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# The Genome Sequence of the Emerging Common Midwife Toad Virus Identifies an Evolutionary Intermediate within Ranaviruses

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**Worldwide amphibian population declines have been ascribed to global warming, increasing pollution levels, and other factors directly related to human activities. These factors may additionally be favoring the emergence of novel pathogens. In this report, we have determined the complete genome sequence of the emerging common midwife toad ranavirus (CMTV), which has caused fatal disease in several amphibian species across Europe. Phylogenetic and gene content analyses of the first complete genomic sequence from a ranavirus isolated in Europe show that CMTV is an amphibian-like ranavirus (ALRV). However, the CMTV genome structure is novel and represents an intermediate evolutionary stage between the two previously described ALRV groups. We find that CMTV clusters with several other ranaviruses isolated from different hosts and locations which might also be included in this novel ranavirus group. This work sheds light on the phylogenetic relationships within this complex group of emerging, disease-causing viruses.**

Global population declines and extinction of multiple amphibian species have been reported over the last 20 years (11). As estimated by the International Union for Conservation of Nature, the *Amphibia* class includes the highest number of critically endangered species among the animal classes examined, with an estimated 41% of its species under threat (23). Therefore, this class seems to be a particularly sensitive indicator of the current biodiversity losses associated with the global warming process and other human-related environmental factors. It has been shown that habitat loss, increased human population density, and increased climatic variability are important factors that compromise the survival of amphibian species (34) and that multiple drivers of extinction are likely to coordinately accelerate amphibian declines in the near future (20).

The role of emerging infectious diseases in the rapid decline of amphibian populations is being increasingly studied. The recent spread of the lethal fungal pathogen *Batrachochytrium dendrobatidis* is well documented and has been associated with long-term amphibian population declines in several locations (6). A second widespread pathogen of amphibians that is now recognized as an emerging infectious disease and therefore likewise included in the list of notifiable diseases by the World Organization for Animal Health is ranaviruses. Although their association with population declines has to be studied further (14), ranavirus infections have been clearly associated with mass mortalities of several amphibian as well as reptile and fish species worldwide (3, 36, 40).

*Ranavirus* is a genus within the *Iridoviridae* family which includes large, icosahedral viruses containing circular, double-stranded DNA genomes with sizes ranging from 105 kbp to 140 kbp and a coding potential of approximately 100 open reading frames (ORFs) (10). The family includes five different genera, two of which infect insects while the others infect cold-blooded vertebrates. Species from the genera *Megalocytivirus* and *Lymphocystivirus* have been found to infect only teleost fish, while ranaviruses are known to infect reptiles, amphibians, and fish, and the reasons for this broad host specificity are yet unknown.

Ranavirus outbreaks have been described from different locations worldwide. However, full-length genome sequences have

been published only for Asian, American, and Australian isolates (18, 21, 24, 28, 35, 37, 41). Recently, it has been proposed that ranaviruses can be subdivided into two distinct groups based on phylogenetic analyses and genome colinearity, grouper iridovirus (GIV)-like ranaviruses and amphibian-like ranaviruses (ALRV) (24). The first group includes GIV and Singapore grouper iridovirus (SGIV), which were isolated in Asia from fish (35, 41). The ALRVs include frog virus 3 (FV3) and *Ambystoma tigrinum* virus (ATV), which were isolated from frogs and salamanders, respectively, in North America, the tiger frog virus (TFV), isolated in China, the epizootic hematopoietic necrosis virus (EHNV), isolated from fish in Australia, and the soft-shelled turtle iridovirus (STIV), isolated in China (21). Within the ALRVs, the degree of genome sequence colinearity is high, although two different groups of viruses can be distinguished: the EHNV/ATV group, which may be closer to a putative most recent common ancestor of the whole group (24), and the FV3/TFV/STIV group, which shows two discrete acquired genomic inversions when compared to the genomes of the former group.

Ranavirus emergence as a pathogen of amphibians and other ectothermic vertebrates is probably linked to their host range plasticity as well as to environmental and ecological factors (17). The first clear evidence that viral infection can be the cause of localized amphibian population declines was reported in Europe, where ranaviruses have caused large-scale mortalities of the common frog *Rana temporaria* in the United Kingdom since 1985 (40). The causative agent is thought to be a variant of FV3, which may have

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been introduced through the movement of infected animals from North America (22).

The common midwife toad virus (CMTV) was first isolated on the European continental mainland in 2007 from diseased tadpoles of the common midwife toad (*Alytes obstetricans*) in a high-altitude permanent water trough in the Picos de Europa National Park in Spain (4). The virus, causing a high mortality rate in this species as well as in juvenile alpine newts in the 2008 outbreak (*Mesotriton alpestris cyreni*) (5), was shown to be responsible for a systemic hemorrhagic disease. Common histological findings were the presence of intracytoplasmic inclusion bodies and the necrosis of endothelial cells, the latter of which results in destruction of several organs, including skin, liver, and kidney. Sequence analyses of DNA fragments belonging to the major capsid protein (MCP) and DNA polymerase genes showed that CMTV clustered more closely with the ALRVs than with the GIV-like ranaviruses within the *Ranavirus* genus. In 2010, a CMTV outbreak in a pond in the Netherlands was described as the cause of a mass mortality event affecting water frogs and common newts (29), showing that both the host range and geographic distribution of CMTV are much wider than previously suspected.

The importance of ranavirus infections in amphibian population declines as well as the lack of knowledge about the nature of circulating European ranaviruses prompted us to determine the complete genome sequence of the emerging CMTV.

## MATERIALS AND METHODS

**Virus and cells.** CMTV isolated from alpine newts (*Mesotriton alpestris cyreni*) without plaque purification (4) was amplified in a single step on zebrafish ZF4 cells (ATCC CRL-2050) at 28°C in Dutch-modified RPMI medium containing 2% fetal calf serum. Supernatants from CMTV-infected ZF4 cells were collected, and viral particles were obtained by ultracentrifugation at  $20,000 \times g$  through a 36% sucrose cushion for 1 h at 4°C.

