Yunnan orbivirus, a new orbivirus species isolated from *Culex tritaeniorhynchus* mosquitoes in China

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An orbivirus designated *Yunnan orbivirus* (YUOV) was isolated from *Culex tritaeniorhynchus* mosquitoes collected in the Yunnan province of China. Electron microscopy showed particles with typical orbivirus morphology. The YUOV genome was sequenced completely and compared with previously characterized orbivirus genomes. Significant identity scores were detected between proteins encoded by the segments (Seg-1 to Seg-10) of YUOV and those encoded by their homologues in insect-borne and tick-borne orbiviruses. Analysis of VP1 (Pol) and VP2 (T2, which correlates with the virus serogroup) indicated that YUOV is a new species of the genus *Orbivirus* that is unrelated to the other insect-borne orbiviruses. The replication of YUOV in mosquito cell lines was restricted to *Aedes albopictus* cells and the virus failed to replicate in mammalian cell lines. However, intraperitoneal injection of virus into naïve mice resulted in productive, non-lethal virus replication and viraemia. Infected mice developed serum neutralizing antibodies and were protected against a new infection challenge. Sequence analysis of clones from the segments encoding outer coat proteins (Seg-3 and Seg-6) of YUOV recovered from mouse blood did not show significant changes in the sequences. The availability of the complete genome sequence will facilitate the development of sequence-specific PCR assays for the study of YUOV epidemiology in the field.

INTRODUCTION

The genus *Orbivirus* includes 21 recognized species and represents one of 12 established genera within the family *Reoviridae* (*Orthoreovirus*, *Orbivirus*, *Rotavirus*, *Coltivirus*, *Aquareovirus*, *Cypovirus*, *Fijivirus*, *Phytoreovirus*, *Oryzavirus*, *Seadornavirus*, *Mycoreovirus* and *Idnoreovirus*). Orbiviruses are transmitted by *Culicoides* midges, ticks, phlebotomine flies and anopheline and culicine mosquitoes, and have genomes consisting of 10 segments of double-stranded RNA (dsRNA). The type species of the genus is *Bluetongue virus* (BTV), which, together with *African horse sickness virus* (AHSV) and *Epizootic hemorrhagic disease virus* (EHDV), represents three economically important vertebrate-pathogen species belonging to this genus. All three (BTV, AHSV and EHDV) are transmitted by *Culicoides* midges (Mertens, 1999; Mertens *et al.*, 2000, 2005). The insect-borne orbiviruses have received more attention in terms of analysis and BTV remains the most-studied orbivirus in terms of structural, functional and phylogenetic aspects. Sequence data are available for many of the insect-borne orbiviruses, but are to date available only for two tick-borne orbiviruses, namely Broadhaven virus (BRDV, a member of the species *Great Island virus*; Moss *et al.*, 1992) and St Croix River virus (SCRV; Attoui *et al.*, 2001). Comparison of the homologous protein sequences of insect-borne and tick-borne orbiviruses shows a considerable divergence (showing only 23–38 % amino acid identity), revealing a considerable genetic diversity within the genus *Orbivirus*.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 10 segments of the YUOV genome determined in this study are AY701509–AY701518.

Tables showing the sequences used in phylogenetic analysis of YUOV and alignments of protein sequences are available as supplementary material in JGV Online.

Orbiviruses may be responsible for human infections. Insect-borne orbiviruses that have been reported to infect humans belong to the species *Changuinola virus*, *Corriparta virus*, *Lebombo virus* and *Orungo virus*, whilst those that are tick-borne belong to the species *Great Island virus*.

We report here the characterization of a new virus with a dsRNA 10-segmented genome that was isolated from the mosquito *Culex tritaeniorhynchus* in China. Analysis of the full-length genome and of the biological characteristics of this virus indicated that it belongs to the genus *Orbivirus* and constitutes a new species, which was designated *Yunnan orbivirus* (YUOV).

METHODS

Virus isolation and propagation. Wild *Culex* mosquitoes collected in the south of China in the Yunnan province were homogenized and inoculated into C6/36 cells (first passage), in an attempt to isolate arboviruses. A homogenate of *C. tritaeniorhynchus* mosquito caused cytopathic effect in C6/36 cells and agarose-gel electrophoresis of RNA extracts showed a segmented profile of dsRNA, suggesting infection by a virus of the family *Reoviridae*. These potentially infected cell cultures were propagated again into C6/36 and AP61 cells (second passages), lyophilized at the Department of Pathology of the University of Texas Medical Branch (Galveston, TX, USA) and further characterized in this study as described below.

The lyophilized product was used to infect C6/36 cells (third passage) in L-15 medium at 27 °C as described previously (Attoui *et al.*, 2000b) and a virus stock was prepared. The virus was subsequently plaque-purified in C6/36 cells, using Seaplaque agarose (2 %) and Trypan blue to identify plaques. One clone was further propagated, purified and used for analysis.

