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Peruvian horse sickness virus and Yunnan orbivirus, isolated from vertebrates and mosquitoes in Peru and Australia

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ABSTRACT

During 1997, two new viruses were isolated from outbreaks of disease that occurred in horses, donkeys, cattle and sheep in Peru. Genome characterization showed that the virus isolated from horses (with neurological disorders, 78% fatality) belongs to a new species the *Peruvian horse sickness virus* (PHSV), within the genus *Orbivirus*, family *Reoviridae*. This represents the first isolation of PHSV, which was subsequently also isolated during 1999, from diseased horses in the Northern Territory of Australia (Elsey virus, ELSV). Serological and molecular studies showed that PHSV and ELSV are very similar in the serotype-determining protein (99%, same serotype). The second virus (Rioja virus, RIOV) was associated with neurological signs in donkeys, cattle, sheep and dogs and was shown to be a member of the species *Yunnan orbivirus* (YUOV). RIOV and YUOV are also almost identical (97% amino acid identity) in the serotype-determining protein. YUOV was originally isolated from mosquitoes in China.

Introduction

The genus *Orbivirus*, which is 1 of 15 genera within the family *Reoviridae*, contains 22 distinct virus species recognized by the International Committee for the Taxonomy of Viruses (ICTV). Like the other orbiviruses, the "type" species of the genus, *bluetongue virus*, contains a genome composed of ten segments of linear dsRNA. The orbiviruses replicate in and are transmitted between their vertebrate hosts by blood-feeding anopheline or culicine mosquitoes, *Culicoides* midges, ticks or phlebotomine flies. They are therefore recognized as arboviruses.

Bluetongue virus (BTV), African horse sickness virus (AHSV) and epizootic hemorrhagic disease virus (EHDV) are all transmitted by adult *Culicoides* and are regarded as the economically most important orbiviruses (Mertens, 1999; Mertens et al. 2005). Bluetongue virus has been studied in greater detail than the other orbiviruses, providing a paradigm for their structure, protein function and replication (Grimes et al. 1998; Mertens, 2004). Although sequence data are available for several of the insect-borne orbiviruses, members of only two of the tick-borne orbivirus species have been analyzed: Broadhaven virus (BRDV) (*Great Island virus* species; Moss et al., 1992) and St. Croix River virus (*St Croix River virus* species; Attoui et al. 2001). Comparisons of sequences from homologous proteins have revealed greater genetic divergence between insect-borne and tick-borne orbiviruses (with 23–38% amino acid (aa) identity) than within either of these groups (Attoui et al. 2001).

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Outbreaks of bluetongue disease can affect sheep, some species of deer and to a lesser extent cattle and goats, with case-fatality rates in naive sheep populations that can sometimes exceed 70% (Gambles, 1949; Elbers et al. 2008). There are 24 distinct serotypes of BTV (with a putative 25th serotype; Hofmann et al. 2008), the identity of which is primarily determined by outer capsid proteins VP2 and to a lesser extent by VP5 (Maan et al. 2007a, 2007b; Singh et al. 2005). Certain other orbiviruses (including AHSV and the equine encephalosis viruses (EEV)), are responsible for equine diseases that can cause very high levels of mortality in horses (AHS fatality rates can exceed 90%; Mellor and Hamblin, 2004). African horse sickness was first recognized as early as 1780, during the early days of the European colonization and importation of horses into southern Africa, resulting in epizootics with high levels of mortality in infected animals. AHSV is usually only considered to be enzootic in sub-Saharan Africa, although historically major epizootics have also occurred in the Middle East, the Indian sub-Continent, North Africa and the Iberian Peninsula. There are nine recognized AHSV serotypes (AHSV-1 to 9), the identity of which is determined by outer capsid proteins VP2 and VP5 (particularly VP2) (Bremer et al. 1990).

Equine encephalosis virus was first identified in 1967, associated with horses that had died from a previously unrecognized peracute illness in southern Africa (Viljoen and Huismans, 1989). Seven different EEV serotypes were subsequently identified in southern Africa, frequently associated with subclinical infection of horses (Howell et al. 2008). Serological investigations during the summer of 1967 showed that antibodies to (EEV) were widespread in South Africa but had not occurred to any appreciable extent in the preceding 10 years. In some of the years after 1967, the disease took on epidemic proportions, corresponding with the seasonal abundance of adult *Culicoides*. Outbreaks of EEV infection have been associated with abortions during the first 5 to 6 months of gestation, and it has been suggested that infection could be misdiagnosed as infertility (Viljoen and Huismans, 1989).

Epizootic and epidemic outbreaks of equine encephalitis, with various degrees of neurological severity, have also been reported in the Americas. These were initially attributed to infections by alphaviruses (*Togaviridae*), including eastern equine encephalitis (EEEV), western equine encephalitis (WEEV) and particularly Venezuelan equine encephalitis (VEEV) (Griffin, 1986; 2001). In 1984, approximately 3000 horses were affected (30 of which died) in the Department of San Martin, in the subtropical upper jungle of Peru. This was the first time that an outbreak of equine disease had occurred in the region, with clinical signs of neurological disease that are compatible with alphavirus infections. However, attempts to isolate an alphavirus were unsuccessful (Méndez et al. 1982; Scherer et al. 1979). In subsequent years (1985 and 1986), 19 horses died in the same region. Again, the virus isolates that were obtained from the dead animals did not match any known alphaviruses. In 1997, a more severe epizootic, although with similar clinical signs, took place in the Department of San Martin. Out of about 8000 animals surveyed, 132 horses showed neurological symptoms and 104 of them died as a consequence.

This paper describes the characterization of two dsRNA viruses isolated from fatalities in equids (particularly horses), cattle, sheep and dogs, which were also detected in wild-caught mosquitoes in San Martin. One of these viruses, isolated during 1997 from equids that died as a consequence of neurological syndrome, was named "Peruvian horse sickness virus" (PHSV). Sequencing and phylogenetic analysis of the full genome of this PHSV isolate indicate that it represents a new *Orbivirus* species (now recognized by ICTV), which is also named as *Peruvian horse sickness virus* (PHSV).

During April and May 1999, two horses, from different locations in the Northern Territory of Australia (Katherine and Elsey Station, Mataranka), showed signs of neurologic disease. The animals were euthanized on humanitarian grounds within 72 h of the onset of illness. Elsey virus (ELSV), which was subsequently isolated from affected horses at both sites, was characterized and its RNA sequenced. ELSV was shown to be almost identical to the Peruvian isolate of PHSV (97–100% identity in individual genes), indicating that they both belong to PHSV serotype 1 (PHSV-1).

The second virus from San Martin, which also has a 10 segmented dsRNA genome, was isolated in 1997 from diseased cattle and sheep and a dog and named Rioja virus (RIOV). The genome segments of RIOV and PHSV gave a different migration pattern (electropherotype) after agarose gel electrophoresis, indicating that they belong to different virus species, as defined by species demarcation criteria set by ICTV (Mertens et al. 2005). However, the RIOV electropherotype is similar to that of Yunnan orbivirus (YUOV), which was isolated from wild-caught mosquitoes collected in Yunnan Province, China, during 1997 (Attoui et al. 2005a). Sequence analyses of RIOV confirmed that it belongs to Yunnan orbivirus serotype 1 (YUOV-1).

Results

A total of 43 orbivirus isolates were obtained during the disease outbreaks in San Martin during 1997: 37 of which were from horses, 1 from a donkey, 2 from cattle, 1 from a sheep, 1 from a dog and 1 from a pool of mosquitoes (Tables 1 and 2).

Electropherotyping of RNA of viruses from the Department of San Martin, Peru

The dsRNA genome segments of viruses isolated in San Martin during 1997 were analysed by agarose gel electrophoresis (AGE), showing two distinct migration patterns (electropherotypes) (Fig. 1). Electropherotype (AGE) is one of the parameters recognized by ICTV for identification of individual virus species within the family *Reoviridae* (Mertens et al. 2005). One of the two patterns observed was obtained from isolates subsequently identified as "Peruvian horse sickness virus" (PHSV).

A second electropherotype was obtained from Rioja virus (RIOV) (isolated from sick cattle in Rioja), which is clearly distinct from PHSV. The second electropherotype was also obtained from multiple further isolates from *Ochlerotatus scapularis* mosquitoes and from cattle, sheep, a dog and a donkey showing clinical signs of disease. The second electropherotype was also identical to that obtained from viruses belonging to the *Yunnan orbivirus* species, previously isolated from mosquitoes from both urban and rural cattle-raising areas in China (Fig. 1; Attoui et al. 2005a). These data indicate that RIOV and YUOV belong to the same *Orbivirus* species.

Cases and virus isolation

Peru

Peruvian horse sickness (PHS; caused by PHSV) was initially recognized in the Department of San Martin after the unexpected deaths of horses during the rainy season. This coincided with the coffee harvest, when equids are used as means of transportation. Between January and July 1997, 104 horses died with a mortality rate of ~1.25% and a case-fatality rate of ~78% (there are ~8000 horses in the Department of San Martin; see Table 5). The epizootic affected eight provinces of the Department, lasting 7 months and reaching its peak during March to April of 1997, which coincided with periods of heavy rain.

