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BRIEF REPORT

Characterisation of the genomes of four putative vesiculoviruses: tench rhabdovirus, grass carp rhabdovirus, perch rhabdovirus and eel rhabdovirus European X

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Abstract The complete coding sequences were determined for four putative vesiculoviruses isolated from fish. Sequence alignment and phylogenetic analysis based on the predicted amino acid sequences of the five main proteins assigned tench rhabdovirus and grass carp rhabdovirus together with spring viraemia of carp and pike fry rhabdovirus to a lineage that was distinct from the mammalian vesiculoviruses. Perch rhabdovirus, eel virus European X, lake trout rhabdovirus 903/87 and sea trout virus were placed in a second lineage that was also distinct from the recognised genera in the family *Rhabdoviridae*. Establishment of two new rhabdovirus genera, "Perhabdovirus" and "Sprivivirus", is discussed.

As the number of fish species being cultured worldwide increases, the number of new emerging virus diseases is also likely to increase. At the time of this writing, several rhabdovirus isolated from fish were tentatively assigned to the genus *Vesiculovirus* by the International Committee on Taxonomy of Viruses (ICTV) based on morphology and the limited sequence data available. Unfortunately, new virus isolates are often given a unique name based on the host fish species from which they were first isolated. This can lead to confusion amongst diagnosticians, particularly where the new viruses are closely related to members of existing virus species that are currently reportable under national and international fish health legislation and subject to control and eradication programs.

Spring viraemia of carp virus (SVCV) causes a serious haemorrhagic disease of carp that is reportable to the World Organisation for Animal Health (OIE). Naturally occurring SVCV infections have been recorded from a wide range of fish species: common carp (*Cyprinus carpio carpio*), koi carp (*Cyprinus carpio koi*), crucian carp (*Carassius carassius*), sheatfish (also known as European catfish or wels; *Silurus glanis*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (white amur; *Ctenopharyngodon idella*), goldfish (*Carassius auratus*), orfe (*Leuciscus idus*), and tench (*Tinca tinca*). Other species such as northern pike (*Esox lucius*) and guppy (*Lebistes reticulatus*) and pumpkinseed (*Lepomis gibbosus*) can be infected experimentally. Pike fry rhabdovirus (PFRV) is the causative agent of an acute haemorrhagic disease in Europe, but PFRV has minimal impact worldwide and is not reportable to the OIE. Although SVCV and PFRV cross-react in some serological assays, they can be distinguished based on the lack of cross-neutralisation with polyclonal antisera and are considered members of distinct virus species [1]. Phylogenetic analysis using the full-length amino acid sequences of the N, P M, G and L genes has confirmed a close genetic relationship between SVCV and other established members of the genus *Vesiculovirus* [2]. An analysis using a partial G-gene sequence has separated SVCV in to four subtypes (a-d), which to a certain extent also correspond to their geographical origins [3]. Subgroup a consists of viruses that originate from Asia; subgroup b, of viruses from Maldova and the Ukraine; subgroup c, of viruses from the

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Ukraine and Russia; and subgroup d, of viruses that are more commonly isolated in Europe.

Several other rhabdoviruses that were identified as SVCV- and PFRV-like using serological methods were divided into distinct lineages by phylogenetic analysis based on partial G gene sequences. These lineages were given the names grass carp rhabdovirus (GrCRV) and tench rhabdovirus (TenRV) based on the fish species from which the viruses were first isolated [3–5]. Both are listed as viruses that may be members of the genus *Vesiculovirus*, but the tentative species to which they have been assigned still need to be approved.

Lake trout rhabdovirus (LTRV isolate 903/87), Swedish sea trout virus (SSTV) and the eel virus European X (EVEX) isolated from eel (*Anguilla anguilla*) have been reported to be closely related to the vesiculoviruses based on morphology and sequence comparisons [6–8]. Perch rhabdovirus (PRV) is also reported to be related to the vesiculoviruses genus based on morphology, protein profiles and partial L gene sequences [9]. PRV has been shown to be genetically distinct from SVCV and PFRV based on partial L-gene sequences, but its relationship to SSTV, LTRV and EVEX is less well defined.