Virus purification and electron microscopy. Culture supernatant was pelleted over a 66 % (w/w) sucrose cushion in 0·2 M Tris/HCl, pH 8·0. The material at the top of the cushion was suspended in 0·2 M Tris/HCl, pH 8·0, and centrifuged at 200 000 ***g*** in a discontinuous caesium chloride gradient in 0·2 M Tris/HCl, pH 8·0 (1 ml 55 % CsCl/3 ml 35 % CsCl), as described by Burroughs *et al.* (1994). The light-blue material at the interface of the CsCl layers was recovered, adsorbed to Formvar/carbon-coated grids and stained with 2 % phosphotungstate for 20 s, rinsed in water and dried prior to being examined by electron microscopy on a Philips Morgagni 268 transmission electron microscope.

Replication of YUOV in various insect and mammalian cell lines. Virus replication was tested in other cell lines by inoculation at an m.o.i. of 1 p.f.u. per cell into the insect cell lines C6/36 and AA23 (both from *Aedes albopictus*), A20 and AE (both from *Aedes aegypti*) and A w-albus (from *Aedes w-albus*). It was also inoculated into the mammalian cell lines L929, BHK-21, Vero, BGM, HEp-2 and MRC5. For this purpose, 100 µl of a C6/36 YUOV-infected culture supernatant was added to the cell monolayers and incubated for 1 h at 28 °C for the mosquito cell lines and at 37 °C for the mammalian cell lines. The cells were washed twice with PBS and culture medium was added. At day 5 post-infection, cells were scraped, lysed with deionized water and used for reinfection of new cells or processed for the extraction of RNA. The dsRNA was extracted by using RNA NOW reagent (Biogentex) and used for both agarose-gel electrophoresis and RT-PCR with specific YUOV primers, as described below.

Replication of YUOV in mice. Ten-week-old mice were inoculated intraperitoneally with 100 p.f.u. YUOV (from a C6/36 cell culture). Blood (30–50 µl) was recovered from the caudal vein at days 0, 1, 3, 5 and 7 in tubes containing 20 µl 10 mM EDTA, pH 8·0. The blood samples were extracted by using the one-component RNA NOW reagent (Biogentex), as described below.

Isolation and purification of nucleic acids. Virus dsRNA was extracted from infected *A. albopictus* C6/36 mosquito cells or mammalian cells by using a commercially available guanidinium isothiocyanate-based procedure (RNA NOW reagent; Biogentex) and further purified by precipitating high-molecular-mass single-stranded RNA in 2 M LiCl, as described elsewhere (Attoui *et al.*, 2000a).

Cloning of the dsRNA segments. The genome segments of YUOV were copied into cDNA, cloned and sequenced according to the single-primer amplification technique described previously (Attoui *et al.*, 2000a, b). Briefly, a defined 3′-amino-blocked oligodeoxyribonucleotide was ligated to both of the 3′ ends of the dsRNA segments by using T4 RNA ligase, followed by reverse transcription and PCR amplification using a complementary primer. PCR amplicons were analysed by agarose-gel electrophoresis, ligated into the pGEM-T cloning vector (Promega) and transfected into competent XL-Blue *Escherichia coli*. Insert sequences were determined by using M13 universal primers, a D-rhodamine DNA sequencing kit and an ABI prism 377 sequence analyser (Perkin Elmer).

Sequence analysis. Analysis of the YUOV amino acid sequence was performed by comparing each segment's sequence with a database constructed from all available sequences of the family *Reoviridae*, using the local BLAST program implemented in the DNATools package (version 5.2.018; S. W. Rasmussen, Valby Data Center, Denmark).

For phylogenetic analysis, the VP1 sequence of YUOV – identified as the virus RNA-dependent RNA polymerase (RdRp) – was compared with the amino acid sequences of putative RdRps of representative strains of viruses representing the 12 genera of the family *Reoviridae*. GenBank accession numbers are provided in Supplementary Table S1 (available in JGV Online). Sequence alignments were performed by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic analyses were carried out with the software program MEGA3 (Kumar *et al.*, 2004) using the p-distance determination algorithm, the Poisson correction or the gamma distance and the neighbour-joining method for tree building.

The relatedness of YUOV to characterized orbivirus species was further analysed by comparing the sequence of VP2 (identified as the T2 protein) with the T2 sequences of additional orbiviruses retrieved from databases or which were published previously [Pritchard *et al.*, 1995; Hooper *et al.*, 1999 (obtained as an electronic version from the authors)]. Because some of these sequences were only partial, the final alignment included aa 393–548 relative to the BTV-10 sequence (GenBank accession no. P12435). Other GenBank accession numbers are provided in Supplementary Table S2, available in JGV Online.