During the 1997 outbreak, seven equids, one dog, two cows and one sheep showed signs of neurological disease. The virus isolates from blood and/or brain of the sick (neurological disease) cattle, donkey and sheep or from mosquitoes all had a similar electropherotype to members of the species YUOV and were therefore identified as RIOV. One isolate was made from the spleen of one of the sick horses (Table 1) and had an identical electropherotype to that of

Table 1
Isolation and serology results during 1997 and 2002 in San Martin (Peru) and during 1999 in Northern territories (Australia).

Case code	Date	Species	Origin		Isolation (on C6/36) from		Serology			Neurological signs	Virus species
			Locality	Province	Blood	Brain or spleen	IgG	IgM	VNT (titer)		
CH011 (Peru)	03/02/97	Horse	Chambira	M. Cáceres	+	N	+	N	ND	+	PHSV
J014 (Peru)	04/03/97	Horse	Jepelacio	Moyobamba	–	+ (Brain)	–	–	ND	+	PHSV
PM015 (Peru)	07/03/97	Horse	Pardo Miguel	Rioja	–	+ (Brain)	–	–	ND	+	PHSV
4056 (Peru)	03/03/97	Horse	Campanilla	M. Cáceres	+	+ (Brain)	+	N	ND	+	PHSV
4561 (Peru)	03/03/97	Horse	Juan Guerra	San Martín	N	–	+	+	ND	–	PHSV
4562 (Peru)	03/03/97	Horse	Juan Guerra	San Martín	N	–	+	+	ND	–	PHSV
4566 (Peru)	03/03/97	Horse	Juan Guerra	San Martín	N	–	+	+	ND	–	PHSV
4568 (Peru)	03/03/97	Horse	Juan Guerra	San Martín	N	–	+	+	ND	–	PHSV
4583 (Peru)	03/03/97	Horse	Juanjui	M. Cáceres	+	+ (Brain)	+	N	ND	+	PHSV
4584 (Peru)	03/03/97	Horse	Sisa	El Dorado	N	N	+	+	ND	+	PHSV
4598 (Peru)	03/03/97	Horse	Sisa	El Dorado	N	N	+	+	ND	+	PHSV
4655 (Peru)	03/03/97	Horse	Lamas	Lamas	N	–	+	+	ND	–	PHSV
SH002 (Peru)	15/03/97	Horse	Shucshuyacu	Moyobamba	–	+ (Brain)	–	–	ND	+	PHSV
4583 (Peru)	15/07/97	Horse	Juanjuí	M. Cáceres	+	N	+	N	ND	+	PHSV
4673 (Peru)	15/07/97	Horse	Rio Mayo	Lamas	N	+ (Brain, spleen)	+	+	ND	+	PHSV
4020 (Peru)*	11/02/97	Horse	Juanjui	Mariscal Cáceres	+	–	–	–	ND	–	PHSV
9R-2002 (Peru)*	06/06/02	Horse	Tupac Amaru	Rioja	+	–	–	–	ND	–	PHSV
07M-2002 (Peru)*	06/06/02	Horse	Santa Cruz	El Dorado	+	–	–	–	ND	–	PHSV
08D-2002 (Peru)*	06/06/02	Horse	San Isidro	El Dorado	+	–	–	–	ND	–	PHSV
14D-2002 (Peru)*	06/06/02	Horse	Shucshuyacu	Moyobamba	+	–	–	–	ND	–	PHSV
99/724 (Australia)	25/04/99	Horse	Elsey station (Mataranka)	Northern territory	+	+ (Spleen)	+	+	+ (20)	+	PHSV
99/861 (Australia)	17/05/99	Horse	Florina road (Katherine)	Northern territory	+	ND	ND	N	+ (≤10)[a]	+	PHSV
CP001 (Peru)	25/03/97	Bovine	Campanilla	M. Cáceres	+	N	ND	ND	ND	+	YUOV
4582 (Peru)	07/07/97	Ovine	Juanjuí	M. Cáceres	+	+ (Brain)	ND	ND	ND	+	YUOV
Rioja (Peru)	25/08/97	Bovine	Pardo Miguel	Rioja	+	+ (Brain)	ND	ND	ND	+	YUOV
4704 (Peru)	23/07/97	Dog	Pampa Hermoza	Yurimaguas/Ucayali[b]	N	+ (Brain)	ND	ND	ND	+	YUOV
4856 (Peru)	26/08/97	Donkey	Suyo	Ayabaca/Piura[c]	+	+ (Brain)	ND	ND	ND	+	YUOV
9712.9 (Peru)	16/03/97	Mosquitoes	Habana	Moyobamba	NA	NA	NA	NA	NA	NA	YUOV

N = negative; (–) = no sample; (*) = asymptomatic; NA = not applicable; VNT = virus neutralization test.
[a]The neutralization assay was realized using the serum from the Elsey horse.
[b]Ucayali: a neighboring department located to the east of San Martin.
[c]Piura: a neighboring department located to the west of San Martin.

PHSV. The isolates from blood and/or brains of the horses all had a different but consistent electropherotype and were identified as PHSV. Twenty-nine of the 37 viruses isolated from horses were from apparently healthy animals (from a total of 743 blood samples tested). These included isolate 4020, which was subsequently selected for full genome sequencing. Four of the PHSV isolates were made from serum samples taken from horses showing signs of neurological disease at 5–10 days post-onset. Isolates 4056 and 4583 were obtained from the brains of horses that showed neurological signs, although anti-PHSV IgM was not detected, indicating a chronic infection (Table 1). In some cases, isolates were obtained from both blood and sera of a single animal (e.g. isolates 4056 and 4583).

During surveillance studies conducted in 2002 in San Martin, four isolates of PHSV were obtained from 60 blood samples taken from healthy horses (Table 1). None of the isolates reacted with antibodies to alphaviruses (VEEV, EEEV and WEEV) or to specific orbiviruses (AHSV, Wallal, Bambari, Itupiranga or EHDV).

Table 2
Number of viral isolates from ill, dead and contact horses in San Martin in 1997.

Month	Number of ill and/or dead animals (no. of animals with one or two isolates in C6/36)	Number of contact animals* (no. of animals with isolates in C6/36)
January	–	–
February	1 (1)	98 (7)
March	12 (5)	43 (1)
April	–	–
May	–	166 (13)
June		148 (3)
July	2 (2)	184 (3)
August	–	104 (2)
Total	15 (8)	743 (29)

*Animals that were in close contact with the infected animal.

Australia

Elsey virus (ELSV) was first isolated in C6/36 cells, from the blood and spleen of the "Elsey" horse (isolate V4834) and subsequently from the spleen of a horse from Katherine. Negative contrast electron

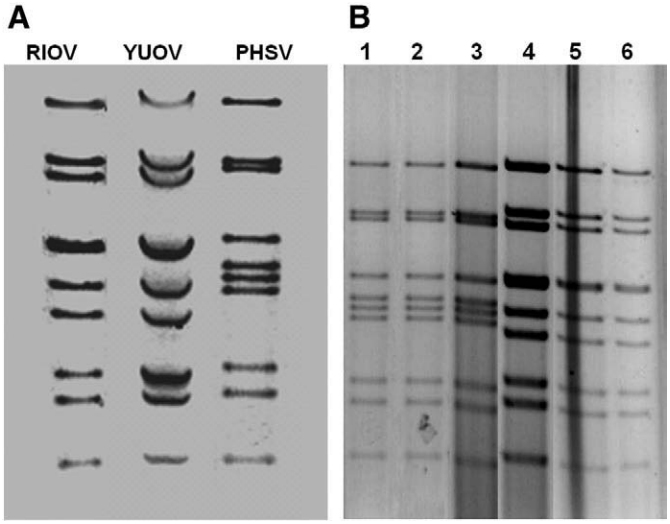

Fig. 1. Comparison of the electropherotypes of Rioja virus (RIOV), Yunnan orbivirus from China (YUOV) and Peruvian horse sickness virus (PHSV). (A) The Rioja virus and Yunnan orbivirus have identical electropherotypes (distinct from that of PHSV), which suggest that they belong to the same species. (B) Agarose gel electropherotypes of 6 isolates made in 1997 and 2002. Lane 1 represent isolate (4020-97) from horse blood (PHSV), lane 2 shows isolate (14) from horse brain (PHSV), lane 3 shows isolate (9R-2002) from horse blood (PHSV), lane 4 shows bovine blood isolate from Rioja (RIOV), lane 5 shows isolate (4856) from donkey's blood (RIOV) and lane 6 shows isolate (9712.9) from *Ochlerotatus scapularis* (RIOV). Samples 1 to 4 were obtained from outbreak in San Martin and samples 5 and 6 were obtained from outbreak in Piura.

microscopy showed icosahedral virus particles (approx 60 nm) within the cytoplasm, budding from plasma membranes, egressing from the plasma membrane and associated with cytoplasmic viral inclusion bodies (VIB) in infected cells. The ultrastructure of the VIB was similar to that described for other orbiviruses, with cores and subcores present within a fibrillar matrix. Tubules (approximately 20 nm in diameter) were also present within the cytoplasm. Orbivirus serogrouping ELISA showed no detectable cross-reactions using antisera against AHSV, BTV, CORV, EHDV, EUBV, EEV, PALV, WALV, WARV and WGRV orbiviruses.

