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Whole genome characterization of a chelonian orthoreovirus strain identifies significant genetic diversity and may classify reptile orthoreoviruses into distinct species

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ABSTRACT

In this study we report the sequence and phylogenetic characterization of an orthoreovirus strain, CH1197/96, isolated from a spur-thighed tortoise (*Testudo graeca*) on chicken embryo fibroblast cells. The 23,957 bp long genome sequence was obtained by combined use of semiconductor and capillary sequencing. Although the genomic characterization showed that the virus was most similar to the bush viper reovirus strain, 47/02, and in phylogenies performed with all segments the two strains formed a monophyletic group, the nucleotide (48.4–70.3%) and amino acid (39.2–80.7%) sequence identity values were moderate between the two reptile origin reoviruses. Based on our results and existing classification criteria for the genus *Orthoreovirus*, the tortoise reovirus strain CH1197/96 might be the first representative of a novel reptilian origin *Orthoreovirus* species.

1. Introduction

Orthoreoviruses are non-enveloped viruses with an icosahedral capsid 70–80 nm in diameter (Attoui et al., 2011). The double-stranded RNA genome of or thoreoviruses consists of 10 segments grouped into three categories based on their electrophoretic mobility: three large (L1-L3), three medium (M1-M3), and four small segments (S1-S4). With the exception of the S1 or S4 segments, which are bi- or tricistronic in different orthoreovirus strains, each genome segment encodes a single open reading frame (ORF) (Day, 2009). Major evolutionary mechanisms of orthoreoviruses are point mutations generated by the viral RNA dependent RNA polymerase lacking proofreading activity and reassortment of cognate genomic segments between members of a particular *Orthoreovirus* species. Based on the currently used classification criteria the genus *Orthoreovirus* is divided into five species: *Mammalian orthoreovirus* (MRV), *Avian orthoreovirus* (ARV), *Nelson Bay orthoreovirus* (NBV), *Reptilian orthoreovirus* (RRV) and *Baboon orthoreovirus* (BRV) (International Committee on Taxonomy of Viruses, ICTV; http://www.ictvonline.org/). A sixth species was proposed for a sole distinct virus, the Broome virus (BroV; Thalmann et al., 2010) and very likely, a number of ARV-related strains can be further divided into additional species; these include the Tvärminne avian virus (TVAV) and the Bulbul orthoreovirus along with the Steller sea lion reovirus (SSRV) and the Psittacine reovirus strain Ge01, respectively (Dandár et al., 2014; de Kloet, 2008; Ogasawara et al., 2015; Palacios et al., 2011).

In the genus *Orthoreovirus* specific sequence identity cut-off values have been defined to classify members into species (Reoviridae Study Group of the ICTV; Attoui et al., 2011). Greater than 75% nucleotide sequence identity between homologous genes is the cut-off value for most genome segments to classify virus strains into the same species, and a nucleotide sequence identity less than 60% is considered to be the cut-off value to demarcate viruses into different virus species. Amino acid sequence identity cut-off values have also been determined by the ICTV, e.g. for the outer capsid proteins, where the amino acid identities greater than 55% indicate that orthoreoviruses belong to the same species, while less than 35% similarity is used to classify virus strains into different species. For the more conserved core proteins these values are higher, in

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Table 1
General features of the tortoise orthoreovirus strain CH1197/96. Nucleotide: nt; amino acid: aa.

Genome segment	Size (nt)	Length (nt) of the 5′ end ORF 3′ end	Sequence at the termini 5′ end/3′ end	Encoded protein	Protein size (aa)
L1	3970	13-3870-87	GUUCUU/UUCAUC	λA (core shell)	1289
L2	3928	14-3870-44	GUUCUU/UUCAUC	λC (core turret)	1289
L3	3852	14-3786-52	GUUCUU/UUCAUC	λB (core RdRp)	1261
M1	2435	27-2334-74	GUUCUU/UUCAUC	μNS (NS factory)	777
M2	2347	13-2283-51	GUUCUU/UUCAUC	μA (core NTPase)	760
M3	2120	27-2022-71	GUUCUU/UUCAUC	μB (outer shell)	673
S1	1509	23-408-66	GUUCAU/UUCAUC	FAST-p14	135
		1050		σC (outer fiber)	349
S2	1315	12-1251-52	GUUCUU/UUCAUC	σA (core clamp)	416
S3	1274	31-1176-67	GUUCUU/UUCAUC	σB (outer clamp)	391
S4	1207	25-1113-69	GUUCAU/UUCAUC	σNS (NS RNAb)	370

particular, more than 85% for strains in the same species and less than 65% for strains belonging to different species (Attoui et al., 2011). For the non-structural proteins cut-off values have not been clearly defined.