Detection of the YUOV genome in mammalian cells and in infected mouse blood. The dsRNA was copied to cDNA by using random hexanucleotide primers as described previously. Briefly, dsRNA was denatured in 15 % DSMO by heating at 99 °C for 1 min and incubated immediately on ice. For reverse transcription, Superscript III reverse transcriptase (Invitrogen) was used at 42 °C. PCR primers designed from segment 7 were used in PCR assays. The resulting cDNA was PCR-amplified using first-round primers YUOVSeg7S1 (positions 200–222; 5′-AGCATTCGGTACGCAGTATCTCG-3′) and YUOVSeg7R1 (positions 603–580; 5′-GCCGAGCCGATCATGTCACG-TGT-3′) to produce an amplicon of 453 bp and second-round primers

YUOVSeg7S2 (positions 235–258; 5′-GAACGACAACGCATTTTGA-AGGAG-3′) and YUOVSeg7R2 (positions 652–630; 5′-CACGTTGT-TCCTGCACTTGGTCTG-3′) to produce an amplicon of 369 bp (Fig. 1).

In order to analyse potential changes in the sequences of outer coat proteins of YUOV (VP3, Seg-3; VP5, Seg-6) after passage in mice, partial sequences of VP3 and VP5 were PCR-amplified. The PCR primers were YUOVSeg3S (positions 774–799; 5′-CGTGATGGAG-AAGAGTGATCCAACTG-3′) and YUOVSeg3R (positions 1378–1353; 5′-TTCTACGTTCATCCGTGACTTTATGG-3′), producing an amplicon of 605 bp, and primers YUOVSeg6S (positions 255–278; 5′-GCAGTGATTGGTAATGTTCTGAAC-3′) and YUOVSeg6R (positions 1014–998; 5′-CAGCTGCCATCACGTCATTCAAATGC-3′), producing an amplicon of 760 bp. Amplicons were cloned into the pGEM-T cloning vector (Promega) and sequenced as described above.

RESULTS

Virus propagation, purification and electron microscopy

During the third passage, the C6/36 cells assumed a fusiform morphology and detached from the culture surface. Agarose-gel electrophoresis of RNA extracts showed a segmented profile of dsRNA containing more than 15 bands, suggesting infection by several different viruses of the family *Reoviridae*. Therefore, the culture supernatant was plaque-purified and used to isolate a clone of *Banna virus* (a human virus belonging to the genus *Seadornavirus*; Attoui *et al.*, 2000b) and a clone of a 10-segmented virus that was plaque-purified twice more and used for experimental analysis.

Virus particles, purified on CsCl, showed a defined surface structure, with ring-shaped capsomeres that were characteristic of orbivirus core particles (Mertens *et al.*, 2000, 2005). They had a small size with an estimated mean diameter of 55 nm, suggesting that they had lost outer capsid components (Fig. 2). Although these size estimates are smaller than those of BTV core particles as determined by X-ray crystallography (Grimes *et al.*, 1998), the particle dimensions observed are comparable to those of BTV and SCRV prepared by similar techniques. This virus was designated *Yunnan orbivirus* (YUOV).

Sequence analysis and taxonomic assignment

Complete sequencing of the genome confirmed the presence of 10 segments within the YUOV genome (Fig. 3) that were cloned and sequenced; the sequences were deposited in GenBank under accession numbers AY701509–AY701518. The length of the segments and their corresponding encoded proteins are given in Table 1. Analysis of the 5′ and 3′ non-coding regions (NCRs) showed that all of the segments share five conserved nucleotides at their 5′ ends and three conserved nucleotides at their 3′ ends (5′-GUUAA-------UAC-3′; Table 1). Moreover, the first and last 2 nt of all of the segments are inverted complements and are identical to those found in other orbiviruses.

The sequence comparison showed that the proteins encoded by YUOV genome segments matched proteins of viruses belonging to the family *Reoviridae*. The highest amino acid identities were with proteins of other orbiviruses. All of the proteins of YUOV showed significant identities with their homologues encoded by genome segments of insect-borne BTV and tick-borne SCRV (Table 2).

Fig. 4 shows a neighbour-joining tree of the amino acid sequence of the YUOV RdRp aligned with all of the RdRp sequences available for different orbiviruses, as well as representative members of other genera within the family *Reoviridae*. Trees with similar topologies were obtained when using the Poisson correction or the gamma distance.

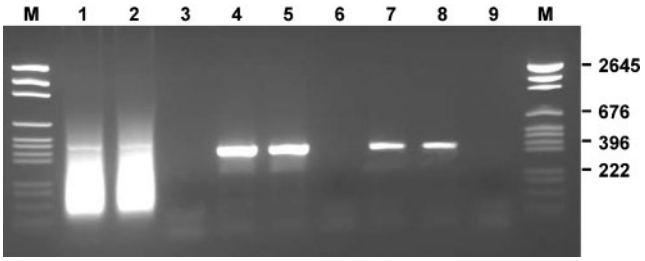

Fig. 1. RT-PCR on YUOV-infected mouse blood using segment 7-specific primers. Lane M, size marker labelled in bp; lanes 1 and 2, first-round PCR on infected mice 1 and 2 (blood recovered at day 3 post-infection); lane 3, first-round PCR on uninfected mouse (blood recovered at day 3); lanes 4 and 5, second-round PCR on infected mice 1 and 2 (blood recovered at day 3 post-infection); lane 6, second-round PCR on uninfected mouse (blood recovered at day 3); lanes 7 and 8, second-round PCR on infected mice 1 and 2 (blood recovered at day 5 post-infection); lane 9, second-round PCR on uninfected mouse (blood recovered at day 5).