Bloods collected since 1998 throughout the Northern Territory, from both sick and healthy horses, showed approximately 11% seroprevalence for antibodies to ELSV by IgG ELISA. Surveys of sera from kangaroos (macropods, family Macropodidae) also detected some weak positive reactions by indirect IgG ELISA, in approximately 11% of tested animals. The causative agent of kangaroo blindness (Wallal virus) has previously been identified as an orbivirus (Hooper, 1999; Hooper et al. 1999). However, Elsey virus showed no significant serological relationship to Wallal virus, as demonstrated by an absence of reactions with specific antibodies by indirect ELISA.

Sera collected in the Northern Territory of Australia from 113 fruit bats, between 1996 and 2003, were also tested for antibodies to ELSV by IgG ELISA and 39 were shown to be positive, with titers higher than those observed in horses (titers up to 160). However, no detectable ELSV-specific antibodies were found in serum samples from 102 rats collected in the Northern Territory of Australia, although a low prevalence of antibody was found in cattle and pigs.

Virus replication, electron microscopy and physicochemical properties

Blood samples and mosquito homogenates (from Peru) did not cause CPE in mammalian cell cultures. Agarose gel electrophoresis (AGE) of mammalian cell culture extracts did not show a poly-segmented dsRNA profile (characteristic of the reoviruses). However, C6/36 cells inoculated with the Peruvian or Australian virus isolates assumed a fusiform morphology, followed by clumping and detachment from the surface of the container. Extracts of the infected C6/36 cells showed a segmented dsRNA profile (by AGE), indicating the presence of a reovirus (a member of the family *Reoviridae*). Suckling mice, adult hamsters and 3-week-old chickens that had been inoculated with isolate 4020 showed no visible signs of infection. However, chickens remained viremic for 2 months after inoculation, as shown by virus isolations.

Examination of ultrathin sections of PHSV-infected C6/36 cells showed icosahedral particles in the cytoplasm, with a mean diameter of 60 nm. These particles were present individually and as para-crystalline arrays (Fig. 2A). Semi-electron-dense granular and fibrillar bodies were also present within the cytoplasm, frequently containing electron dense virus-core-like particles (Fig. 2A). Virus-like particles were frequently associated with putative intermediated filaments and were observed within vesicles (Fig. 2B). PHSV infectivity, as assayed by $TCID_{50}$ in C6/36 cells (isolate 4020, titer 10^5), was unaffected by treatment with ether or chloroform (Tesh et al., 1986), and the virus did not cause agglutination of goose, human group O, chicken or guinea pig erythrocytes, as described previously (Porterfield, 1954).

Sequence analysis and taxonomic assignment

Complete sequencing of the PHSV genome confirmed the presence of 10 dsRNA segments. These data have been deposited in the GenBank database under accession numbers DQ248057 to DQ248066 (for details, see Table 3). The length of the individual genome segments and the proteins for which they code are given in Table 3. Analysis of the 5′ and 3′ non-coding regions (NCRs) showed that all of the segments have seven conserved nucleotides at their 5′ ends but only two conserved nucleotides at their 3′ ends (+ve, 5′-GUUAAAA−−−−−AC-3′; Table 3). The first and last two nucleotides of all of the segments are inverted complements and are identical to those found in other orbiviruses.

Sequence comparisons showed that the proteins encoded by the PHSV isolates match proteins from other “reoviruses”. The highest amino acid identities detected were with proteins of the orbiviruses, with significant identities to those of YUOV and BTV (insect-borne) and with lower levels of identity to those of SCRV (tick-borne) (Table 4).

Fig. 3 shows a neighbor-joining tree for aa sequences of VP1, the RdRp of PHSV (VP1(Pol)), aligned with available RdRp sequences for other orbiviruses (Fig. 3A), as well as representative other reoviruses (Fig. 3B). Sequence identity levels of 36–69% (aa) were detected with the other orbiviruses, while the highest identity with the other reoviruses was detected with the seadornaviruses (15%). Attoui et al. (2002a) previously reported identity values of greater than 30% in the polymerase sequences of viruses belonging to a single genus within the family *Reoviridae*.

The level of aa identity detected between VP1(Pol) sequences from the insect-borne orbiviruses AHSV, BTV and PALV is 55–64%, while that between the insect-borne viruses and SCRV (tick-borne) was only ~35%. The VP1(Pol) of PHSV is ~47–69% identical to that of the other insect-borne orbiviruses (lower value corresponds to BTV and higher value corresponds to YUOV) but only 36% to the tick-borne SCRV.

As a consequence of its important functional role in virus protein/RNA structure and assembly, the T2 protein of the orbiviruses is also highly conserved (Grimes et al. 1998; Gouet et al. 1999). Sequence comparisons identified VP2 of PHSV as the subcore shell “T2” protein, with significant levels of homology to VP2 of BRDV (Moss and Nuttall,

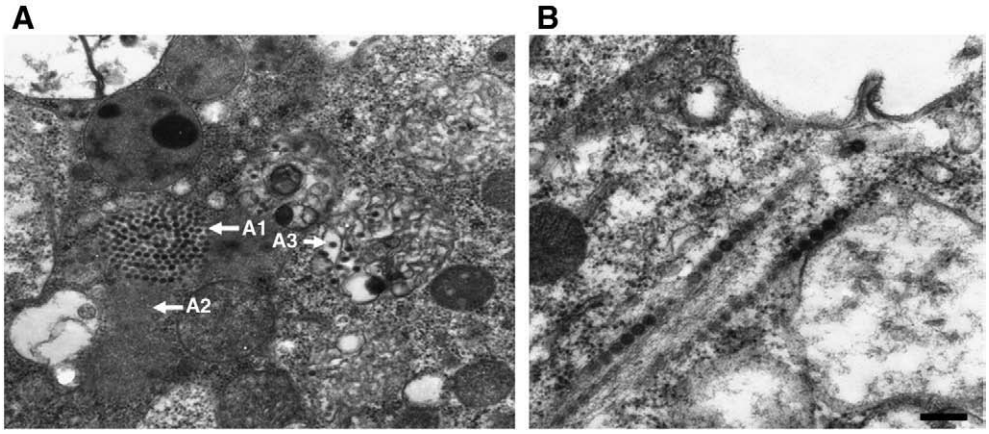

Fig. 2. Thin sections of PHSV-infected C6/36 cells. (A) Negatively stained C6/36 cells showing PHSV core particles in a para-crystalline array (A1) and adjacent to a viral inclusion body (A2). Viruses with vesicles are also in A3. Bar represents 200 nm. (B) Negatively stained C6/36 cells showing aligned viral particles associated with putative intermediate elements.

Table 3
Lengths of dsRNA segments, encoded putative proteins, 5′ and 3′ non-coding regions (NCR) of PHSV and comparison to YUOV.

	Accession number	Segment length (bp)	Protein length (aa)	Protein size (Da)	Length of 5′ NCR	Conserve termini 5′————————3′	Length of 3′ NCR
PHSV							
Segment 1	DQ248057	3987	1311	151250	14	5′-GUUAAAA————————UUAAGAUAC-3′	40
Segment 2	DQ248058	2856	925	105406	11	5′-GUUAAAA————————UUAAGGUAC-3′	70
Segment 3	DQ248059	2747	881	103797	18	5′-GUUAAAA————————UAAGGAUAC-3′	86
Segment 4	DQ248060	1996	646	74409	7	5′-GUUAAAA————————UAAAGAUAC-3′	51
Segment 5	DQ248064	1784	554	63744	30	5′-GUUAAAA————————UAAGGAUAC-3′	92
Segment 6	DQ248061	1695	529	59113	44	5′-GUUAAAA————————UAGAGAUAC-3′	64
Segment 7	DQ248065	1613	435	48190	90	5′-GUUAAAA————————UAGUGAUAC-3′	218
Segment 8	DQ248063	1180	353	39529	19	5′-GUUAAAA————————UCGUAGCAC-3′	102
Segment 9	DQ248062	1071	334	36846	16	5′-GUUAAAA————————UAAAGAUAC-3′	53
Segment 10	DQ248066	819	255	28162	12	5′-GUUAAAA————————UAAAGAUAC-3′	42
Consensus						5′-GUUAAAA————————UHRDRRYAC-3′	
ELSV							
Segment 2	FJ225396	Partial (364 bp)	Partial	NA	NA	NA	NA
Segment 3	FJ225397	Partial (606 bp)	Partial	NA	NA	NA	NA
Segment 9	FJ225398	1071	334	36846	16	5′-GUUAAAA————————UAAAGGUAC-3′	53
Segment 10	FJ225399	819	255	28098	12	5′-GUUAAAA————————UAAAGAUAC-3′	42
YUOV							
Segment 1	AY701509	3993	1315	150971	13	5′-GUUAAAU————————AAGUAC-3′	32
Segment 2	AY701510	2900	940	107049	11	5′-GUUAAAA————————CGAUAC-3′	66
Segment 3	AY701511	2688	873	100164	18	5′-GUUAAAA————————AGAUAC-3′	48
Segment 4	AY701512	1993	645	74180	7	5′-GUUAAAA————————AAGUAC-3′	48
Segment 5	AY701513	1957	574	66820	30	5′-GUUAAAA————————UGAUAC-3′	202
Segment 6	AY701514	1683	535	58950	41	5′-GUUAAAA————————AGAUAC-3′	34
Segment 7	AY701515	1504	435	48143	29	5′-GUUAAAA————————AGAUAC-3′	167
Segment 8	AY701516	1191	355	39159	19	5′-GUUAAAA————————GCAUAC-3′	104
Segment 9	AY701517	1082	338	37193	17	5′-GUUAAAA————————CGGUAC-3′	48
Segment 10	AY701518	825	253	28438	15	5′-GUUAAAA————————CGGUAC-3′	48
Consensus						5′-GUUAAAW————————NVRUAC-3′	
RIOV							
Segment 2	FJ225400	Partial (778 bp)	Partial	NA	NA	NA	NA
Segment 3	FJ225401	Partial (483 bp)	Partial	NA	NA	NA	NA
Segment 9	FJ225402	1082	338	37286	17	5′-GUUAAAA————————CGGUAC-3′	48

Highly conserved terminal sequences are shown in upper case letters. In consensus sequences, R represents A, G; Y represents C or U; H represents A, C or U; D represents A, U or G ; V represents A, G or C; W represents A or U; and N represents A, U, G or C. Mass,* calculated theoretical molecular mass; bp, base pairs; aa, amino acids; Da, Daltons; NA, not applicable (sequences are partial).