**Isolation of viral DNA.** The virus particles were treated with DNase I and S7 micrococcal nuclease to digest free DNA. After proteinase K treatment, viral DNA was extracted with phenol-chloroform and precipitated with sodium acetate and ethanol in the presence of 10  $\mu$ g of glycogen (Roche) as the carrier. Extracted DNA was randomly amplified using Phi29 DNA polymerase (GenomiPhi V2; GE Healthcare).

**Sequencing and assembly of the viral genome.** Amplified viral DNA was pyrosequenced on a 454 GS FLX instrument housed in the Parque Científico de Madrid. The output consists of 40,271 sequences with an average size of 375 bp. Reads were assembled in two steps with Newbler 2.5.3 (Roche-454 Life Science). First, a *de novo* assembly under stringent parameters (97% minimum overlap identity in at least 250 bp) generated 2 contigs flanked by repeated sequences. Then a unique contig was obtained by aligning the reads to the *de novo* genome under more relaxed parameters (98% identity in at least 50 bp). PCR amplification and Sanger sequencing were carried out in order to define homopolymer sequencing ambiguities at positions 73,736 and 3,392 and a polymorphism at position 11,812 as well as the precise number of TGTGAAGCGTAAGTCCCC repeats at position 66,494. The number of repetitions of the microsatellite region detected at position 38,092 was determined by running a specific PCR product in a 4% agarose gel (see Fig. 2). Sequences of primers are available upon request.

**Genome analysis and annotation.** Open reading frames (ORFs) were numbered consecutively from the same arbitrary start point as in ATV and EHNV (24, 28), and transcriptional sense was indicated by R (right) or L (left). The genome was annotated with the genome annotation transfer utility (GATU) software (39) using the genome of STIV as a template and further refined manually by using the similarity search algorithm BLASTP (<http://blast.ncbi.nlm.nih.gov/>) on all unassigned ORFs longer than 120 bp. Overlapping ORFs were annotated only if they had been

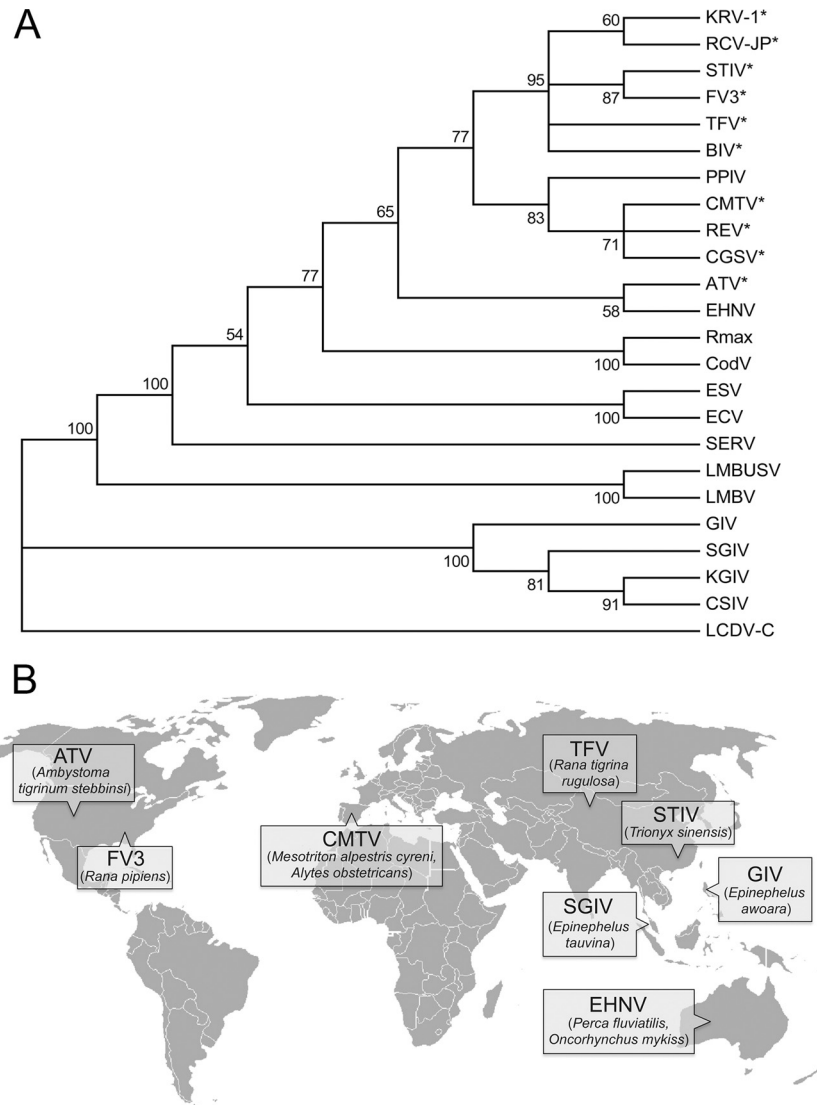
previously annotated in other ranaviral genomes, preserving the same number for both R and L orientations (CMTV ORFs 35 and 54). Nucleotide-to-nucleotide comparisons between the CMTV genome and other iridoviral genomes as well as among different ranaviruses were calculated using the JDotter (Java Dot Plot Alignments) software with the default settings. The algorithm employed by this software is described in detail in reference 9. In the dot plots generated, a straight line represents a stretch of similarity between the two sequences compared on the *x* and *y* axes. The full-length genome sequences (accession numbers) used in the dot plot analyses were FV3 (AY548484), TFV (AF389451), ATV (AY150217), EHNV (FJ433873), GIV (AY666015), SGIV (AY521625), and STIV (NC012637). Phylogenetic trees were constructed using MEGA5 software. For the phylogenetic analysis of ranavirus MCPs, the following sequences were used: ranavirus KRV-1 (KRV-1; accession no. ADO14139), *Rana catesbeiana* virus-JP (RCV-JP; no. BAH80413), soft-shelled turtle iridovirus (STIV; no. ABC59813), frog virus 3 (FV3; no. ACP19256), tiger frog virus (TFV; no. AAK55105), Bohle iridovirus (BIV; no. ACO90022), pike-perch iridovirus (PIV; no. ACO90019), *Rana esculenta* virus (REV; no. ACO90020), Chinese giant salamander virus (CGSV; no. ADZ47908), *Ambystoma tigrinum* virus (ATV; no. TP003785), epizootic hematopoietic necrosis virus (EHNV; no. AAO32315), *Ranavirus maxima*/9995205/DNK (Rmax; no. ADI71344), cod iridovirus/15/04.11.92/DNK (CodV; no. ADI71343), European sheatfish virus (ESV; no. ACO90018), European catfish virus (ECV; no. ACO90017), short-finned eel ranavirus (SERV; no. ACO90021), largemouth bass ulcerative syndrome virus (LMBUSV; no. ADB77863), largemouth bass virus (LMBV; no. CBW46836), grouper iridovirus (GIV; no. AEI85923), Singapore grouper iridovirus (SGIV; no. AAS18087), king grouper iridovirus (KGIV; no. AEI85924), crimson snapper iridovirus (CSIV; no. AEI85915), and lymphocystis disease virus from China (LCDV-C; no. YP\_025102).