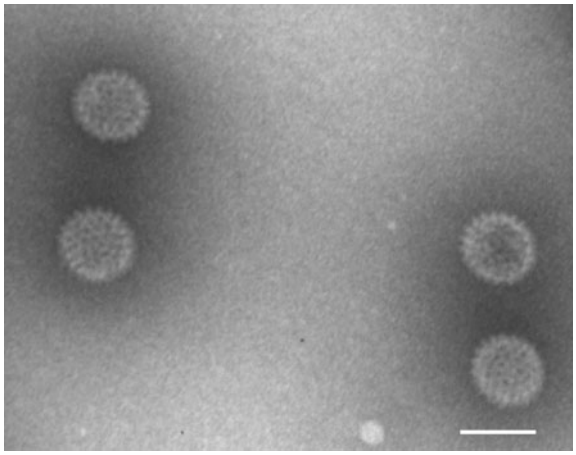

Fig. 2. Electron micrograph of YUOV core particles negatively stained with 2% potassium phosphotungstate. The virus was purified on a caesium chloride gradient. One particle shown was more permeable to the stain. Bar, 50 nm.

Fig. 3. Agarose-gel electrophoretic profile of the YUOV genome. The dsRNA was run on a 1·2 % agarose gel containing 0·5 μg ethidium bromide ml^{-1} in TAE buffer. The genome was separated into nine distinct bands; the fourth band from the top was found to contain segments 4 and 5.

Between 36 and 47 % amino acid sequence identity was detected in VP1 (Pol) between YUOV and the other orbiviruses. In a previous study (Attoui *et al.*, 2002), we reported that the polymerase sequences of viruses belonging to a single genus within the family *Reoviridae* have identity values of >30 %.

The data presented here on the number of segments, the terminal nucleotides and the sequence relatedness to orbivirus proteins, therefore, confirm that YUOV belongs to the genus *Orbivirus*.

Within the genus *Orbivirus*, amino acid identity detected between the VP1 (Pol) sequences from the insect-borne species AHSV, BTV and PALV are 55–64 % and those between these viruses and the tick-borne SCRV are ~35 %. The VP1 (Pol) of YUOV is approximately 47 % identical to insect-borne orbiviruses and 36 % identical to SCRV. Accordingly, YUOV is related only distantly to both the insect- and the tick-transmitted species of the genus *Orbivirus* described to date and probably represents a new species of this genus.

Analysis of VP2 of YUOV showed it to be the 'T2' protein, which forms the subcore shell of the orbivirus capsid [VP2 (T2) of BRDV (Moss & Nuttall, 1994), VP2 (T2) of SCRV (Attoui *et al.*, 2001) and VP3 (T2) of BTV (Grimes *et al.*, 1998)]. As a consequence of its important functional role in virus protein–RNA structure and assembly, the T2 protein is highly conserved (Grimes *et al.*, 1998; Gouet *et al.*, 1999), exhibiting very high levels of sequence identity. T2 amino acid identity within a single species of the genus *Orbivirus* (serogroup) is >91 % and this value can be used for delineation of species (Attoui *et al.*, 2001). The level of amino acid identity that was detected in the 'T2' protein between YUOV and the other orbiviruses ranges between 24 and 52 %, showing that YUOV is a member of a new species within the genus *Orbivirus*.

Replication of YUOV in insect cells, mammalian cells and mice

Virus replication in mosquito and mammalian cells was tested. In the *A. albopictus* mosquito cells C6/36 and AA23, virus replication was demonstrated by detection of the segmented genome of the virus in agarose gels and by

Table 1. Lengths of dsRNA segments 1–10, encoded putative proteins and 5′ and 3′ non-coding regions (NCRs) of YUOV

Highly conserved terminal sequences are shown in upper-case letters. In consensus sequences, R represents A/G, V represents A/G/C, W represents A/U and N represents A/U/G/C. Mass, calculated theoretical molecular mass.

Segment	Length (bp)	Protein		5′ NCR		3′ NCR	
		Length	Mass (Da)	Length (bp)	Terminal sequences	Terminal sequences	Length (bp)
1	3993	1315	150 971	13	5′-GUUAAAU---	---AAGUAC-3′	32
2	2900	940	107 049	11	5′-GUUAAAA---	---CGAUAC-3′	66
3	2688	873	100 164	18	5′-GUUAAAA---	---AGAUAC-3′	48
4	1993	645	74 180	7	5′-GUUAAAA---	---AAGUAC-3′	48
5	1957	574	66 820	30	5′-GUUAAAA---	---UGAUAC-3′	202
6	1683	535	58 950	41	5′-GUUAAAA---	---AGAUAC-3′	34
7	1504	435	48 143	29	5′-GUUAAAA---	---AGAUAC-3′	167
8	1191	355	39 159	19	5′-GUUAAAA---	---GCAUAC-3′	104
9	1082	338	37 193	17	5′-GUUAAAA---	---CGGUAC-3′	48
10	825	253	28 438	15	5′-GUUAAAA---	---CGGUAC-3′	48
				Consensus	**5′-GUUAAAw---**	**---nvrUAC-3′**	