1994), VP2(T2) of SCRV (Attoui et al. 2001) and VP3(T2) of BTV (Grimes et al. 1998). The T2 aa identity within a single *Orbivirus* species (serogroup) is usually more than 91%, suggesting that value can be used to identify members of a the same virus species (Attoui et al. 2001). The level of aa identity detected in the "T2" protein between PHSV and the other orbiviruses that have been characterized ranges between 24% and 66%, identifying it as a member of a distinct species (Fig. 4).

Partial sequences of genome segments 2 and 3 and full-length sequences for segments 9 and 10 were determined for ELSV. These sequences have been deposited in GenBank under accession numbers FJ225396 to FJ225399 (Table 3). The aa identities detected between PHSV and ELSV were 100% for VP2(T2), 99% for VP3 (the larger outer coat protein), 100% for VP6(Hel) and 96% for NS3. The nucleic acid identities detected between PHSV and ELSV were 99% for segments 2, 3 and 9 and 98% for segment 10. Amino acid identities between the different isolates of PHSV from Peru were 95–99% for VP2(T2) and 97–99% for the VP3 (outer coat proteins), indicating that they all belong to a single serotype (PHSV-1).

PHSV VP3 has a low level of similarity to the equivalent outer coat proteins of other orbiviruses, which belong to other virus species and different serotypes (e.g., 29% with BTV VP2 and 27% with YUOV VP3). The phylogenetic tree constructed using the outer coat proteins (involved in serum neutralization) of different orbiviruses also

Table 4
Correspondence between segments (and their encoded proteins) of Peruvian horse sickness virus (PHSV) and other orbiviruses.

PHSV	YUOV (% aa identity)	BTV10 (% aa identity)	SCRV (% aa identity)	Putative function of PHSV proteins[a]
S1, VP1(Pol)	S1, VP1(Pol) (69%)	S1, VP1(Pol) (45%)	S1, VP1(Pol) (36%)	RNA-dependent RNA polymerase
S2, VP2(T2)	S2, VP2(T2) (66%)	S3, VP3(T2) (36%)	S2, VP2(T2) (24%)	Major subcore protein (equivalent VP3, BTV)
S3, VP3	S3, VP3 (27%)	S2, VP2 (17%)	S3, VP3 (22%)	Similar to outer shell protein VP2 of BTV
S4, VP4(CaP)	S4, VP4(CaP) (61%)	S4, VP4(Cap) (42%)	S4, VP4(Cap) (35%)	Minor core and capping enzyme
S5, NS1(TuP)	S5, NS1(TuP) (52%)	S5, NS1(Tup) (22%)	S6, NS1(Tup) (22%)	Tubules
S6, VP5	S6, VP5 (59%)	S6, VP5 (31%)	S5, VP5 (34%)	Outer capsid protein
S7, NS2(ViP)	S7, NS2(ViP) (47%)	S8, NS2(ViP) (25%)	S7, NS2(ViP) (16%)	Viral inclusion bodies
S8, VP7(T13)	S8, VP7(T13) (71%)	S7, VP7(T13) (22%)	S8, VP7(T13) (22%)	Major core surface protein
S9, VP6(Hel)	S9, VP6(Hel) (38%)	S9, VP6(Hel) (26%)	S9, VP6(Hel) (21%)	Minor core protein, Helicase
S10, NS3	S10, NS3 (45%)	S10, NS3 (28%)	S10, NS3 (18%)	Virus release

[a] The putative functions of PHSV proteins by comparison to the already established functions of BTV. The functions and abbreviations (shown in brackets) used to indicate these roles are from the *Reoviridae* section in the eight taxonomy report of the ICTV (Mertens et al. 2005).

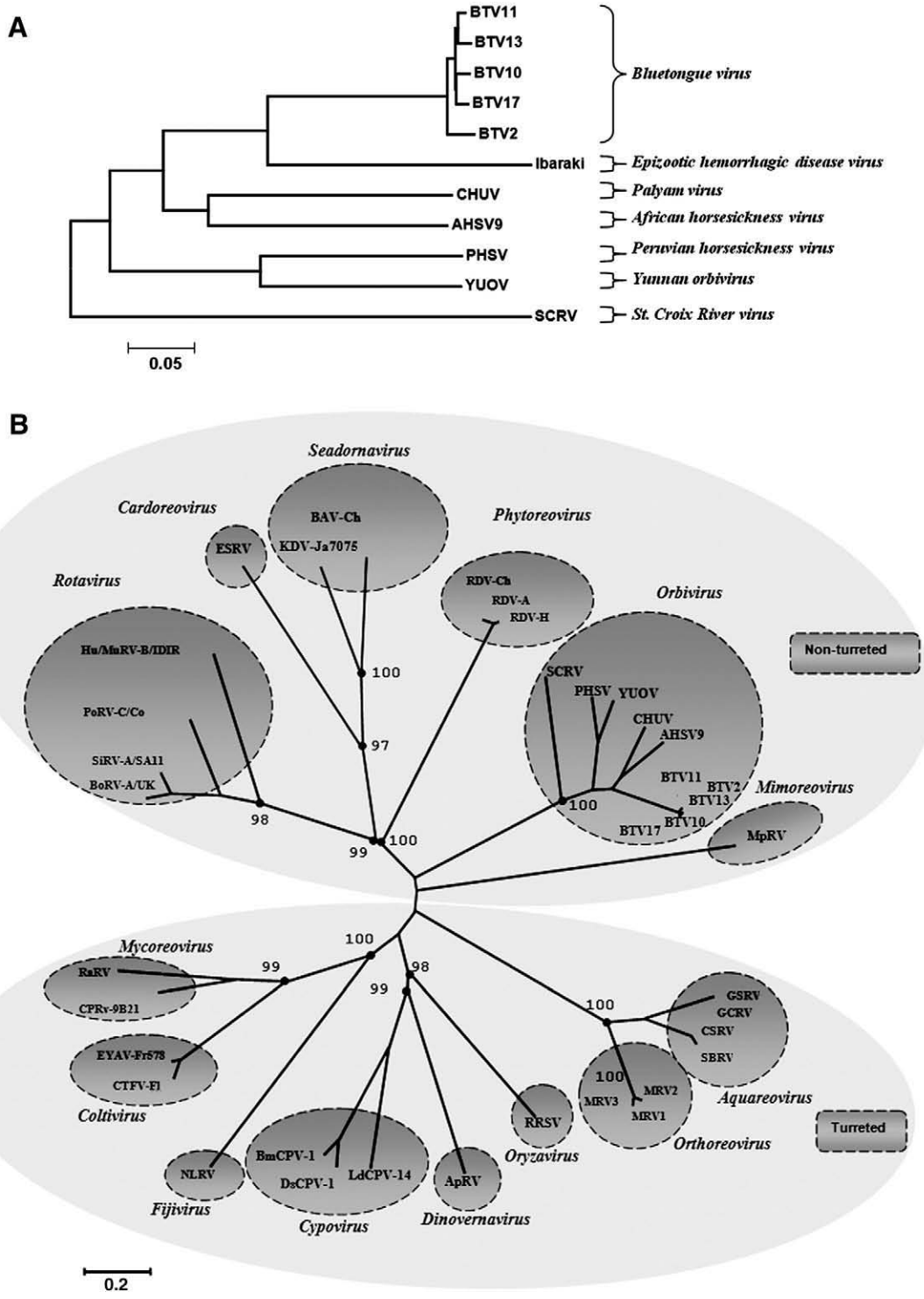

Fig. 3. Phylogenetic comparison of the viral polymerase VP1(Pol) proteins of PHSV, other *Orbivirus* species (A) and members of other genera (14 out of the 15 recognized genera) within the family *Reoviridae* (B). The tree was constructed with the help of the MEGA4 program, using the neighbor-joining method and the P-distance algorithm. Accession numbers and further detail of the sequence and viruses used are given in Supplementary Table A. Scale bars at lower left of the trees indicate the number of substitutions per site. Bootstrap values are indicated at the nodes. The names in italics indicate the genera.

showed that PHSV and the ELSV cluster together as a single serotype (Fig. 5). The VP7(T13) of PHSV exhibited a relatively low aa identity to its homologues in the *Culicoides*-borne BTV (20% with AHSV or EHDV VP7; 22% with BTV VP7; 24% with EEV or Palyam virus VP7) but a higher aa identity with the mosquito-borne YUOV (71%).