To assist in the classification of the fish vesiculo-type viruses, the sequences of the complete coding regions of four putative vesiculoviruses, TenRV isolate S64 [10], GrCRV isolate V76 [11], the original EVEX [8], and the original PRV [12], were determined by 454 *de novo* sequencing. We report on the genetic relationship of the four viruses genomes to each other and to members of other recognized rhabdoviruses species.

EVEX, PRV, TenRV S64 and GrCRV V76 were grown on either bluegill fry caudal trunk (BF-2) cells or epithelial papilloma of carp (EPC) cells. Virus was precipitated from the supernatant from two 75-cm^2 flasks using the polyethylene glycol method [13], and the virus pellet was resuspended in 2.0 ml of GMEM. The viral RNA (vRNA) was extracted from 100 µl of purified virus using TRIzol (Invitrogen) following manufacturer's instructions and dissolved in 40 µl of RNase-free water. To check the quality of the RNA for each virus sample an RT-PCR was performed using a generic primer set. Briefly, reverse transcription was performed at 37 °C for 1 h in a 20-µl volume consisting of 1x Moloney murine leukaemia virus reverse transcriptase (M-MLV-RT) reaction buffer, 1.25 mM dNTP mix, 100 µM primer Ves Gen 1 (5'-GGRMGDTTYTTYTCHYTRA TGTC-3'), 200 U M-MLV-RT, 40 U RNasin (Promega), and 4 µl of vRNA. PCR was performed in a 50-µl reaction volume consisting of 1x Go Taq Flexi PCR buffer, 5 mM $MgCl_2$, 0.25 mM dNTPs mix, 50 µM primers Ves Gen1 and Ves Gen 2 (5'- ATCAGRTCNGGYCTNCCRTTRTARTA-3'), 1.25 U of Go Taq polymerase (Promega) and 2.5 µl of the reverse transcriptase reaction. The 50-µl reaction was overlaid with mineral oil and subjected to 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for one min, followed by an extension of 72 °C for 5 min. The resultant amplicons were separated on a 2 % agarose gel stained with ethidium bromide. Having confirmed the suitability of the RNA for RT-PCR, a first-strand cDNA synthesis was performed in a 20-µl reaction. A 13-µl mix consisting of 40-µM primer A (GTT TCC CAG TCA CGA TCN NNN NNN NNN NNN NN), 0.8 mM dNTPs and 11 µl vRNA was heated to 65 °C for 5 min followed by 1 min on ice. Seven microlitres of Superscript III (Invitrogen) mix containing 4 µl of 5x RT buffer, 7 mM DTT, 40 U RNase inhibitor and 200 U Superscript III Reverse Transcriptase was then added, and the mix was heated to 25 °C for 5 min, followed by 1 h at 50 °C, and finally, 15 min at 70 °C to stop the reaction. A second strand was synthesised using Sequenase (GE Healthcare). The first-strand reaction mix was heated to 94 °C for 2 min and then held at 10 °C for 5 min. The reaction volume was adjusted to 30 µl by the addition 10 µl of a Sequenase mix consisting of 1x Sequenase buffer and 4 U Sequenase. The temperature of the reaction mix was increased from 10 °C to 37 °C over 8 min and then held at 37 °C for a further 8 min. The mix was then heated to 94 °C for 2 min and cooled to 10 °C. A further 1.2 µl of Sequenase was added, and the mix was heated again from 10 °C to 37 °C over 8 min and held at 37 °C for a further 8 min. Finally, the mix was incubated at 94 °C for 8 min. A final amplification step was performed using conventional PCR using a Tag primer (5'- GTT TCC CAG TCA CGA TC-3'). The amplified products were purified using a QIAquick PCR Kit (QIAGEN), and then blunt ended using T4 DNA polymerase (Promega) following the manufacturer's instructions. Whole-genome shotgun pyrosequencing was performed by generating a standard DNA fragment library (Roche Applied Sciences, Indianapolis, IN) and sequencing with a GS-FLX System using Titanium chemistry (454 Life Sciences, Roche Applied Sciences). The resulting sequence reads were assembled with Newbler (v2.3 Roche Applied Sciences), and the results were compared to a viral protein database downloaded from NCBI using BlastX. Gaps in the sequence data were closed using a conventional PCR and sequencing approach. The 3' leader and 5' trailer regions of the genome were obtained using sequence-specific primers and a 5'/3' RACE kit (Roche) using the manufacturers recommended protocols. The amplification products were sequenced using the ABI PRISM Big Dye Terminator v3.1 cycle sequencing kit in an ABI 3130xl Avant Genetic analyser. Sequences were analysed using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Analysis of the full genome sequence, open reading frames (ORFs), and orthologous protein families was conducted by using Artemis [14] and Glimmer software [15].