Table 2. Correspondence between YUOV and other orbiviruses

YUOV	BTV-10 (amino acid identity)	SCRV (amino acid identity)	Putative function of YUOV protein*
S1, VP1 (Pol)	S1, VP1 (Pol) (47 %)	S1, VP1 (Pol) (36 %)	RNA-dependent RNA polymerase
S2, VP2 (T2)	S3, VP3 (T2) (38 %)	S2, VP2 (T2) (24 %)	Major subcore protein (equivalent VP3, BTV)
S3, VP3	S2, VP2 (29 %)	S3, VP3 (28 %)	Similar to outer shell protein VP2 of BTV
S4, VP4 (CaP)	S4, VP4 (CaP) (41 %)	S4, VP4 (CaP) (36 %)	Minor core and capping enzyme
S5, NS1 (TuP)	S5, NS1 (TuP) (23 %)	S6, NS1 (TuP) (22 %)	Tubules
S6, VP5	S6, VP5 (31 %)	S5, VP5 (34 %)	Outer capsid protein
S7, NS2 (ViP)	S8, NS2 (ViP) (33 %)	S7, NS2 (ViP) (15 %)	Viral inclusion bodies
S8, VP7 (T13)	S7, VP7 (T13) (20 %)	S8, VP7 (T13) (25 %)	Major core surface protein
S9, VP6 (Hel)	S9, VP6 (Hel) (26 %)	S9, VP6 (Hel) (26 %)	Minor core protein, helicase
S10, NS3	S10, NS3 (24 %)	S10, NS3 (15 %)	Virus release

*Putative functions of YUOV proteins by comparison to the already established functions of BTV. The functions and abbreviations (shown in parentheses) used to indicate these roles are from Mertens *et al.* (2000).