**Nucleotide sequence accession number.** The genome sequence of CMTV was deposited into GenBank under accession no. JQ231222.

## RESULTS

**CMTV is a distinct ranavirus isolated in Europe.** To better understand the taxonomic position of the CMTV, we sequenced its MCP gene and performed a phylogenetic analysis comparing the MCP gene to that of 23 other ranaviruses. As shown in Fig. 1A, the CMTV clustered very closely with the Chinese giant salamander virus, isolated in China in 2010 (16), and the *Rana esculenta* virus, isolated from edible frogs (*Pelophylax esculentus*) in Italy and Denmark in 2009 (3). Together with the pike-perch iridovirus, isolated from pike-perch fry in Finland in 1998 (38), all four viruses formed a distinct group that was included within the previously described ALRVs but clearly separated from its two proposed major branches, represented by FV3 and EHNV. Collectively, the CMTV-like viruses are found to be more closely related to the FV3 group, suggesting both may have diverged from a common ancestor. Finally, the CMTV was distantly related to other ranaviruses, such as *Ranavirus maxima*/9995205/DNK, cod iridovirus/15/04.11.92/DNK (2), European sheatfish virus (1), and European catfish virus (32), that were isolated from fish on the European continent. As no full-length genome sequences for ranaviruses isolated in Europe have been described to date (Fig. 1B), and given the apparently distinct position of CMTV within the ALRVs, we decided to sequence its complete genome.

**CMTV is a typical ALRV.** The complete genomic sequence of the CMTV was obtained using pyrosequencing combined with Sanger sequencing of PCR products for unresolved regions. After assembly, we obtained a final genome size of 106,878 bp, with an average coverage of 128 reads per position. The genome size of CMTV is smaller than that of the EHNV (127,011 bp) and slightly

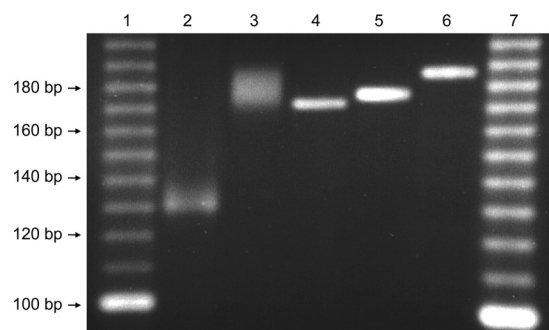


**FIG 1** CMTV is a novel amphibian-like ranavirus isolated in Europe. (A) Phylogenetic tree of ranavirus major capsid protein sequences. The sequences were aligned with ClustalW and trimmed manually to the 450-amino-acid sequence available for the Chinese giant salamander virus (CGSV) MCP, and phylogenetic reconstruction was obtained by neighbor-joining with 1,000 bootstrap replicates. The consensus bootstrap tree is shown, with confidence values indicated on the branches. Branches with bootstrap values below 50% are collapsed. Lymphocystis disease virus from China (LCDV-C) was used as the outgroup. Viruses infecting amphibian or reptile hosts are marked with an asterisk. The GenBank accession numbers of the *Iridovirus* MCP sequences used for this analysis are listed in Materials and Methods. (B) World map showing the locations of isolation and the hosts of the published full-length ranavirus genome sequences and CMTV.

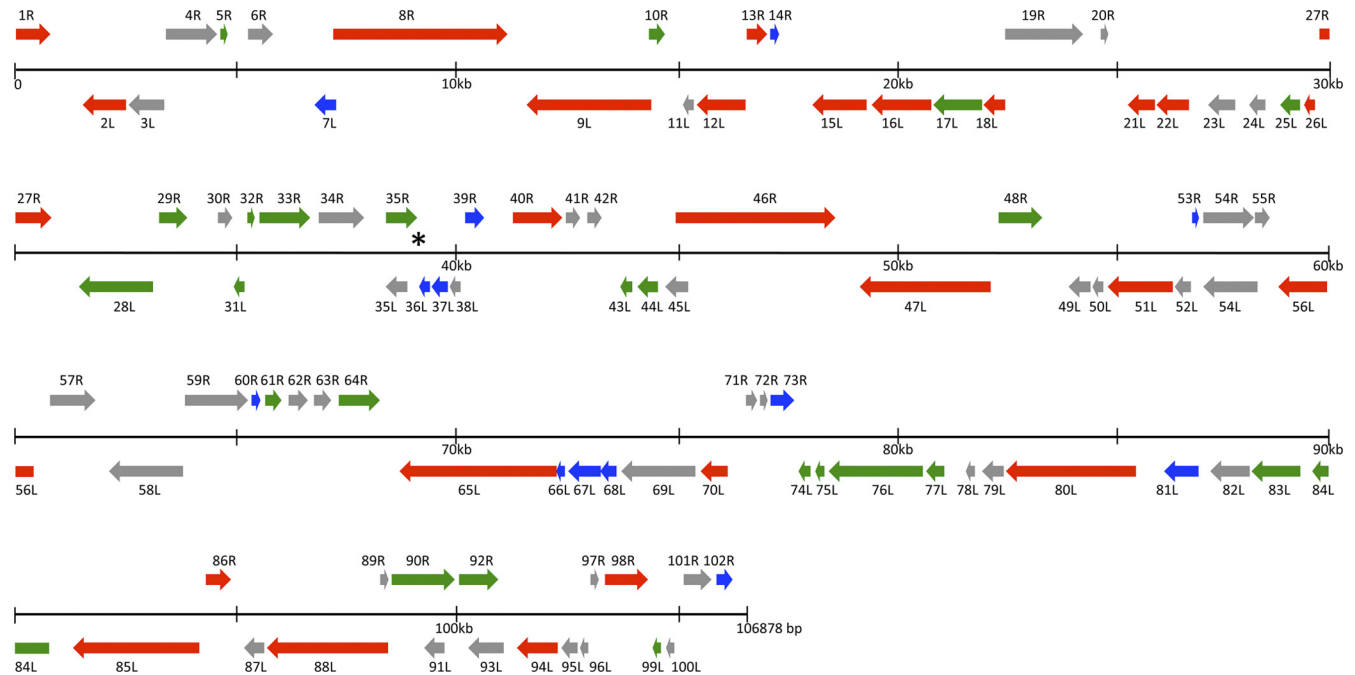
larger than those of the other fully sequenced ALRVs ATV (106,332 bp), FV3 (105,903 bp), STIV (105,890 bp), and TFV (105,057 bp). The 55.3% GC content of the CMTV genome is similar to those of the other ALRVs, which range from 54% to 55%, but higher than those of the GIV (49%) and SGIV (48%) ranaviruses.