The EVEX sequence was 11,778 nucleotides in length (JX827265) and coded for five genes, separated by short intergenic regions of 2-6 nucleotides in length and flanked by a 3' leader of 52 nucleotides and a 5' trailer sequence of 40 nucleotides (Fig. 1a). The five open reading frames were predicted to encode the nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, glycoprotein (G) and RNA-dependent RNA polymerase (L) protein in the order 3' N-P-M-G-L-5', consistent with other vesiculoviruses. The PRV sequence was 11,487 nucleotides in length (JX679246), the intergenic regions ranged from 4 to 6 nucleotides in length, and the 3' leader and 5' trailer sequences were 100 and 65 nucleotides in length, respectively. The TenRV (KC113517) and GrCRV (KC113518) sequences were 11,082 and 11,096 nucleotides in length, respectively, the intergenic regions ranged from 2-4 nucleotides in length for both genomes, and the 3' leader and 5' trailer sequences were 54 and 15 nucleotides in length for TenRV and 53 and 19 nucleotides for GrCRV. The encoded genes and gene orders were the same as those observed for EVEX.

(a)

	N-P	P-M	M-G	G-L
EVEX	GAAA	ACAA	GA	GAUAAG
PRV	GAAA	AGACAA	GAAA	GCAA
SCRV	GAAA	GAAA	GAA	GAAA
LTRV	GAAA	GGUCAA	GACG	GUCGA
SVCV	GA	GA	GA	GAUA
PFRV	GA	GA	GA	GAUA
GrCRV	GA	GA	GA	GAUA
TenRV	GA	GA	GA	GAUA
VSIV	GA	CA	GA	G
VHSV	G/A	G/A	G/A	G/A

(b)

SVCV and PFRV (NC002803 and AJ318079)
3′ TGCTTCTGTTTATTTGGTAA
   ||||||||
5′ ACGAAGACTACAAA

GrCRV
3′ ------TGTTTATTTGGTAA
        ||
5′ ACGAAGACTACAAA

TenRV
3′ -----TGTTTATTTGGTAA
        ||
5′ ---AGACTACAAA

EVEX(FN557213)
3′ TGCTCTTTTTGTTTTGGGTAATTTGCTGAA
   ||||||||||||||||||
5′ ACGAGAAAAACAAAACCATGCCCCC

EVEX
3′ ----------TTTTGGGTAATTTGCTGAA

5′ -----------------ATGCCCCC

PRV
3′ --CTCTTTTTGGTTTGGTTCGAATT
     ||||||||||||||||
5′ --GAGAAAAACCAAACC

Fig. 1 Schematic diagram showing (**a**) the variation in the intergenic regions of the fish vesiculo-type viruses compared to vesicular stomatitis Indiana virus (VSIV), viral haemorrhagic septicaemia virus (VHSV) and other fish rhabdoviruses (vRNA sense) and (**b**) the inverse complementarity of the 3' leader and 5' trailer sequences in the SVCV, EVEX PRV, GrCRV and TenRV genomes. "–" indicates the nucleotides believed to be missing from the complete genome sequence determined in this study. The sequences are presented as vRNA sense

