outbreaks of viral diseases which cause severe economic losses to fish farmers. Taking into account the cumulative mortality

registered during the epidemics here described, and the fact that all the 14 historical strains were isolated during clinical outbreaks, the IcRV can certainly be considered as a serious threat for black bullhead catfish, in addition to the already known ECV and IcHV infections.

DATA ACCESS

MiSeq raw data have been submitted to the NCBI Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/Traces/sra/) under the accession number SRR6122530. The partial N gene nt and the full-length G gene nt sequences obtained for IcRV strains isolated in the period 1993–2016 are available under the GenBank accession numbers MF960871-MF960900.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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