The microsatellite identified in FV3 (12) and STIV was also found in CMTV. The use of primers specific for the flanking sequences produced a larger PCR amplification product on CMTV DNA than on FV3 DNA, showing that the region in CMTV contains 60 dinucleotide repeats, compared to the 34 copies observed in the other two viruses, and that it can therefore be used for differentiation of these viruses (Fig. 2).

The CMTV genome was found to contain 104 ORFs (Fig. 3) encoding putatively expressed proteins with predicted molecular masses ranging from 5.2 kDa to 144.2 kDa and with conserved



**FIG 2** PCR amplification of the microsatellites in CMTV and FV3. The microsatellites of FV3 (lane 2) and CMTV (lane 3) were PCR amplified using specific primers and run on a 4% agarose gel together with reference PCR products of known sizes (lane 4, 170 bp; lane 5, 174 bp; lane 6, 184 bp). Lanes 1 and 7, molecular weight markers.



**FIG 3** Linear schematic organization of CMTV genome. Predicted open reading frames (ORFs) are represented as arrows indicating the size and direction of transcription. The black lines represent the CMTV genome and are divided into 30-kb segments. ORFs in the forward strand are drawn above the genome line and those in the complement strand are drawn below. *Iridovirus* core genes, ranavirus-specific genes, and amphibian-like ranavirus-specific genes are indicated in red, green, and blue, respectively. The asterisk shows the position of the microsatellite repetition.

domains assigned to structural proteins as well as proteins potentially involved in replication, transcription, and host response modification (Table 1). For 101 CMTV-predicted proteins, orthologues were readily found in other ranavirus (24) genomes, with identities ranging from 65% to 100%. Only three ORFs (11L, 96L, and 100L) had not been previously annotated in other ranaviruses, but no significant similarities to other known proteins were found in the databases. Early stop codons or frameshift mutations account for the absence of annotated protein orthologues for these three ORFs in other ALRVs in spite of their relatively high conservation at the nucleotide level. Additionally, a genomic inversion in the FV3 group split the CMTV ORF 100L in these three ranaviruses. Whether these proteins are truly expressed and functional during CMTV infection remains to be addressed.

Analyses of all sequenced iridovirus genomes have identified 26 conserved genes, which make up the core iridovirus genes (15). As expected, these genes are conserved in CMTV. Additionally, 27 more genes were described to be conserved throughout the *Ranavirus* genus based on the EHNV gene content (24). These include a multigene family with five members in EHNV of which only two are retained in ATV and the FV3 group as well as in CMTV (ORFs 82L and 83L). Finally, the set of 13 genes conserved in all ALRVs (24) was also found in CMTV. Altogether, these results show that the CMTV can be classified as a typical ALRV in terms of gene content.

**CMTV represents an intermediate in ALRV evolution.** Due to the >90% identity among ranavirus MCP sequences, phylogenetic studies based exclusively on these sequences may be insufficient for differentiating among ranavirus types. To further establish the phylogenetic relationships between CMTV and other fully sequenced ranaviruses, we performed an analysis of the concate-

nated protein sequences derived from the 26 iridoviral core genes (15). As previously reported, the Asian fish viruses GIV and SGIV formed a separated branch within the fully sequenced ranaviruses (Fig. 4). CMTV was found to cluster very confidently within the FV3-like group, which was clearly resolved from the EHNV-like group. Furthermore, CMTV sequences were found to have a slightly higher similarity to the FV3/STIV group than to TFV.

To determine the degree of colinearity of the CMTV genome with those of other ranaviruses, we performed dot plot analyses (Fig. 5 and data not shown). As expected, CMTV did not show major colinearity stretches when compared to the GIV-like fish ranaviruses (data not shown). As described before, ALRVs can be divided into two separate groups in which colinearity is maintained, one including EHNV and ATV and the second including FV3, STIV, and TFV. The CMTV genome was found to not be colinear with either of these groups. Specifically, compared to EHNV, an inversion of the CMTV genomic segment located between positions ~62,000 and ~91,000 was observed, while in comparison to FV3, a single different inversion affecting positions ~15,000 and ~105,000 was detected. These sites of genomic rearrangements correspond exactly to those of the double genomic inversion identified previously when FV3 was compared to EHNV (24). This suggests that CMTV or its ancestor may occupy an intermediate position in the evolutionary process that gave rise to the FV3/STIV/TFV group from the EHNV-like ancestor. Thus, inversion of one segment from an EHNV-like precursor may have produced a CMTV ancestor. A further inversion might have then produced the genomic structure found in the FV3/TFV group.

Upon closer inspection of the corresponding dot plots, CMTV was found to contain no major deletions and only three DNA sequence insertions of about 500 bp each compared to FV3 (Fig.