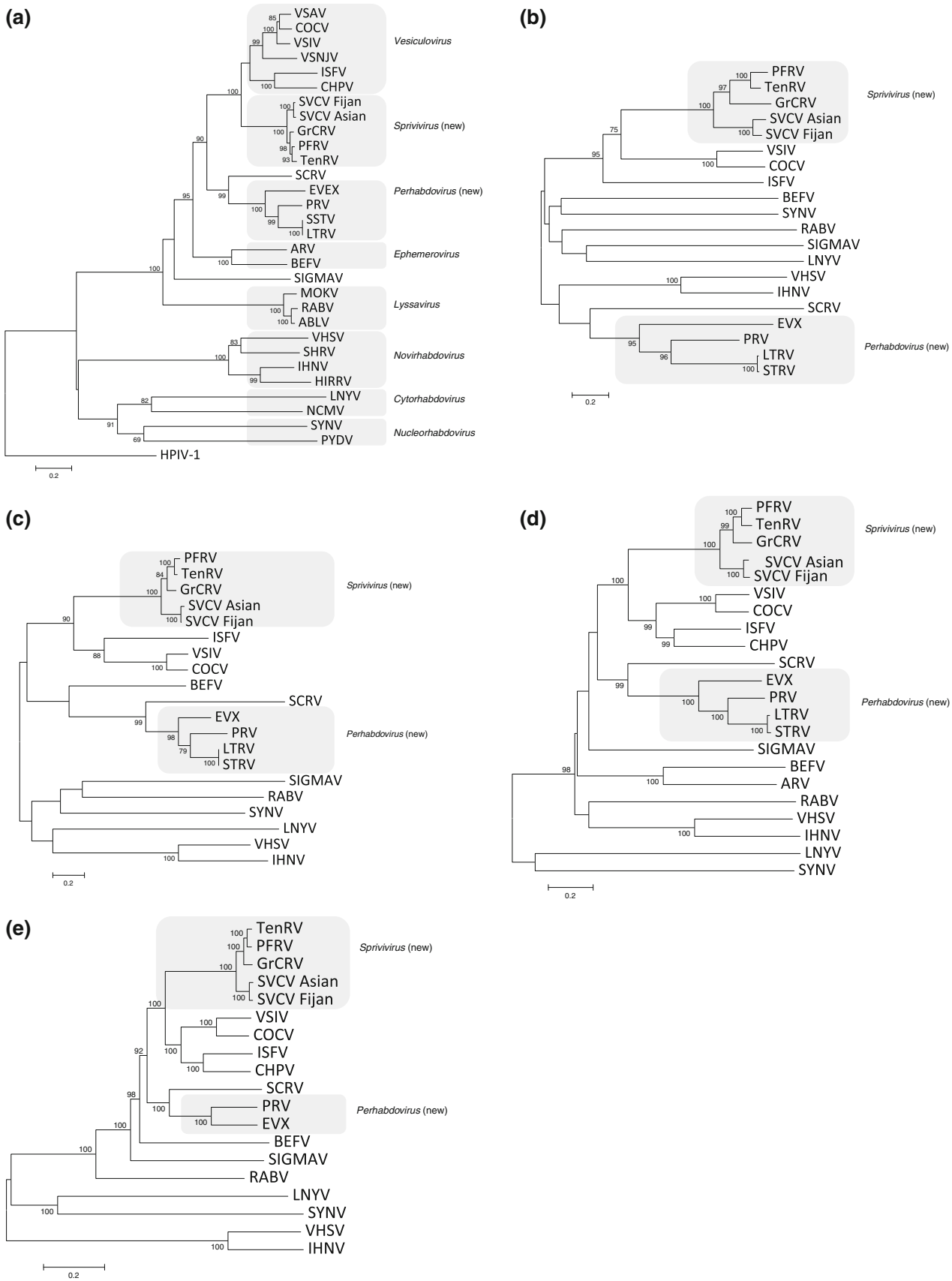
The transcriptional start and termination/polyadenylation signals for all genes of all four viruses were 3'-UUGUC and 3'-AU(A/G)C$(U)_{6-7}$, which is consistent with other viruses in the genus *Vesiculovirus* [16] However, the intergenic sequences differ significantly from those found in vesiculovirus and novirhabdovirus genomes. The intergenic sequence in the GrCRV and TenRV genomes is a conserved dinucleotide, 3'-GA, with the exception of the G-L intergenic region, which is 3'-GAUA. This is identical to the intergenic regions found in the SVCV and PFRV genomes [17]. This 3' GA is also found between both the N and P genes and the M and G gene of vesicular stomatitis Indiana virus (VSIV), the type member of the genus *Vesiculovirus*. However, VSIV has a 3'-CA between the P and M genes and a single 3'-G between the G and L genes. Viral haemorrhagic septicaemia virus (VHSV) and infectious haemotopeietic necrosis virus (IHNV) have a single 3'G/A in all intergenic regions. The intergenic region between the N and P genes of PRV and EVEX was 3'-GAAA. This is also conserved in the SSTV and LTRV genomes, but the remaining intergenic regions were highly variable in both length and in sequence (Fig. 1a).