Although reoviruses of reptiles are frequently detected and can be readily isolated from squamates and terrapins, our knowledge about their genetic diversity and evolutionary relationships is limited. The single complete RRV genome sequence has been only very recently determined for 47/02, a reovirus strain isolated from a green bush viper (*Atheris squamigera*) (Bányai et al., 2014). Additional sequence data have been determined for the L3 and S1 genome segments of a handful of reptile origin RRVs (Bányai et al., 2014; Duncan et al., 2004; Marschang, 2011; Wellehan et al., 2009). Evolutionary analysis of the bush viper reovirus uncovered a number of shared features with other orthoreoviruses and clustered this strain into a monophyletic group with BRV and BroV suggesting the common origin of this clade.

In this study we performed whole genome sequencing of a reovirus strain, CH1197/96, isolated from a spur-thighed tortoise (*Testudo graeca*) originated from Switzerland. Our results indicate significant genetic diversity between strains detected in scaled reptiles and the single isolate from a spur-tighed tortoise. The taxonomic implications are discussed in the study.

2. Materials and methods

The spur-tighed tortoise (*T. graeca*) died spontaneously at a zoo facility in Switzerland. Pathologic examination revealed cachexy, epithelial necrosis of the tongue and splenomegaly; virological examinations were performed later in Germany. Strain CH1197/96 was detected by applying consensus nested PCR (Wellehan et al., 2009) and isolated from the tongue, lung, liver, esophagus and kidney samples of the tortoise on chicken embryo fibroblast cells. Cytopathic effect, syncytium formation was seen within 4–7 days for all tissues tested; no blind passages were necessary. After isolation the strain could also be propagated on a continuous viper heart cell line (VH-2).

To further characterize the isolated reovirus strain, it was subjected to whole genome sequencing using the protocol described in detail elsewhere (Bányai et al., 2014). In brief, RNA was extracted from polyethylene glycol precipitated cell culture supernatant using TRIzol Reagent (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's recommendations. Random primed reverse transcription was followed by amplification of complementary DNA (cDNA). A cDNA library was prepared using the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, Beverly, MA, USA) using the Ion Torrent Xpress barcode adapters (Life Technologies, Carlsbad, CA, USA). The emulsion PCR and subsequent templated bead enrichment were performed with a OneTouch v2 instrument and Ion OneTouch™ES, respectively. Sequencing was carried out on a 316 chip using the Ion Torrent Personal Genome Machine® (Life Technologies). Sequences were assembled and aligned with the software CLC Genomics Workbench (http://www.clcbio.com). To determine the sequences of the segment termini, a short oligonucleotide (Bányai et al., 2011) was ligated to the 3′ ends of the genomic RNA and a primer complementary to the ligated oligonucleotide was combined with gene specific primers to amplify and sequence these short missing regions. Direct sequencing was carried out applying the BigDye cycle sequencing kit v1.1 (Life Technologies) and run on an automated sequence analyser (ABI PRISM® 3100-Avant Genetic Analyzer). Contigs were aligned with Sanger sequencing reads using MultAlin online software (http://multalin.toulouse.inra.fr/multalin/) and were edited in Genedoc (Nicholas et al., 1997). Phylogenetic analysis was performed and sequence identity values were calculated by applying the MEGA6 program package (Tamura et al., 2013) based on multiple sequence alignments generated by the TranslatorX online platform (Abascal et al., 2010); the best-fit substitution models (General Time Reversible, Gamma distributed for λA, λB, μA, μB, and σNS; General Time Reversible, Gamma distributed with Invariant sites for λC, μNS, and σB; Tamura 3-parameter, Gamma distributed for σA; Kimura 2-parameter, Gamma distributed with Invariant sites for σC; complete deletion for all genes) were selected for each gene-specific dataset based on the Bayesian information criterion. Maximum-likelihood trees were generated and tree topologies were validated by bootstrap analysis (500 replicates).

3. Results and discussion

Similar to other reptilian reoviruses, the tortoise orthoreovirus strain CH1197/96 induces cell–cell fusion and syncytium in cell culture (data not shown). High-throughput sequencing runs revealed only reoviral reads, indicating that reovirus was the sole virus isolated in cell-culture. The complete genome of CH1197/96 was 23,957 bp in length and the size of the individual genome segments ranged from 3970 bp (L1) to 1207 bp (S4) (Table 1). The GenBank accession numbers for the complete genomic sequence of CH1197/96 are KT696547-KT696556. The G + C content was 48.7%. The genomic organization of CH1197/96 was similar and corresponded with that of the bush viper reovirus (47/02). All segments, except S1, were monocistronic and the following orthoreoviral proteins were identified by ORF prediction and sequence comparison: λA, λB, λC, μA, μB, μNS, σA, σB, σC, σNS, and p14. The 5′ and 3′ untranslated regions were 13–31 and 44–84 nt long, (Table 1) and were found to be highly conserved at the termini (5′ end GUUCUU; 3′ end UUCAUC) with the exception of the fifth nucleotide, which differed in the 5′ UTR sequences in two S-class genome segments (S1 and S4: GUUCAU).

The sequence similarity values of homologue genes (Supplementary material, Table 1) and the proteins (Supplementary material, Table 2) they encode ranged between 48.4–70.3% and