Partial sequences of genome segments 2 and 3 and full-length sequence of segment 9 were determined for RIOV from Peru. These sequences have been deposited in GenBank under accession numbers FJ225400 to FJ225402 (Table 3). Amino acid identities between YOUV and RIOV were 96% for VP2(T2), 93% for VP6(Hel) and 97% for VP3 (outer coat), again indicating that they belong to the same serotype within the same *Orbivirus* species. However, aa identity levels between middle point orbivirus (MPOV) (a YUOV isolate from the Northern Territory of Australia; Cowled et al. 2007) and RIOV were 97% in the VP2(T2) gene but only 72% within the VP3 gene. The amino acid identities between MPOV and YUOV were 98% within VP2(T2)

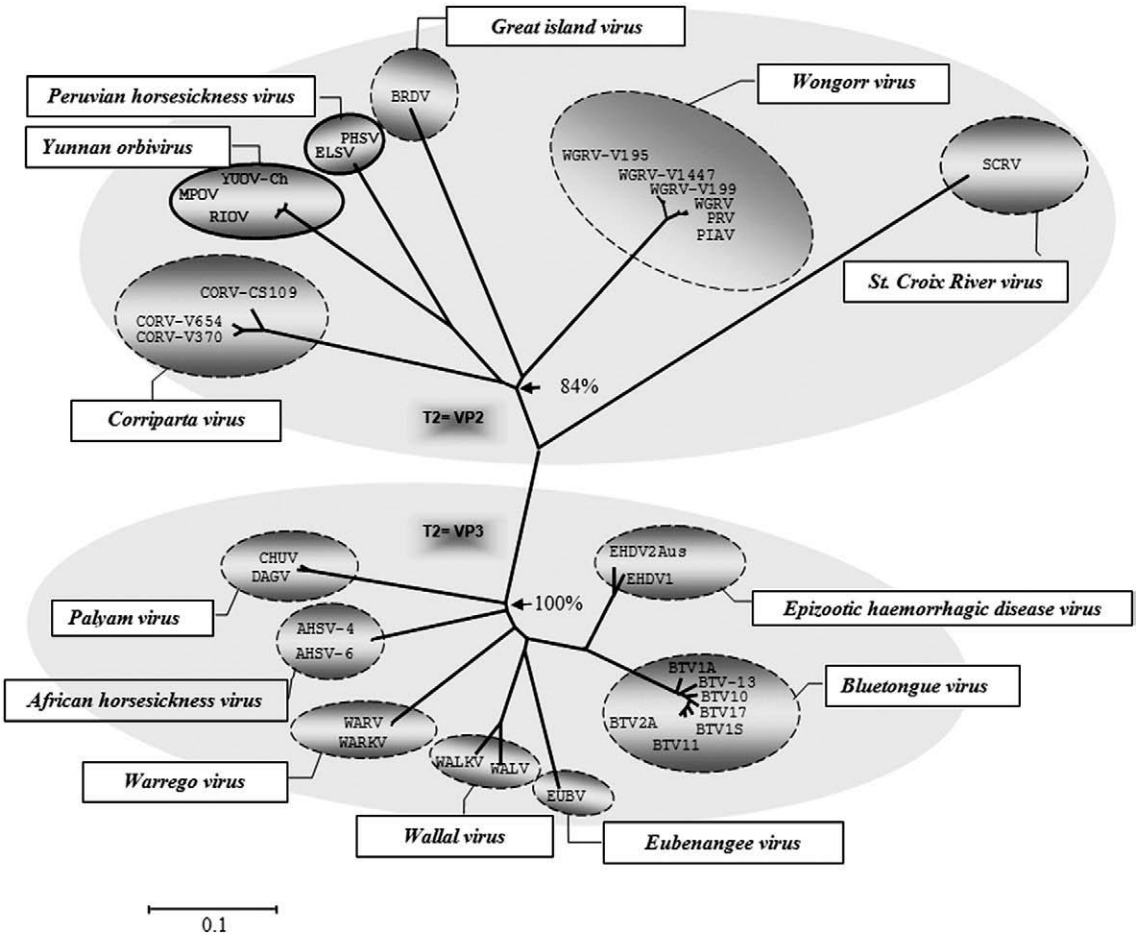

Fig. 4. Phylogenetic comparison of the T2 proteins (the major component of the subcore shell) of PHSV and other orbivirus species. This protein is equivalent to the VP3(T2) protein of BTV, the prototype *Orbivirus* species and to the VP2(T2) of two tick-borne orbiviruses: St. Croix river virus (SCRV) and Broadhaven virus (BRDV). Since many of the available sequences are incomplete, the analysis (presented as radial tree) is based on partial sequences (aa 393 to 548 relative to BTV-10 sequence). The tree shows the viruses having their VP2 protein as the T2 (the mosquito-borne and the tick-borne viruses) form a separate group from those having the VP3 as the T2 protein (*Culicoides*-borne viruses). Accession numbers and further detail of the sequence and viruses used are included in Supplementary Table B. Scale bars at lower left of the trees indicate the number of substitutions per site. The names within the rectangular boxes indicate virus species.

but only 62% within the VP3 (outer coat), indicating in this case that although they belong to the same *Orbivirus* species, MPOV and RIOV represent different serotypes. Amino acid identities between the different isolates of RIOV from Peru were 98–100% for the VP2(T2) and 94–99% for the VP3 (outer coat protein), indicating that they all represent a single serotype (YUOV-1).

The phylogenetic tree constructed using the outer coat protein of different orbiviruses showed that YUOV and RIOV cluster together as a single serotype, while MPOV clearly clustered as a separate serotype (Fig. 5).

Nucleic acid identities between YUOV and RIOV were 84% in segment 2 and 89% in segment 3. Nucleic acid identities between RIOV and middle point orbivirus were 97% within segment 2 but only 69% in segment 3. Nucleic acid identities between middle point orbivirus and YUOV were 83% within segment 2 but again only 64% within segment 3. These results confirm that all three viruses, YUOV, RIOV and MPOV, are members of the same species but belong to two different serotypes (see above).

PCR identification of animal and mosquito isolates using sequence-specific primers to segments 2 and 3 of PHSV or YUOV

Sequence comparisons of prototype strains of PHSV from Peru and YUOV from China, Australia and Peru have made it possible to design RT-PCR primers that can be used to specifically amplify and distinguish genome segments from the Peruvian YUOV and PHSV isolates. All of the primer sets listed in Table 6 amplified PCR products of the expected sizes from either YUOV or PHSV (as indicated).

Discussion

This paper describes a new orbivirus, PHSV, which has been associated with neurological disease in horses in Peru, from a region where encephalitic alphaviruses are also known to cause clinical illness. The resistance of PHSV to organic solvents, its possession of a genome composed of 10 segments of linear dsRNA, electron microscopic observations showing 50–55 nm diameter core particles and the conserved terminal nucleotides confirm the status of PHSV as an orbivirus (family *Reoviridae*).

The genus *Orbivirus* contains both insect-borne and tick-borne viruses as well as viruses with no known vectors (no genetic data are presently available for these). The insect-borne orbiviruses have been studied intensively and full-length genome sequences have been determined for the major veterinary pathogens BTV, AHSV, EEV and EHDV. Viruses of two species of tick-borne orbiviruses have also been sequenced, Broadhaven virus (BRDV, partial sequence; Moss et al., 1992) and St. Croix River virus (SCRV, complete sequence; Attoui et al. 2001). Full-length genome characterization of PHSV establishes its genetic relationship with the reoviruses and its identity as an orbivirus. The PHSV genome comprises 10 segments of dsRNA, each with conserved terminal sequences that are similar but not completely identical to those of other orbiviruses. All of the PHSV proteins