During the course of this study, the complete genome sequence for EVEX 153311 (FN557213) was published by Galinier et al. [18]. This complete genome sequence was 11,806 nucleotides in length with a 3' leader of 63 nucleotides and a 5' trailer of 58 nucleotides, indicating that the sequence we present here lacks the complete 3' and 5' termini. This would explain why inverse complementary was not observed between the 3'leader and 5' trailer sequences. Nonetheless, based on the sequence data available, the two virus isolates share 100 % and 97.5 % nucleotide sequence identity in the 3' leader and 5' trailer, respectively, and they share 99.0 % nucleotide sequence identity over the genome as a whole. Inverse complementarity was observed between the 3' leader and 5' trailer sequences of the PRV genome; however, alignment with the published sequence for EVEX (FN557213) suggests that two nucleotides may be missing from both the 3' leader and 5' trailer sequences (Fig.1b). Inverse complementarity was not observed between the ends of the 3' leader and 5' trailer sequences of the TenRV or the GrCRV genome, indicating that the 3' and 5' ends were also incomplete (Fig. 1b). In this case, alignment with the

complete genome sequence of the SVCV (NC002803 and AJ318079) and PFRV (FJ872827) genomes suggests 6 nucleotides were missing from the 3' leader of the GrCRV sequence and that 5 and 3 nucleotides were missing from the 3' leader and 5' trailer, respectively, of the TenRV sequence. Further work will be required to provide the complete the 5' and 3' sequences.

The replication temperature range of the fish viruses is lower than that of the mammalian rhabdoviruses, which reflects the aquatic poikilothermic nature of the natural hosts. In addition, the fish viruses are typically isolated on cultured fish cell lines at 15-25 °C. This ecological separation of the fish viruses rhabdoviruses from those isolated from mammals is also supported by the level of sequence divergence. Multiple sequence alignments and phylogenetic analysis were performed using predicted amino acid sequences for the five predicted ORFs. When analysing the N protein sequences, the N protein sequence of human parainfluenza virus 1 (HPIV-1) was used as an outgroup. Multiple alignments were performed using Clustal W [19] with a gap-opening penalty of 15 and gap-extension penalty of 6.66. Phylogenetic analyses were conducted using MEGA version 4 [20, 21]. A neighbour-joining tree was constructed using a maximum composite likelihood model, and the robustness of the tree was tested using 1000 bootstrap replicates. Analysis based on the predicted amino acid sequences of the N, P, M G and L proteins consistently placed SVCV (NC002803 and AJ318079), PFRV (FJ872827), TenRV (this study, KC113517) and GrCRV (this study, KC113518) in a lineage that is distinct from those containing the viruses of the genus *Vesiculovirus* and the other recognised genus containing fish viruses, *Novirhabdovirus*. This separation is supported by bootstrap analysis values of 100 %. GrCRV and TenRV shared 94.3 % amino acid sequence identity with each other based on the complete N potein sequence, 95.0 % and 96.2 % amino acid sequence, respectively, identity with PFRV, and 90.5 % and 89.7 % with SVCV. This compares to 54.0 % and 52.0 % amino acid sequence identity with the N protein sequence of VSIV. Similar identities were observed for the M, G and L protein, but much lower levels of identity in the P protein. Phylogenetic analysis also consistently placed PRV (this study, JX679246) and EVEX (this study, JX827265) in a separate monophyletic lineage together with sea trout virus (AF434992) and trout rhabdovirus 903/87 (AF434991), which was supported by bootstrap values of >95 %. Again, this lineage is distinct from the lineages corresponding to the genera currently containing fish viruses (*Novirhabdovirus*) and viruses of terrestrial animals (*Vesiculovirus*) (Fig. 2). PRV shares 72.8 % and 60.3 % amino acid sequence identity with LTRV and EVEX respectively based on the complete N protein sequence. EVEX and LTRV share 59.3 % amino acid sequence identity and LTRV and SSTV share 100 % amino acid sequence identity. This compares with only 30.2 % amino acid sequence identity between PRV and VSIV in the same region of the genome. On the basis of these findings, two proposals (ref: *2012.001adV.A.v2. Perhabdovirus* and *2012.002a-fV.U.v2.Sprivivirus*) have been submitted to the ICTV to create two new genera, "Perhabdovirus" and "Sprivivirus". In addition, the proposals seek to establish four new species. "Pike fry rhabdovirus" is proposed as a new species whose members are related to, but distinct from, SVCV, and it has been recommended that both viruses be assigned to the new genus "Sprivivirus". Due to the close genetic relationship between GrCRV, TenRV and PFRV, it was felt that it is more appropriate for TenRV and GrCRV to be considered members of the same new species, "Pike fry rhabdovirus", rather than separate species. The creation of three further species, "Perch rhabdovirus", "Anguillid rhabdovirus" and "Sea trout rhabdovirus" within the new genus "Perhabdovirus" was also proposed. The SSTV (AF434992) and LTRV (AF434992) share a high degree of sequence homology throughout the genome and were therefore considered isolates of the same species, "Sea trout rhabdovirus". EVEX was considered a member of the proposed species "Anguillid rhabdovirus". This classification supports the proposition of recent studies on perch