TABLE 1 Predicted CMTV open reading frames<sup>a</sup>

ORF	Position (nt range)	Product size (aa)	Predicted function and conserved domain or signature <sup>b</sup>	Best BLASTP hit <sup>c</sup>			EHNV orthologue <sup>d</sup>		FV3 orthologue <sup>d</sup>	
				ORF	% ID	Accession no.	ORF	% ID	ORF	% ID
1R	16–786	256	Putative replication factor; pfam04947	FV3 1R	98	YP_031579	100R	98	1R	98
2L	1,497–2,510	337	Putative myristylated membrane protein	STIV 2L	90	YP_002854233	1L	91	2L	90
3L	2,548–3,387	279		EHNV 2L	97	ACO25192	2L	97	NA	
4R	3,419–4,633	404		TFV 4R	98	ABB92272.1	3R	97	3R	96
5R	4,674–4,856	60		FV3 4R	97	YP_031582.1	4R	92	4R	97
6R	5,291–5,893	200	cl03568	FV3 5R	86	YP_031583.1	NA	NA	5R	86
7L	6,823–7,251	142		STIV 9L	95	YP_002854240.1	6L	78	NA	
8R	7,246–11,211	1,321	Largest subunit of DNA-dependent RNA polymerase; cl11429	TFV 8R	99	AAL77794.1	7R	98	8R	98
9L	11,566–14,412	948	Helicase; smart00487	FV3 9L	98	YP_031587.1	8L	98	9L	98
10R	14,428–14,841	137		ATV p8	99	YP_003779.1	9R	97	10R	96
11L	15,222–15,455	77					NA		NA	
12L	15,499–16,593	364	Putative DNA repair protein; cl14812, cl14815	Rana grylio virus 9808 XPG	98	AAV43134.1	10L	97	95R	98
13R	16,689–17,156	155	pfam10881	FV3 94L	97	YP_031673.1	11R	97	94L	97
14R	17,217–17,432	71		STIV 98L	85	YP_002854329.1	12R	95	93L	96
15L	18,167–19,354	395	Immediate early protein ICP-46	STIV 97R	99	YP_002854328.1	13L	98	91R	98
16L	19,478–20,869	463	Major capsid protein; pfam04451	PPIV MCP	99	ACO90019.1	14L	99	90R	98
17L	20,962–22,041	359		STIV 95R	88	YP_002854326.1	15L	88	89R	86
18L	22,109–22,561	150	Thiol oxidoreductase; pfam04777	STIV 94R	100	YP_002854325.1	16L	97	88R	99
19R	22,594–24,393	599		TFV 93L	95	ABB92341.1	17R	94	87L	96
20R	24,746–24,961	71		TFV 92L	80	ABB92340.1	NA		86L	75
21L	25,406–25,993	195	Thymidine kinase; cd01673	TFV 91R	98	ABB92339.1	18L	96	85R	96
22L	26,068–26,805	245	Proliferating cell nuclear antigen	EHNV 19L	99	ACO25209.1	19L	99	84R	98
23L	27,222–27,866	214	Cytosine DNA methyltransferase; cl12011	FV3 83R	99	YP_031662.1	20L	99	83R	99
24L	28,233–28,520	95	Thymidylate synthase; cl00358	EHNV 21L	94	ACO25211.1	21L	94	NA	
25L	28,816–29,289	157	Putative immediate early protein; cl12687	EHNV 22L	97	ACO25212.1	22L	97	82R	93
26L	29,418–29,696	92	Transcription elongation factor SII; cl02609	FV3 81R	97	YP_031660.1	23L	97	81R	97
27R	29,752–30,870	372	RNase III; cd00593, TIGR02191	EHNV 24R	99	ACO25214.1	24R	99	80L	99
28L	31,495–33,213	572		STIV 86R	95	YP_002854317.1	25L	83	79R	95
29R	33,298–33,987	229		STIV 85L	94	YP_002854316.1	26R	95	78L	95
30R	34,687–35,034	115		TFV 82L	99	ABB92335.1	27R	96	77L	98
31L	35,031–35,252	73		TFV 81R	99	ABB92334.1	28L	96	76R	96
32R	35,315–35,569	84	LITAF-like protein; cl02754	FV3 75L	100	YP_031654.1	29R	99	75L	100
33R	35,626–36,807	393		EHNV 30R	97	ACO25220.1	30R	97	74L	85
34R	36,947–37,999	350	Putative NTPase/helicase	EHNV 31R	98	ACO25221.1	31R	98	73L	97
35R	38,496–39,212	238		TFV 77L	98	ABB92331.1	32R	100	72L	97
35L	38,502–38,984	160		EHNV 33L	96	ACO25223.1	33L	96	NA	
36L	39,269–39,502	77		FV3 71R	96	YP_031650.1	34L	90	71R	96
37L	39,542–39,916	124		FV3 70R	98	YP_031649.1	35L	97	70R	98
38L	39,934–40,200	88		FV3 69R	100	YP_031648.1	36L	99	69R	100
39R	40,318–40,782	154	cl00060	EHNV 37R	97	ACO25227.1	37R	97	NA	
40R	41,407–42,570	387	Ribonucleotide reductase, small subunit; cd01049	ATV p39	99	YP_003810.1	38R	98	67L	98
41R	42,625–42,978	117		STIV 70L	97	YP_002854301.1	39R	90	66L	91
42R	43,115–43,459	114		EHNV 40R	89	ACO25230.1	40R	89	65L	96
43L	43,852–44,139	95	CARD-like caspase; cl14633	STIV 67R	97	YP_002854298.1	41L	96	64R	93
44L	44,250–44,744	164	dUTPase; cd07557	STIV 66R	98	YP_002854297.1	42L	97	63R	96
45L	44,861–45,403	180		STIV 65R	92	YP_002854296.1	NA		NA	
46R	45,123–48,776	1,217	DNA-dependent RNA polymerase B subunit; cd00653, cl04593, COG0085	FV3 62L	98	YP_031641.1	43R	96	62L	98

(Continued on following page)

TABLE 1 (Continued)