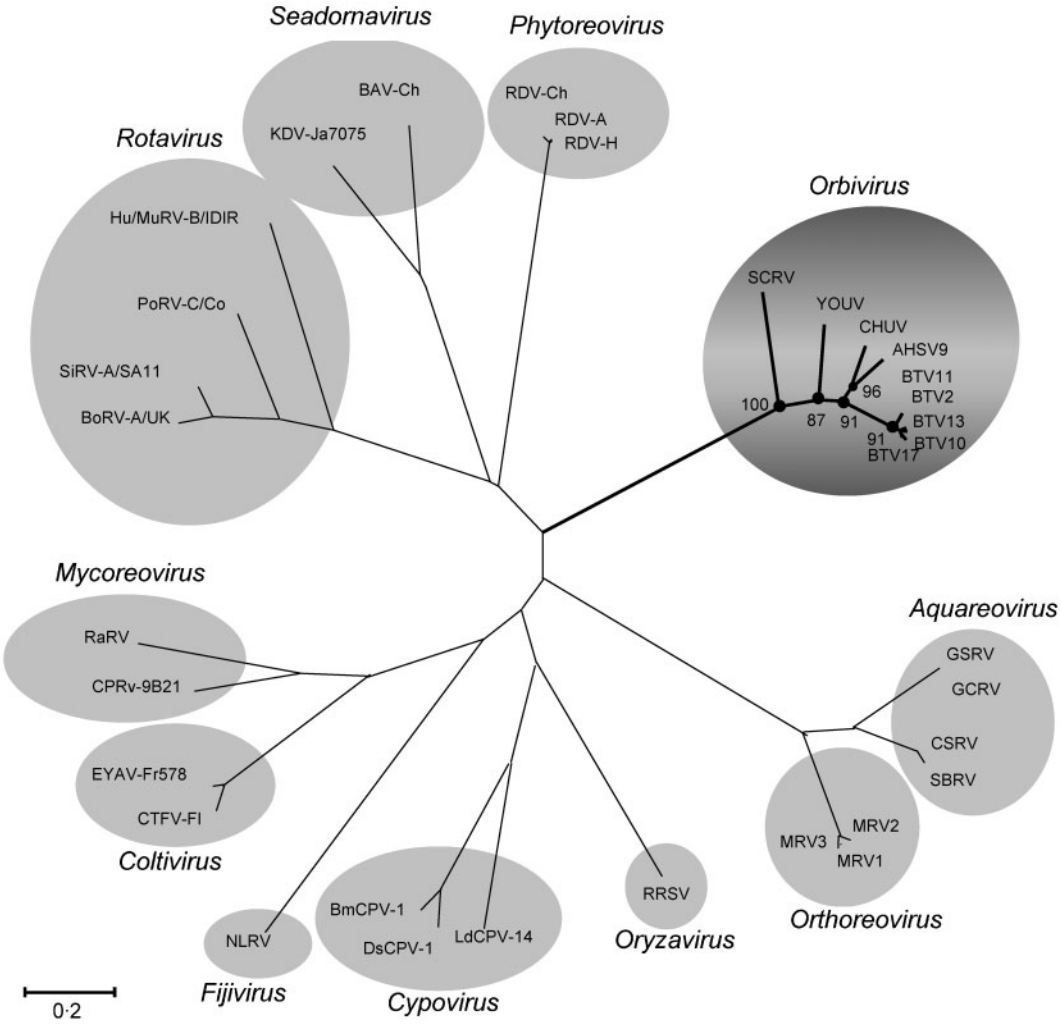

Fig. 4. Phylogenetic comparison of the viral polymerase VP1 (Pol) proteins of YUOV, other orbivirus species and members of other genera within the family *Reoviridae*. The analysis (presented as a radial tree) was constructed by using the MEGA3 program, using the p-distance algorithm. The cluster of orbiviruses is presented at the upper right of the tree and bootstrap values >85 % support the branching within this cluster. GenBank accession numbers and further details of the sequences and viruses used are given in Supplementary Table S1, available in JGV Online.

RT-PCR, which generated amplicons identical to the original sequence. It did not replicate in any of the A w-albus, A20 or AE cells or in any of the mammalian cell lines tested: agarose-gel electrophoresis and RT-PCR failed to show the presence of virus genome.

In mice injected intraperitoneally with YUOV, virus genome was detected in blood by 3 days post-infection until day 5. At day 15 post-infection, the serum of the mouse was tested for the presence of antibodies by Western blot. A 1/100 dilution could detect YUOV proteins in infected C6/36 cells, without any non-specific reactivity against non-infected cells (data not shown). These anti-YUOV antibodies were shown to seroneutralize virus infection: (i) inhibition of virus replication in C6/36 cells was observed in an antibody concentration-dependent manner and (ii) a second injection of the virus into immunized mice failed to produce a productive infection.

Analysis of PCR products from infected mouse blood at day 5 post-infection was performed. Amplicons from the NS2 protein gene (Seg-7), VP3 (Seg-3) and VP5 (Seg-6) were cloned and sequenced. A total of 35 clones from each PCR were analysed (approx. 14 000 nt per segment). Assuming that the error rate of *Taq* polymerase ranges between $0{\cdot}27 \times 10^{-4}$ and $0{\cdot}4 \times 10^{-4}$ per nucleotide per cycle (Smith *et al.*, 1997), it was expected that, in 40 cycles of amplification, 15–22 errors could occur in every 14 000 nt sequenced.

In VP3, 12 single changes were identified in 12 separate clones, whilst six changes were identified in six separate clones of VP5 and 10 changes were identified in 10 separate NS2 clones. The number of changes detected in each of the PCR products from segments 3, 6 and 7 of YUOV could hence be compatible with errors of *Taq* polymerase. Moreover, for all of the segments studied, these single mutations seemed to be distributed randomly along the sequence, with no identified 'hot spot'. The alignments of the PCR products are shown in Supplementary Figs S1, S2 and S3, available in JGV Online. Similar data were obtained from virus grown only in cell culture. Accordingly, these nucleotide changes do not reflect changes due to the viral RdRp in the nucleotide sequences of the tested segments and this result is not evocative of the existence of a quasispecies in the viral population infecting mice at day 5 post-infection.

DISCUSSION

The genus *Orbivirus* contains insect-borne viruses, tick-borne viruses and viruses with no known vectors (no genetic data are currently available for the latter viruses). The insect-borne orbiviruses have been studied intensively and full-length genome sequences have been determined for the major veterinary pathogens BTV, AHSV and EHDV (Roy & Mertens, 1999). Two species of tick-borne orbivirus have been sequenced to date: BRDV (partial sequence; Moss *et al.*, 1992) and SCRV (complete sequence; Attoui *et al.*, 2001).

Many arguments suggest the classification of YUOV in the family *Reoviridae*. These include the size and morphology of the virion particles, the segmented nature of the dsRNA genome, the genetic relatedness of the YUOV polymerase to those of other members of the family *Reoviridae* and the identification in this protein of the signature motifs of RdRps of viruses belonging to this family.

The full-length genome characterization of YUOV has facilitated the analysis of its genetic relationships to previously reported members of the genus *Orbivirus*. First, the viral genome is made of 10 segments with conserved terminal sequences similar to those of other orbiviruses. However, in many orbiviruses, such as BTV, AHSV, *Palyam virus* and *Equine encephalosis virus*, the six terminal nucleotides at the 3′ end are conserved among the 10 segments (Mertens *et al.*, 2005). This is not the case for YUOV, which shows only three conserved nucleotides at the 3′ end. The analysis of these conserved ends is one of the species-defining parameters of the orbiviruses (Mertens *et al.*, 2005). Second, all YUOV proteins have a significant identity to proteins of other orbiviruses. In particular, the amino acid identity observed in the polymerase confirms the status of YUOV as an orbivirus according to previously defined criteria (amino acid identity >30 % with all characterized orbiviruses). Third, the sequence of YUOV VP2 (T2) protein clearly identifies the virus as a distinct species: amino acid identity to those of previously characterized orbiviruses is significantly lower (24–38 %) than the threshold value proposed (91 %; Attoui *et al.*, 2001). Previously, YUOV has been classified as a tentative species within the genus *Orbivirus*. The present study shows that it should be considered as a new and distinct species within this genus.