Fig. 2 Phylogenetic relationships between rhabdoviruses based on (**a**) the complete N protein using human parainfluenza virus 1 (HPIV-1; NC_003461) as an outgroup. The rhabdoviruses used in the analysis were vesicular stomatitis Alagoas virus (VSAV; EU373658), cocal virus (COCV; EU373657), vesicular stomatitis Indiana virus (VSIV; NC001560), vesicular stomatitis New Jersey virus (VSNJV; JX121111), Isfahan virus (ISFV; AJ810084), Chandipura virus (CHPV; GU212858), grass carp rhabdovirus (GrCRV; KC113518), tench rhabdovirus (TenRV; KC113517), pike fry rhabdovirus (PFRV; FJ872827), European and Asian spring viraemia of carp virus (SVCV Fijan AJ318079 and SVCV Asian DQ097384), Siniperca chuatsi rhabdovirus (SCRV; NC_008514), eel rhabdovirus Eurpoean X (EVEX; JX827265), perch rhabdovirus (PRV; JX679246), Swedish sea trout virus (STRV; AF434992), European lake trout rhabdovirus (LTRV; AF434991), Adelaide river virus (ARV; U10363), bovine ephemeral virus (BEFV; NC_002526), sigma virus (SIGMAV; GQ410979), Mokola virus (MOKV; NV006429), rabies virus (RABV;NC001542), Australian bat lyssavirus (ABLV; NC_003243), potato yellow dwarf virus (PYDV; NC_016136), Sonchus yellow net virus (SYNV; NC_001615), lettuce necrotic virus (LNYV; NC_007642), northern cereal mosaic virus (NCMV; NC_002251), viral haemorrhagic septicaemia virus (VHSV; NC_000855), snakehead rhabdovirus (SHRV; NC_000903), infectious haematopoietic necrosis virus (IHNV; NC_001652), and hirame rhabdovirus (HIRRV; NC_005093). Trees showing the relationship between rhabdoviruses based on the complete (**b**) P protein (**c**) M protein (**d**) G protein and (**e**) L protein are presented. The trees were generated using neighbor-joining distance methods as described in Materials and methods, and bootstrap values >70 % are shown at the branch points. The existing and newly proposed genera are indicated by the shaded areas