ORF	Position (nt range)	Product size (aa)	Predicted function and conserved domain or signature <sup>b</sup>	Best BLASTP hit <sup>c</sup>			EHNV orthologue <sup>d</sup>		FV3 orthologue <sup>d</sup>	
				ORF	% ID	Accession no.	ORF	% ID	ORF	% ID
47L	49,315–52,356	1,013	DNA polymerase; cl10023, cl10012	STIV 63R	99	YP_002854294.1	44L	99	60R	99
48R	52,518–53,576	352		FV3 59L	98	YP_031638.1	45R	97	59L	98
49L	54,062–54,616	184		EHNV 46L	98	ACO25236.1	46L	98	NA	
50L	54,667–54,912	81	cl08321	EHNV 47L	88	ACO25237.1	47L	88	NA	
51L	54,995–56,491	498	Putative phosphotransferase	STIV 60R	99	YP_002854291.1	48L	98	57R	98
52L	56,532–56,936	134		EHNV 49L	98	ACO25239.1	49L	98	NA	
53R	56,973–57,122	49		EHNV 50R	100	ACO25240.1	50R	100	NA	
54R	57,130–58,425	431	Helicase; cd00046, COG1061	TFV 56L	98	AAL77803.1	51R	98	55L	97
54L	57,142–58,281	379		FV3 55R	97	YP_031634.1	p40	97	55R	97
55R	58,338–58,730	130		STIV 56L	98	YP_002854287.1	NA		NA	
56L	58,875–60,443	522	Myristylated membrane protein; pfam02442	FV3 53R	99	YP_031631.1	53L	98	53R	99
57R	60,780–61,847	355	3-beta-hydroxysteroid dehydrogenase; pfam01073	STIV 54L	99	YP_002854285.1	54R	97	52L	98
58L	62,104–63,789	561		TFV 53R	98	ABB92313.1	84R	98	51R	98
59R	63,868–65,379	503	cl02640, PTZ00108	STIV 52L	96	YP_002854283.1	83L	97	49L	98
60R	65,427–65,738	103		ATV p78	88	YP_003849.1	82L	98	48L	98
61R	65,741–66,157	138		TFV 49L	99	ABB92309.1	81L	96	47L	98
62R	66,282–66,749	155	Neurofilament triplet H1-like protein; PTZ00449	STIV 49L	94	YP_002854280.1	80L	83	46L	83
63R	66,859–67,269	136		FV3 45L	98	YP_031623.1	79L	98	45L	98
64R	67,396–68,364	322		TFV 46L	80	ABB92307.1	78L	96	42L	98
65L	68,753–72,331	1,192		FV3 41R	98	YP_031619.1	77R	98	41R	98
66L	72,321–72,461	46		ATV p71	91	YP_003842.1	76R	70	NA	
67L	72,647–73,330	227		ATV p70	84	YP_003841.1	75R	80	40R	79
68L	73,346–73,696	116		EHNV 74R	97	ACO25264.1	74R	97	39R	94
69L	73,805–75,502	565	Ribonucleoside-diphosphate reductase, large subunit; cd01679, PRK09102	TFV 41R	99	AAL77800.1	73R	99	38R	98
70L	75,641–76,276	211	Putative NIF/NLI interacting factor; cl11391	EHNV 72R	99	ACO25262.1	72R	99	37R	98
71R	76,657–76,956	99		FV3 36L	89	YP_031614.1	NA		36L	89
72R	77,010–77,228	72		FV3 36L	93	YP_031614.1	NA		36L	93
73R	77,268–77,846	192		EHNV 71L	65	ACO25261.1	71L	65	35L	64
74L	77,831–78,151	106		EHNV 70R	94	ACO25260.1	70R	94	34R	95
75L	78,293–78,484	63		EHNV 69R	97	ACO25259.1	69R	97	33R	97
76L	78,567–80,717	716	Neurofilament triplet H1-like protein; cl06505, cl06430	STIV 35R	84	YP_002854266.1	68R	85	32R	81
77L	80,767–81,186	139	cl10444	EHNV 67R	98	ACO25257.1	67R	98	31R	95
78L	81,674–81,940	88		EHNV 66R	100	ACO25256.1	66R	100	NA	
79L	82,077–82,565	162		STIV 32R	99	YP_002854263.1	63R	92	28R	98
80L	82,614–85,544	976	Putative tyrosine kinase; cl14933	STIV 31R	98	YP_002854262.1	62R	96	27R	97
81L	86,043–86,855	270	eIF2a-like protein; cl09927	EHNV 61R	95	ACO25251.1	61R	95	26R	83
82L	87,180–88,094	304	p31K protein; cl12506	EHNV 60R	97	ACO25250.1	60R	97	25R	99
83L	88,158–89,255	365		FV3 24R	98	YP_031602.1	57R	96	24R	98
84L	89,680–90,828	382		FV3 23R	98	YP_031601.1	56R	97	23R	98
85L	91,206–94,133	975	Putative D5 family NTP/ATPase; cl11759, cl07361, cl07360	STIV 25R	99	YP_002854256.1	85L	98	22R	99
86R	94,258–94,926	222		ATV p82	95	YP_003853.1	86R	95	21L	95
87L	95,163–95,609	148		STIV 23R	99	YP_002854254.1	88L	98	20R	96
88L	95,657–98,443	928	cl07414	TFV 19R	96	ABB92284.1	89L	88	19R	94
89R	98,232–98,468	78		FV3 18L	97	YP_031596.1	NA		18L	97
90R	98,505–100,013	502		STIV 18L	99	YP_002854249.1	90R	99	17L	99
91L	99,224–99,730	168		STIV 19R	99	YP_002854250.1	NA		NA	
92R	100,050–100,997	315		STIV 17L	97	YP_002854248.1	91R	98	NA	
93L	100,254–101,081	275	Putative integrase homologue	FV3 16R	99	YP_031594.1	NA		16R	99
94L	101,361–102,308	315	Putative A32-like virion packaging ATPase; cl04659, smart00382	STIV 16R	99	YP_002854247.1	92L	97	15R	97

(Continued on following page)

TABLE 1 (Continued)