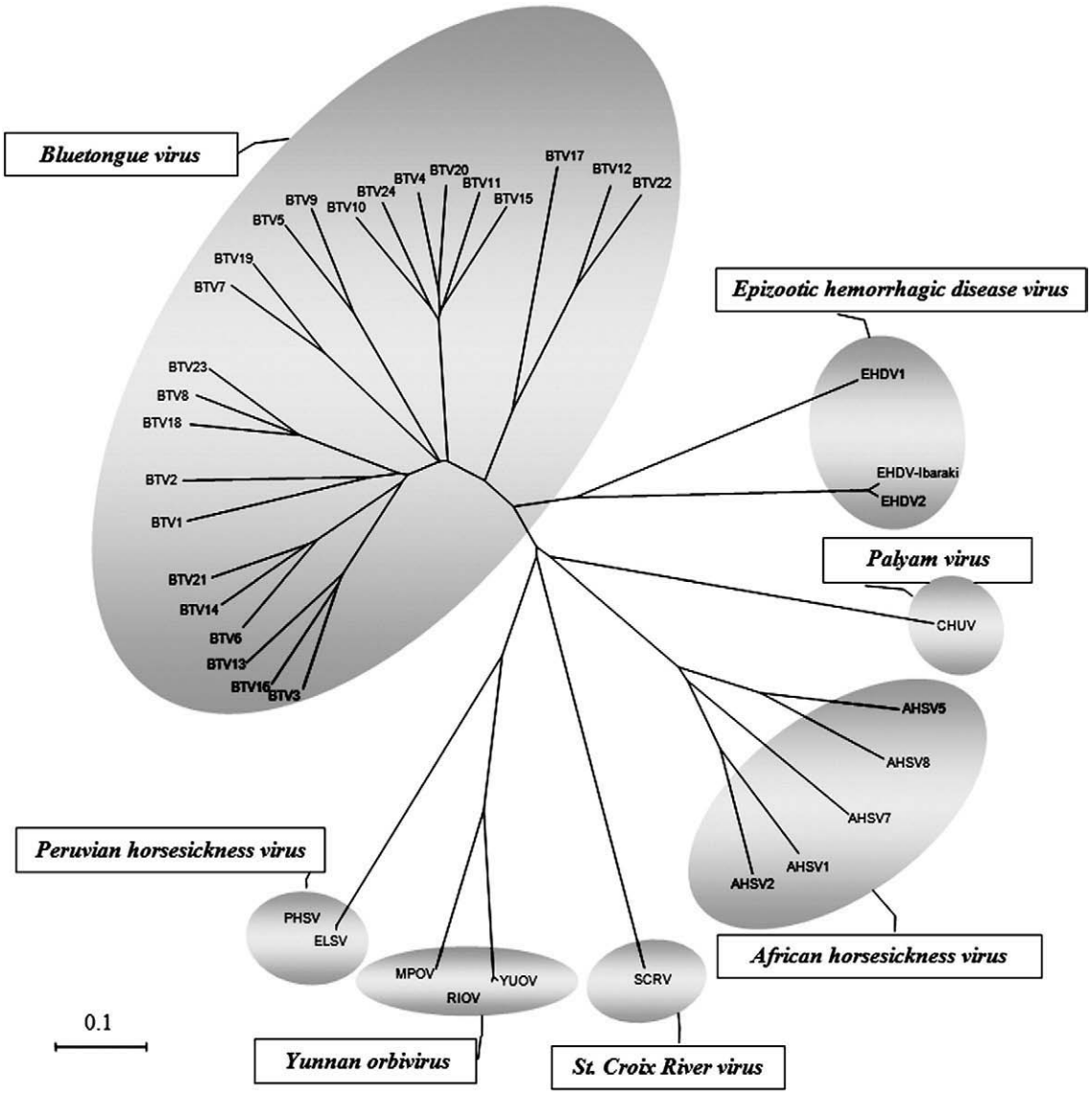

Fig. 5. Phylogenetic tree constructed using the serotype-determining outer coat protein of orbiviruses (the VP3 of the Culicoides-borne viruses and VP2 of the tick-borne and mosquito-borne viruses). PHSV and Elsey virus are shown as members of a single serotype within species *Peruvian horse sickness virus*. Rioja virus (RIOV) and Yunnan orbivirus (YUOV) are shown as members of single serotype (YUOV-1), while middle point orbivirus (MPOV) belongs to a separate serotype (YUOV-2) within species *Yunnan orbivirus*. Scale bar at lower left of the trees indicates the number of substitutions per site. The names within the rectangular boxes indicate virus species.

also show significant identity with those of other orbiviruses. In particular, the aa identity observed in the VP1 polymerase confirms the status of PHSV as an orbivirus, according to accepted criteria (aa identity >30% with other characterized orbiviruses).

The sequence of the PHSV VP2(T2) protein indicates that it is a distinct *Orbivirus* species, with aa identities to previously characterized orbiviruses that are significantly lower (24–66%) than those proposed to distinguish different virus species (>91%; Attoui et al. 2001). PHSV, which is the first orbivirus from the Americas that has been associated with a neurological syndrome in horses, was first isolated in Peru in 1997 from blood, brain or spleen samples collected from sick (based on neurological signs) or dead horses. No other viruses were isolated from these animals, and PHSV is considered likely to be the etiologic agent of the disease. However, the virus was also isolated from animals that had resolved their neurological manifestations and from healthy animals. Approximately 64% of the animals that were tested showing clinical signs (Table 1) were found to be viremic. The failure to detect IgM antibodies in some of the viremic animals indicates a chronic infection typical of a number of dsRNA viruses, including the coltiviruses and some other orbiviruses, possibly reflecting an intra-erythrocyte location (Attoui et al. 2005b). This would explain why experimentally infected chickens remained viremic for 2 months because viruses could be sheltered within the erythrocytes, which protects them from immune clearance (as shown previously with coltiviruses).

In 1999, ELSV was isolated in the Katherine region of the Northern Territory, associated with neurological disease in horses. The aa sequence of this orbivirus is almost identical to that of PHSV, particularly the outer coat proteins (99%), indicating that they belong to a single serotype. However, serum neutralization assays need to be carried out to fully confirm these sequence findings. Retrospective serological surveillance of horses in the Katherine region over a period of 10 years showed 11% seroprevalence of antibodies to PHSV (Elsey virus). These findings support the hypothesis that PHSV is responsible for neurological syndromes in horses in both Peru and Australia.

All of the viruses described in this paper were isolated from vertebrates or invertebrates from similar latitudes and topographies, in areas with similar rainfall and temperatures. Climate and particularly temperature are important factors affecting insect vectors and transmission of arboviruses (Purse et al. 2004; Gould and Higgs,

2009). Changes in climate may therefore be particularly important factors in the spread and emergence of these viruses (Purse et al. 2004; Gould and Higgs, 2009).

The proteins of the insect-borne orbiviruses that are involved in determination of serotype and serogroup include VP2 (outer coat) and VP7(T13) (outer core), respectively, of BTV, AHSV and EHDV. Sequence comparison shows that these proteins are equivalent to VP3 (outer coat) and the VP7(T13) of PHSV, respectively. Both VP3 and VP7(T13) of PHSV exhibited a relatively low aa identity to their homologues in the *Culicoides*-borne orbiviruses but a higher aa identity with the mosquito-borne YUOV. However, the high amino acid identity values (>96%) between PHSV and ELSV in the VP2(T2), VP3 (outer coat), VP6(hel) and the NS3 confirm that these two viruses are not only members of the same virus species but also members of the same serotype.

The evolutionary relationship of PHSV to 11 other orbivirus species was investigated based on the analysis of the T2 subcore shell protein that correlates with virus species. PHSV is within the group of mosquito-borne and tick-borne orbiviruses (in which the T2 protein is identified as VP2). The more distantly related *Culicoides*-borne viruses have their VP3 as the T2 protein. The level of aa identity detected in the "T2" protein between PHSV and the other orbiviruses (24% and 66%) identifies it as a member of a distinct (new) *Orbivirus* species (although more closely related to the other insect-transmitted than to the tick-transmitted orbiviruses).

Yunnan orbivirus was first isolated from wild-caught mosquitoes collected in 1997 during investigations of arboviruses in China. The virus was shown to replicate in insect, but not in mammalian, cell cultures. However, it infects adult mice, which became viremic 3 days post-inoculation (Attoui et al. 2005a). Rioja virus was isolated in 1997 from cattle and sheep in San Martin, from donkeys in Piura and from a dog in Ucayali, Peru. The virus was found to also be a member of the *Yunnan orbivirus* species, which was not previously associated with a vertebrate host. Rioja virus also failed to grow in mammalian cells but replicated in C6/36 cells. Sequence analysis identified RIOV as belonging to YUOV serotype 1 (YUOV-1).

Middle point orbivirus (MPOV) was isolated in 1998 from a healthy cow at Beatrice Hill farm, located 50 km east of Darwin in Australia's Northern Territory. Sequence analysis of segments 2 and 3 confirmed that this is a new serotype of YUOV (YUOV-2) (Cowled et al. 2007).

Although MPOV was successfully propagated in BSR cells (a clone of BHK-21 cells) (Cowled et al. 2007), YUOV and RIOV did not replicate in mammalian cells including L929, BHK-21, Vero, BGM, HEp-2 and MRC-5 (Attoui et al. 2005a). However, a Chinese isolate of YUOV was recently shown to replicate in BSR cells (unpublished data). MPOV (YUOV-2) from Australia was isolated from cattle but not associated with cases of clinical disease, while RIOV (YUOV-1) from Peru was associated with encephalitis, mainly in cattle, but it also appears to be capable of infecting and causing neurological signs in sheep and dogs. These findings suggest that YUOV may be an emerging pathogen of these animals.

The RT-PCR assay for PHSV that was developed in this study provides a simple and direct method to distinguish them from Yunnan orbiviruses. The availability of sequence data for conserved and variable proteins (neutralization antigens) of these viruses provides a basis for the development of additional diagnostic assays (real-time, conventional RT-PCR or serological assays). These could be used to monitor the global distribution and spread of PHSV and YUOV and to better characterize the pathogenesis and pathology of these emerging viruses. Finally, both PHSV and YUOV infect equids (PHSV infects horses, while YUOV was isolated only from a donkey); however, only YUOV infects cattle, sheep and dogs.

The arthropod vector of PHSV has not been identified during this study. Phylogenetic analyses showed the clustering of the PHSV T2 among the VP2(T2) group constituted by mosquito-borne and tick-borne orbiviruses. However, as shown for other viruses (Attoui et al. 2002b), the lower G+C content of PHSV genome suggests that it is not transmitted by ticks. It therefore appears likely that the vector is a mosquito. Interestingly, significant titers of anti-ELSV antibodies were identified in fruit bats, which could be regarded as potential reservoirs in Australia.