(a)
VSAV
COCV
VSIV
VSNJV
ISFV
CHPV
Vesiculovirus
SVCV Fijan
SVCV Asian
GrCRV
PFRV
TenRV
Sprivivirus (new)
SCRV
EVEX
PRV
SSTV
LTRV
Perhabdovirus (new)
ARV
BEFV
Ephemerovirus
SIGMAV
MOKV
RABV
ABLV
Lyssavirus
VHSV
SHRV
IHNV
HIRRV
Novirhabdovirus
LNYV
NCMV
Cytorhabdovirus
SYNV
PYDV
Nucleorhabdovirus
HPIV-1
0.2
(b)
PFRV
TenRV
GrCRV
SVCV Asian
SVCV Fijan
Sprivivirus (new)
VSIV
COCV
ISFV
BEFV
SYNV
RABV
SIGMAV
LNYV
VHSV
IHNV
SCRV
EVX
PRV
LTRV
STRV
Perhabdovirus (new)
0.2
(c)
PFRV
TenRV
GrCRV
SVCV Asian
SVCV Fijan
Sprivivirus (new)
ISFV
VSIV
COCV
BEFV
SCRV
EVX
PRV
LTRV
STRV
Perhabdovirus (new)
SIGMAV
RABV
SYNV
LNYV
VHSV
IHNV
0.2
(d)
PFRV
TenRV
GrCRV
SVCV Asian
SVCV Fijan
Sprivivirus (new)
VSIV
COCV
ISFV
CHPV
SCRV
EVX
PRV
LTRV
STRV
Perhabdovirus (new)
SIGMAV
BEFV
ARV
RABV
VHSV
IHNV
LNYV
SYNV
0.2
(e)
TenRV
PFRV
GrCRV
SVCV Asian
SVCV Fijan
Sprivivirus (new)
VSIV
COCV
ISFV
CHPV
SCRV
PRV
EVX
Perhabdovirus (new)
BEFV
SIGMAV
RABV
LNYV
SYNV
VHSV
IHNV
0.2

rhabdoviruses and eel rhabdoviruses [18, 22] that PRV LTRV, SSTV and EVEX belong to a clade that is close to, but distinct from, the vesiculoviruses and that SVCV and PFRV belong to a second clade that positioned on the interface of the first clade and that of the mammalian vesiculoviruses. These recent studies [23] also suggest that Siniperca chuatsi rhabdovirus (SCRV) should be assigned to the same clade as the SSTV, LTRV 903/87 and EVEX. However, there is insufficient amino acid sequence identity throughout the SCRV genome, and in particular the P, M and G proteins, when compared to EVEX (<28.4 %) to justify inclusion of SCRV in the same genus.

The taxonomic changes proposed here have not been endorsed by the ICTV Executive Committee, and they may differ from any new taxonomy that is ultimately approved by the ICTV. Creating two new genera for the fish viruses raises some issues regarding the uniformity of the phylogenetic distance ruler used to demarcate different genera in the family *Rhabdoviridae*. For example, based on the complete N protein sequence, the genetic distance between SVCV and vesicular stomatitis Indiana virus (VSIV), the type member of the genus *Vesiculovirus*, is similar to the distance between members of two different species (*Adelaide river virus* and *Bovine ephemeral virus*) currently assigned to the genus *Ephemerovirus.* However, genetic distance is only one characteristic that can be used when attempting to demarcate genera, and other characteristics such as the natural host range, tissue tropism, and replication temperature can be important and often useful features when trying to resolve such issues. In the case of SVCV, the proposed type member of the proposed genus "Sprivivirus", the replication temperature range is lower than that of the mammalian rhabdoviruses, which reflects the aquatic poikilothermic nature of its natural hosts. Also, SVCV, and the other fish viruses are typically isolated on cultured fish cell lines at 15-25 °C compared to a permissive temperature range for VSIV of 30-39 °C. Although in the case of the proposed genera "Sprivivirus" and "Perhabdovirus" the use of both genetic and phenotypic information to define the taxonomic position can be justified, the uniformity of approach when redefining other taxonomic structures within the family *Rhabdoviridae* will need to be reviewed.

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