The proteins involved in determination of serotype and serogroup in the studied insect-borne orbiviruses are VP2 (outer coat) and VP7 (T13), respectively. In YUOV, sequence comparison showed that these proteins are VP3 and VP7 (T13), respectively. VP7 (T13) of YUOV exhibits a low amino acid identity to its homologues in insect-borne orbiviruses (e.g. 20 % to BTV VP7). This indicates that YUOV belongs to a distinct serogroup and confirms that it should be classified as a new and distinct species. VP2 of insect-borne orbiviruses is the protein involved in cell attachment and serum neutralization and it is highly variable in a manner that correlates with virus serotype (Mertens *et al.*, 2000, 2005). It is therefore not surprising that the corresponding YUOV VP3 has a low level of similarity to homologous orbivirus outer coat proteins (e.g. 29 % to BTV VP2).

YUOV was isolated from the mosquito *C. tritaeniorhynchus*, a known vector of *Japanese encephalitis virus* in Asia (Das *et al.*, 2004). Virus replication was shown to be restricted to a small number of mosquito cell lines. Among those tested here, only *A. albopictus* cell lines supported virus replication. In addition, none of the mammalian cell lines tested supported virus replication. This is a different situation from that known for the well-studied arboviruses BTV,

AHSV and EHDV. However, the virus replicated efficiently when it was injected into mice, resulting in a non-lethal infection that elicited protective antiserum. Accordingly, it is not known whether this virus has the potential to infect natural vertebrate hosts and complete a host–vector arboviral cycle. Further investigations (including prospective serological studies) are required to clarify this question.

The evolutionary relationship of YUOV to 11 other species of the genus *Orbivirus* was investigated based on analysis of the T2 protein. YUOV is found within a group containing two insect-borne viruses (*Wongorr virus* and *Corriparta virus*) and one tick-borne virus (BRDV). It is noteworthy that in tick-borne orbiviruses, such as BRDV and SCRV, the T2 protein is, as it is the case of YUOV, encoded by genome segment 2 (VP2). The more distantly related insect-borne viruses, such as BTV, AHSV, EHDV, *Wallal virus*, *Eubenangee virus*, *Warrego virus* and *Palyam virus*, have their T2 encoded by genome segment 3 (VP3). The genome segment encoding T2 of *Corriparta virus* and *Wongorr virus* is segment 2 (VP2) (Parkes & Gould, 1996). This is an interesting finding, showing that those viruses with T2 proteins encoded by segment 2 form a separate cluster (Fig. 5) that is supported by bootstrap values of $>85\,\%$. Similar trees were obtained with the Poisson correction and the gamma distance. The position of SCRV in the T2 tree is also of interest. The branch of SCRV was found to dissect the phylogenetic tree and forms a separate cluster. SCRV was