Previous studies of BTV have indicated infections of carnivores in Africa (Alexander et al. 1994) and more recently in Belgium (Jauniaux et al. 2008). In 1995, AHSV has also been isolated from dogs and the virus infection was attributed to ingestion of meat and organs from virus-infected prey species (Alexander et al., 1995). This route of infection could potentially explain the isolation of Rioja virus (YUOV-1) from a dog in Ucayali department in Peru.

A major epidemiological question arising from this work is the occurrence of almost identical viruses on two continents, separated by approximately 16,000 km. However, recent events in northern Europe (incursions of BTV serotype 8 into Netherlands, Germany and the UK) have clearly demonstrated that orbiviruses can be moved around the globe very quickly. They are also able to establish in new regions, possibly as a result of either animal or vector movements. Similar mechanisms have also been suggested for the detection of two similar rhabdoviruses, one in Australia and the other in North America (Gibbs et al., 1989). Results of experimental infections of candidate vector species with PHSV might shed more light on the probable routes and mechanisms of virus movement and transmission. Epidemiological surveys are being conducted to assess the current situation with PHSV and YUOV isolates.

Materials and methods

Regional topography

The Department of San Martin in Peru is located east of the Peruvian Andes (7° South, 76° 30′ West) at an altitude that ranges from 300 to 800 m above sea level. The topography of the region is irregular with large valleys and is crossed from south to north by the Huallaga River. The region has a subtropical rainforest climate, with an annual rainfall between 2000 and 2500 mm and temperatures ranging from an annual maximum of 34 to 38 °C to a minimum of 10 to 12 °C. There are abundant natural pastures that are used to raise both cattle and equids (an important means of transportation for people and crops). The dense forest provides an environment that supports several endemic arboviruses, including yellow fever virus, VEEV, EEEV and dengue viruses.

Elsey Station is situated at Mataranka, 14° 56′ South, 133° 08′ East, 151 m above sea level and 420 km southeast of Darwin, in the Northern Territory of Australia. It has a subtropical climate, in a transitional region between the dryness of the "red centre" and the tropical lushness and rainforest of the "top end" of Australia. The topography of the region corresponds to tropical savannah, with average annual rainfall of approximately 800 to 1000 mm and temperatures ranging from an annual maximum of 29.3 to 37.9 °C, and minimum of 12.4 to 24.5 °C. Katherine is a significant regional centre, situated 14° 28′ South, 132° 16′ East, 320 km southeast of Darwin (i.e., about 100 km north of Mataranka), at 209 m above sea level. The town is named after the nearby Katherine River and is situated in rugged outback country with deep rainforests, rocky cliffs and escarpments. It has an average annual rainfall of 1112 mm, with temperatures ranging from an annual maximum of 30 to 38 °C to a minimum of 13 to 25 °C. The region is predominantly tropical savannah, woodland, open eucalypt forest and rocky escarpment, with isolated pockets of monsoon rainforest and vine thickets.

Test samples and clinical case definition

"Disease cases" included equines with fever (>39 °C), anorexia, lack of motor coordination, sagging jaw, tooth grinding and/or a stiff

neck and usually died 8–11 days after onset (78% fatality rate). Surveys of equine mortality, conducted from January to July 1997 by the Peruvian National Service of Animal Health, identified 132 horses with these clinical signs (Table 5). The animals that survived took ~3 months for complete recovery. Sera and/or organs (brains and/or spleens) from 15 sick and/or dead horses and sera from 743 apparently healthy horses were tested for viruses. Thirty-seven out of a total of 758 horses, were identified as positive, producing cytopathic effects (CPE) in C6/36 cells (8 from sick and/or dead animals and 29 from asymptomatic horses). During the outbreak, some animals (from other vertebrate species) showed similar clinical signs and some died as a consequence. Blood and/or brain or spleen isolates were selected from 1 donkey, 2 cattle, 1 sheep and 1 dog for molecular studies. Another four virus isolations were made during 2002 from whole blood samples collected for surveillance from 60 healthy horses in the same region.

Samples collected from two horses showing signs of neurological disease in the Northern Territory of Australia were tested for virus (one horse from Mataranka, and one horse from Katherine).

Virus isolation and propagation

Blood or brain from equids (horses or a donkey), cattle and sheep from Peru, were homogenized in a tissue grinder with Eagle's minimum essential medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics (EMEM), at a ratio of 1 volume of sample to 5 volumes of medium.

Insects were collected in the Department of San Martin during 1997, including 1193 mosquitoes and 400 biting midges (*Culicoides* spp.). A further four hundred and three horse ticks (*Anocentor nitens*) were also collected in San Martin. Pools of 10–15 mosquitoes, 30–50 *Culicoides* or 5–7 adult ticks were homogenized in 3 ml EMEM. The homogenates were centrifuged for 5 min at 1500 rpm at 4 °C. The supernatant was recovered and centrifuged for a further 15 min at 7000 rpm at 4 °C. Fractions of 100 µl, of each of the Peruvian samples (Equids, cattle or sheep, insects or ticks) were inoculated onto mammalian cells (Vero, BHK-21, LLCMK2), insect cells (C6/36 cells; an *Aedes albopictus* cell line) and intracranially, intraperitoneally or intramuscularly into 2-day-old suckling mice, 1-month-old hamsters and 3-week-old chickens in Peru. Cells were monitored for CPE for 10 days pi. Samples were inoculated into the main vein of embryonated chicken eggs.

Samples of sera and spleen necropsies were obtained from two symptomatic horses in the Northern Territory. Spleen samples were homogenized in brain heart infusion broth containing antibiotics penicillin (6 mg/ml), streptomycin (20 mg/ml) and amphotericin B (2.5 mg/ml). The resulting suspensions were transferred to a 10-ml polypropylene centrifuge tubes and held at 4 °C overnight. Then homogenates were clarified by centrifugation at 2000×*g* at 4 °C for 15 min) and inoculated to two C6/36 cell culture tubes (EMEM 10% fetal calf serum) and then incubated for seven days prior to two further passages in BSR cell cultures (grown in BME supplemented with 5% FCS). Virus was also cultured from blood samples of the same animals.

Serological testing

C6/36 cells were infected with a virus isolate obtained from horse blood during the outbreak of 1997 (isolate 4020). Cells were

Table 5
Number of cases and dead horses as a consequence of equine neurological disease in San Martin during 1997 epizootic.

Province	District	Locality	Month	No. of ill horses	No. of dead horses
M. Cáceres	Campanilla	Ampato	January	5	5
M. Cáceres	Campanilla	Campanilla	January	6	6
Huallaga	El Eslabón	El Eslabón	January	1	0
Huallaga	Sacanche	Moscú-Miraflores	January	4	4
Moyobamba	Habana	Habana	February	1	1
Moyobamba	Calzada	Calzada	February	6	4
Moyobamba	Jepelacio	Jepelacio	February	1	1
M. Cáceres	Juanjuí	Chambira	February	3	2
M. Cáceres	Juanjuí	Ledoy	February	1	1
M. Cáceres	Pajarillo	Costa Rica	February	1	1
Rioja	Yorongos	Bella Florida	February	3	3
Picota	Tingo de Ponaza	Tingo de Ponaza	February	5	2
M. Cáceres	Juanjuí	Chambira	March	2	2
M. Cáceres	Pajarillo	Cuñumbuza	March	3	3
Moyobamba	Moyobamba	Pacayzapa	March	4	4
Moyobamba	Moyobamba	Moyobamba	March	3	3
Moyobamba	Moyobamba	Indañe	March	1	1
Moyobamba	Habana y Calzada	Habana y Calzada	March	16	16
Rioja	Pardo Miguel	Pioneros Alto	March	3	0
Rioja	Pardo Miguel	Aguas Claras	March	4	4
Rioja	Pardo Miguel	Pardo Miguel	March	11	3
M. Cáceres	Pajarillo	Sector Clara	April	2	1
M. Cáceres	Pajarillo	Shumanza	April	1	1
M. Cáceres	Juanjuí	Cunchihuillo	April	1	1
San Martín	Juan Guerra	Juan Guerra	April	18	13
Lamas	Pinto Recodo	Mishquiyacu	April	3	3
Rioja	Pardo Miguel	Mirador	April	4	4
Moyobamba	Jepelacio	Ramirez	April	2	2
San Martín	Juan Guerra	Juan Guerra	May	7	7
Huallaga	Alto Saposoa	Alto Saposoa	May	2	1
Moyobamba	Calzada	Calzada	May	1	1
El Dorado	Santa Rosa	Santa Rosa	June	2	1
M. Cáceres	Pajarillo	Dos Unidos	July	1	1
Lamas	Lamas	Churucayu	July	2	1
Moyobamba	Moyobamba	Moyobamba	July	1	1
Huallaga	Saposoa	Saposoa	July	1	0
Total				132	104

harvested at 24 h post-infection and immediately fixed in methanol. The virus was identified by immunofluorescence staining with a variety of mouse immune ascitic fluids (MIAF) (Wu et al. 2000). These included three anti-alphavirus MIAFs (anti-VEEV, anti-EEEV and anti-WEEV) and five anti-orbivirus MIAFs (antibody to AHSV, Wallal (WALV), Bambari, Itupiranga and EHDV). All MIAFs were prepared at the Department of Pathology at the University of Texas Medical Branch, Galveston. Convalescent horse sera were tested at 1:10 and 1:20 by immunofluorescence staining of PHSV-infected (isolate 4020) C6/36 cells.