ORF	Position (nt range)	Product size (aa)	Predicted function and conserved domain or signature <sup>b</sup>	Best BLASTP hit <sup>c</sup>			EHNV orthologue <sup>d</sup>		FV3 orthologue <sup>d</sup>	
				ORF	% ID	Accession no.	ORF	% ID	ORF	% ID
95L	102,405–102,764	119		STIV 15R	100	YP_002854246.1	93L	98	14R	100
96L	102,841–103,035	64					NA		NA	
97R	103,076–103,270	64		TFV 13L		ABB92279.1	NA		NA	
98R	103,382–104,425	347		STIV 14L	98	YP_002854245.1	95R	97	12L	98
99L	104,491–104,703	70		STIV 13R	99	YP_002854244.1	96L	99	11R	97
100L	104,769–104,990	73					NA		NA	
101R	105,187–105,873	228		EHNV 98R	97	ACO25288.1	98R	97	96R	94
102R	105,939–106,352	137		STIV 103R	99	YP_002854334.1	99R	96	97R	99

<sup>a</sup> nt, nucleotides; aa, amino acids; ID, identity.

<sup>b</sup> Predicted function is based on conserved domains and/or previous annotation in other ranaviruses. LITAF, lipopolysaccharide-induced tumor necrosis factor alpha (TNF- $\alpha$ ).

<sup>c</sup> Significant hits using conserved domain search at NCBI BLASTP are shown.

<sup>d</sup> NA, not applicable (BLASTP *E* value > 0.001).

5). These insertions corresponded to sequences that are also found in EHNV and to different degrees in ATV and TFV but that are specifically lost in FV3 and STIV. In particular, the insertions affect CMTV 24L, CMTV 39R, and CMTV 81L, which encode a truncated thymidylate synthase protein, a putatively secreted protein containing an intact FGF domain, and an eIF2 $\alpha$ -like protein, respectively. Conversely, compared to EHNV, CMTV presents at least 14 deletions and two insertions, which affect CMTV 6R as well as CMTV 71R and CMTV 72R, that are also found in TFV, FV3, and STIV. This pattern of sequence gain and loss among ALRVs is also consistent with the intermediate position of CMTV in the evolution of EHNV-like viruses toward FV3-like viruses, since CMTV retains EHNV-like characteristics which are lost in FV3-like viruses and has already acquired sequences found previously only in FV3-like viruses. Therefore, the genomic content and structure of CMTV may resemble those of the last common ancestor of TFV, STIV, and FV3 viruses.

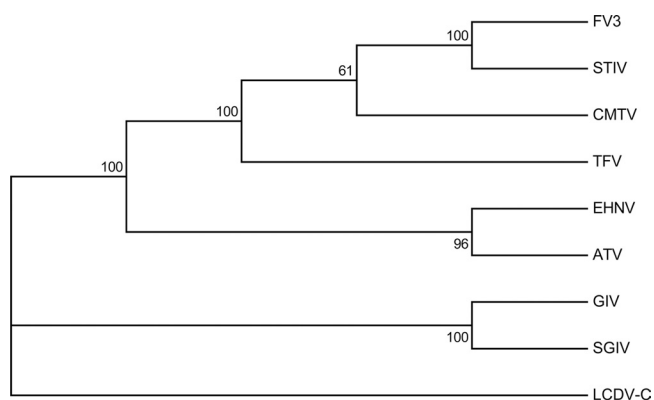
## DISCUSSION

In this report, we have shown that the CMTV, the first European ranavirus to be completely sequenced, should be classified as an ALRV. Based on our analyses, we propose that the CMTV might

represent, in terms of gene content and genomic structure, a very recent ancestor of the previously described group of FV3-like viruses (24). As the CMTV forms a cluster with ranaviruses isolated from different species and locations worldwide, including fish and amphibian viruses, we believe that these may form a single, novel group of ALRVs. The information provided by this report should therefore be useful to specifically address the relationship of these viruses with CMTV and to further define the taxonomic positions of different ranavirus isolates, which have been poorly described at the genetic level.

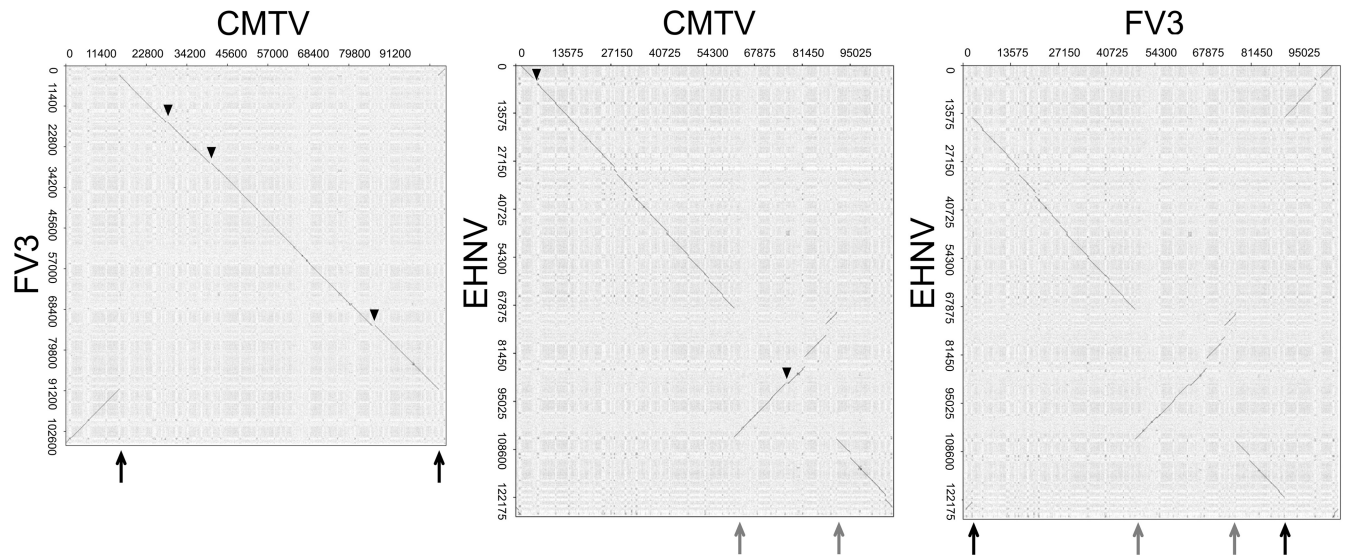
One of the most outstanding features of ranaviruses is their wide host range, which includes anuran and urodele amphibians, reptiles, and teleost fish. It has been proposed that the most recent common ancestor of all ALRVs was most probably a fish virus, with independent events giving rise to isolates infecting different hosts (24). Experimental evidence suggests that ATV may infect only urodele hosts, while neither fish nor frogs are susceptible to ATV infection (25). As CMTV can infect both anuran and urodele amphibians in the wild (4, 5, 29), it is tempting to speculate that CMTV may have derived from an ATV-related virus, acquiring the ability to infect frogs. Potentially, further divergence into the FV3-like viruses may have produced frog- and reptile-specific viruses. However, FV3 infections of both fish and salamander species have been reported (30), and the Bohle iridovirus, isolated from the ornate burrowing frog in Australia, is also known to infect fish (31). Therefore, both data from experimental infections and a clearer taxonomic description of ranaviruses based on complete genome information will help address this issue.