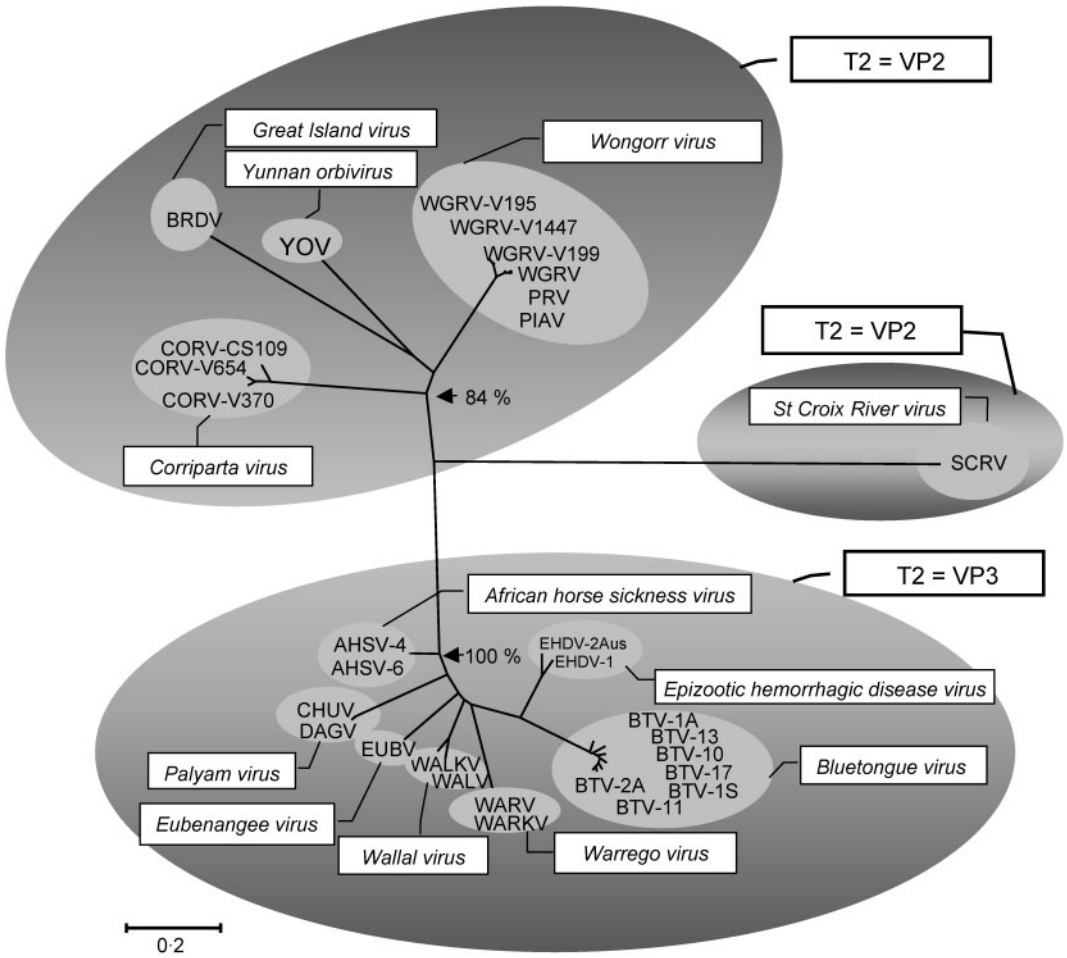

Fig. 5. Neighbour-joining phylogenetic tree (using the Poisson correction algorithm or the gamma distribution) of the T2 proteins (the major component of the subcore shell) of YUOV and other orbivirus species. This protein is equivalent to the VP3 (T2) protein of BTV, the prototype species of the genus *Orbivirus*, and to the VP2 (T2) of two tick-borne orbiviruses, St Croix River virus (SCRV) and Broadhaven virus (BRDV). Many of the available sequences are incomplete; therefore, the analysis (presented as a radial tree) is based on partial sequences (aa 393–548 relative to the BTV-10 sequence; GenBank accession no. P12435). Two clusters that are supported by bootstrap values $>80\,\%$ are identified: the cluster of viruses with the T2 protein encoded by segment 2 (VP2) and the cluster of viruses with the T2 protein encoded by segment 3 (VP3). SCRV dissects the tree and forms a distinct phylogenetic group. GenBank accession numbers and further details of the sequences and viruses used are included in Supplementary Table S2, available in JGV Online.

found to be the most divergent orbivirus from insect-borne and other tick-borne orbiviruses. Its position within the T2 and polymerase trees reflects this divergent and ancient character. A taxonomic proposal has been made to the International Committee on Taxonomy of Viruses (ICTV) to recognize *Yunnan orbivirus* as a new species within the genus *Orbivirus*.

Sequence variation in RNA viruses can result from the error-prone nature of RdRps and selective pressure, resulting in adaptation and evolution. The generally observed mean mutation rates of viral RNA genomes range between 10^{-4} and 10^{-5} mutations per nucleotide per round of RNA replication (although rates between 10^{-3} and 10^{-6} mutations per nucleotide per round of RNA replication have been reported; Pugachev *et al.*, 2004). The high mutation frequencies of most RNA viruses might lead to the generation of genetically heterogeneous populations, which may reflect a virus quasispecies. One of the most obvious examples is the case of *Hepatitis C virus* , where the mutation rate was estimated to be as high as 8×10^{-2} mutations per nucleotide per round of RNA replication within the hypervariable region of the genome (Herring *et al.*, 2005). The sequence variation of YUOV was investigated in infected mice. A clonal virus obtained by plaque purification (and in which genetic homogeneity was controlled by RT-PCR and cloning) was injected into mice and possible sequence variation was investigated 5 days after infection. Sequence analysis of amplicons obtained from mouse blood showed that changes in the nucleotide sequences of segments 3, 6 and 7 were compatible with misincorporations during PCR amplification by *Taq* polymerase. This suggests that infection was not followed by the emergence of new variants or of a quasispecies. Whether this low variability is a consequence of the characteristics of the viral polymerase or of the absence of an efficient selective pressure is not known. Further understanding of the determinants of dsRNA virus evolution is hampered by the paucity of similar available information. New models are required to better address this question.

Finally, the sequence characterization of YUOV has revealed its genetic relationship to other sequenced orbiviruses. In particular, analysis of the sequence of VP2 (T2) (the protein that correlates with serogroup or species) and VP1 (Pol) has formed the basis of its identification as a new species of the genus *Orbivirus*. The availability of the complete genome sequence will facilitate the development of sequence-specific PCR assays for the study of YUOV epidemiology in the field.

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