ELSV (isolate 99/724) was assessed in Australia, using an orbivirus serogrouping ELISA (Blacksell et al. 1994) with a panel of antisera to viruses: AHSV, BTV, Corriparta (CORV), EHDV, Eubenangee (EUBV), EEV, Palyam (PALV), WALV, Warrego (WARV) and Wongorr (WGRV). Horse serum (84661), from the ELSV affected horse (99/724), was included in the serum panel.

Sera from affected horses in Australia were also tested for antibody to ELSV in a virus neutralization test (VNT), using an input dose of 100 $TCID_{50}$ ELSV and C6/36 cells. Sera were tested commencing at dilutions of 1/10 and neutralization titers were assigned as the dilution of serum causing complete inhibition of virus growth. Sera of species other than horses were also tested for antibodies to ELSV. These included fruit bats (black flying foxes and red flying foxes), macropods, rats, cattle and pigs.

IgM antibody in equine sera was assessed using sucrose gradient fractionated serum (Lunt et al. 1994) in an indirect ELISA, using plates coated with a semipurified virus antigen (Burroughs et al. 1994). IgM antibodies were also assessed by an indirect immunofluorescence assay, using fixed PHSV-infected C6/36 as antigen. Sera from asymptomatic horses in the Lima area in Peru (located approximately 700 Km away from San Martin) were used as negative controls, and cells infected with VEEV or EEEV were used as positive controls in conjunction with anti-VEEV or anti-EEEV IgM containing sera.

Electron microscopy

Virus was inoculated onto C6/36 cells. When CPE was apparent, supernatant was adsorbed onto formvar-filmed carbon-coated grids and stained with 2% (w/v) phosphotungstic acid (pH 6.8) for 1 min. Scraped cells were pelleted by low speed centrifugation and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 300 mOsm/kg) for 40 min, rinsed in the same buffer (3×20 min), post-fixed with 1% (w/v) osmium tetroxide in the same buffer (1 hour) and rinsed with milliQ water (3×5 min). Following washing, the cells were dehydrated through graded alcohol (70–100%) and infiltrated and embedded in Spurr's epoxy resin. Ultrathin sections were double stained in uranyl acetate and lead citrate and examined in a Philips CM120 transmission electron microscope at 120 kV.

Isolation of nucleic acids

Infected C6/36 cells were harvested at 7 days pi. The cells were lysed by three successive freeze–thaw cycles and centrifuged at 2000×*g* at 4 °C for 5 min. The supernatant from the lysate was centrifuged at 200,000×*g* for 3 h. Virus dsRNA was extracted from the pelleted virus material using a guanidinium isothiocyanate-based procedure (Trizol; Invitrogen, UK) as described elsewhere (Attoui et al. 2000) or the RNeasy kit (Qiagen). The extracted RNA was analysed by 1% agarose gel electrophoresis (AGE) containing 0.5 μg/ml ethidium bromide. Bands of dsRNA were visualized by UV trans-illumination.

Cloning of the dsRNA segments

The PHSV genome segments were copied into full-length cDNAs and amplified (FLAC), as previously described (Maan et al. 2007a, 2007b). The amplicons were purified by agarose gel electrophoresis, recovered using the Genclean kit (Qbioegen, UK), ligated into the pGEM-T cloning vector (Promega, UK) and transfected into competent XL-blue *E. coli*. Insert sequences were determined using M13 universal primers, the D-Rhodamine DNA sequencing kit and an ABI prism 377 sequence analyzer (Applied Biosystems, UK). For genome segments longer than 1.5 kb, internal primers were designed from the initial sequences obtained using the M13 primers to allow further sequencing of the full-length of the segment. Four clones of each segment were fully sequenced.

RT-PCR amplifications of RNA extracted from virus isolates collected from mosquitoes or other mammals

RT and PCR primers were designed based on alignments of sequence of PHSV and ELSV (from Peru and Australia, respectively; both isolates belong to species *Peruvian horse sickness virus*) or YUOV and RIOV isolates (from China and Peru, respectively; both isolates belong to species *Yunnan orbivirus*). Two sets were designed for each species (for first round and nested PCRs), targeting sequences of genome segment 2 (encoding the T2 subcore shell protein) and segment 3 (encoding the outer capsid and cell attachment protein). The designation and sequences of these primers are listed in Table 6.

Table 6
Primers used to amplify the genome segments 2 and 3 of isolates of *Peruvian horse sickness virus* and *Yunnan orbivirus* from Peru.

Primer name	Sequence (5′→3′)	Segment	Position (relative to YUOV or to PHSV)	Orientation	Amplicon size (bp)
YUOVSeg2S1	GATATTGCBATTTGGGTGAATTC	2	2109–2131	Forward	785
YUOVSeg2R1	TACACCACGTCCCGAAGGACTCGC	2	2870–2893	Reverse	
YUOVSeg2S2	GCAGTGTTTRGACACTCCAAATG	2	2180–2202	Forward	670
YUOVSeg2R2	CTATCCGTGRGACCACTACGCCTCC	2	2825–2849	Reverse	
YUOVSeg3S1	TATTMRTAGRTTGCGMATRAARTAT	3	2205–2229	Forward	471
YUOVSeg3R1	TACAAACATCCGCGTTGATGTAGC	3	2675–2651	Reverse	
YUOVSeg3S2	RTAGRTTRCGMATAAAYTATGWWGAAAT	3	2210–2237	Forward	437
YUOVSeg3R2	AGCATACTACTCCGCGCCAGCAAT	3	2646–2623	Reverse	
PHSVseg2s1	GTTTTAATCGATATTAGACAGGAACC	2	1125–1150	Forward	359
PHSVseg2r1	CTTCTACTAACGTGTGAATAAGG	2	1483–1461	Reverse	
PHSVseg2s2	GGAAAGATGATGTTTTCATATGG	2	1197–1219	Forward	258
PHSVseg2r2	CCTTCTCCTTAAACTATCAACTC	2	1554–1432	Reverse	
PHSVseg3s1	GCAAAATTGAATTATGTTCAATGC	3	1244–1267	Forward	488
PHSVseg3r1	CAATTCTACGATCTCGTAGGTTGG	3	1731–1708	Reverse	
PHSVseg3s2	TGGTAGAATGTCTGGCCTGAGAG	3	1308–1330	Forward	373
PHSVseg3r2	GCTATATAACTCTGATAAATATGG	3	1680–1657	Reverse	

B is T, G or C; R is A or G; M is A or C; and W is A or T.

Sequence analysis and comparisons

The sequences derived for PHSV genome segments were compared to those from other members of the family *Reoviridae*, using the local BLAST program in the DNATools package (version 5.2.018, S.W. Rasmussen; Valby Data Center, Valby, Denmark).

The aa sequence of VP1 from PHSV (identified as the viral RNA-dependent RNA polymerase (RdRp)) was compared with the sequences of RdRps from viruses representing 14 genera of the family *Reoviridae*. GenBank accession numbers are provided in Supplementary Table A. Sequence alignments were performed using the Clustal W software program (Thompson et al. 1994). Phylogenetic analyses were carried out with the software program MEGA version 4 (Kumar et al. 2004) using the *p*-distance determination algorithm or the Poisson correction and the neighbor-joining method for tree building.

Phylogenetic analysis of the "T2" (subcore shell) proteins of orbiviruses identified two major clusters. One cluster includes viruses having their T2 being the VP2 (encoded by genome segment 2). This includes three mosquito-borne viruses (Wongorr virus, Corriparta virus and Yunnan orbivirus) and the tick-borne orbiviruses (Broadhaven and St. Croix River viruses). The second group includes the more distantly related *Culicoides*-borne viruses such as BTV, AHSV, EHDV, Wallal virus, Eubenangee virus, Warrego virus and Palyam virus, which have their T2 encoded by genome segment 3 (VP3). The relationship of PHSV to other previously characterized orbiviruses was also analysed by comparing the sequence of VP2(T2) to orbivirus T2 sequences retrieved from databases or published (Pritchard et al. 1995; Hooper et al. 1999; Hooper, 1999). However, because some of these sequences are incomplete, the final alignment includes only amino acids positions 393 to 548 (relative to the BTV-10 sequence). GenBank accession numbers are provided in Supplementary Table B.

Sequence comparison of the PHSV VP3 (identified as the outer capsid protein, carrying neutralization epitopes) was also used to investigate relationship to other orbiviruses. GenBank accession numbers are provided in Supplementary Table C.

Sequence analysis of the outer capsid proteins of other orbiviruses, in particular BTV (Maan et al. 2007b, 2008) and EHDV (Anthony, 2007), has shown phylogenetic grouping that correlates with the virus serotype (Mertens et al. 2005). In BTV, variations of up to 27% were detected in the amino acid sequences of VP2 (the major determinant of virus serotype) within a single serotype. In contrast, viruses from different BTV serotypes consistently showed >24% amino acid variation in VP2 (Maan et al. 2007a, 2007b). This may reflect the involvement of multiple epitopes on VP2 that are involved in serotype determination (Gould and Eaton, 1990; White and Eaton, 1990). The outermost capsid protein (VP3) of the PHSV or YUOV strains analysed here exhibited amino acid variations of <3% within each virus species, and it is therefore considered very likely that in each case they are members of a single serotype.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.08.032.

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