As described above, CMTV has retained some features from the EHNV-like group which are lost in FV3-like viruses and has acquired potential coding sequences which were previously identified only in the FV3-like viruses, further supporting their intermediate position in the evolution between these groups of ALRVs. Interestingly, some of these coding regions may play a role in host range or virulence. In particular, a potentially functional protein belonging to the fibroblast growth factor family is present only in EHNV, ATV, and CMTV. In baculoviruses, such an activity was shown to be able to induce cell migration, suggesting a role in immunomodulation or viral spread (13). Additionally, the eIF2 $\alpha$ -like protein (vIF2 $\alpha$ H) present in most ALRVs, including CMTV, but truncated in the FV3 and STIV has been demonstrated to act as an inhibitor of the antiviral protein kinase PKR (33). Thus, ATV mutants lacking



**FIG 4** CMTV is closely related to the FV3-like viruses. Phylogenetic analysis of the concatenated sequences of the 26 iridovirus core proteins (15) from the indicated viruses. The sequences were aligned with ClustalW, and the phylogenetic reconstruction was obtained by neighbor-joining with 1,000 bootstrap replicates. Consensus bootstrap confidence values are indicated above the branches. LCDV-C was used as the outgroup.





**FIG 5** CMTV is an evolutionary intermediate in ALRVs. Dot plot comparisons of the CMTV genome and the EHNH and FV3 genomes. For clarity, the reverse complement sequence of the FV3 genome was used in these comparisons. As a reference, a dot plot of EHNH versus FV3 is shown in the right panel. Black arrows indicate the sites of inversion in the CMTV genome compared to the FV3 genome, and gray arrows indicate the sites of inversion in the CMTV genome compared to the EHNH genome. Triangles indicate sequences present in CMTV but absent in FV3 or EHNH.

vIF2 $\alpha$ H activity were shown to be more sensitive to the antiviral activity of interferon (IFN) and displayed an increased time to death in infected salamanders, demonstrating the protein's role as an important virulence factor *in vivo* (27). Conversely, CMTV 6R, which is found in ALRVs except EHNH and ATV, shows significant similarity to DNA and protein sequences from frog species, suggesting a recent acquisition event during the process of adaptation to novel amphibian hosts. Implications of these specific events of sequence gain and loss among ALRV groups on virus host range or pathogenicity remain to be addressed.

Whether the broad host range of ranaviruses, compared to that of other double-stranded DNA viruses like orthopoxviruses, is an inherent feature of the iridoviruses or related to a more recent evolutionary history of this virus family is an important topic for study. While gene loss has been established as one of the main driving forces in poxvirus evolution (19), we observe very little differences in terms of global gene content among ALRVs, which might indicate that this group of viruses is only on the verge of an evolutionary radiation. This may relate to the human-mediated exchange of host species or viruses between distant regions, which increases the number of potential hosts to infect and is consistent with their established character as emerging infectious-disease-causing agents.

As pathogens of wildlife, ranaviruses have been found to cause mass mortalities mainly in amphibian populations worldwide. One of the earliest amphibian population declines to be reported was that affecting the common midwife toad and other amphibian species, including salamanders, during the last decade in Spain (7, 8). These declines have been associated with the emergence of chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis*. Recent studies have shown the widespread yet heterogeneous distribution of *Batrachochytrium dendrobatidis* across the Iberian Peninsula and predict further spread of the disease in the future (42). However, the isolation of CMTV from diseased amphibians in Spain suggests that ranavirus infection, too, may be having an impact on population declines, as reported for other locations. In the Nether-

lands, animals affected with CMTV showed no evidence of *Batrachochytrium dendrobatidis* infection (29), suggesting that the two pathogens did not occur simultaneously. Whether an interaction between the pathogens is important for amphibian extinction dynamics remains to be addressed. Mass mortality events affecting the urodele *Triturus marmoratus* in northwest Portugal in 2003 are probably also related to ranavirus outbreaks, and recently, a novel ranavirus closely related to FV3 was isolated from lizards in Portugal (12). The nature of these viruses and whether they may represent different isolates of the CMTV are currently unknown. Although the distribution and degree of prevalence of CMTV in different host species need to be studied in greater detail, the presence of CMTV in the Netherlands (29) suggests that this virus may show a wide geographic distribution across the European mainland. In the case of tiger salamander infections in North America, it was shown that while ATV has coevolved with its host in their geographic range, human-mediated transfer of ATV-infected bait salamanders introduced novel, more virulent strains to discrete locations, resulting in disease emergence (26, 36). It will be important to determine the mechanisms of emergence of CMTV-caused disease and whether it may be linked to environmental factors, virus spread, or both.

As advances in sequencing technologies make more genomic information on the ranavirus isolates available, a better understanding of the evolutionary history of this virus family will be gained. In particular, a better knowledge of the ranavirus species and strain structure will be crucial to set up detection methods and surveillance strategies in order to minimize their impact on amphibian wildlife and cultivated species.

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