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Discovery of an orthoreovirus in the aborted fetus of a Steller sea lion (*Eumetopias jubatus*)

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Abstract

An aborted mid-gestational male Steller sea lion fetus with an attached placenta was recovered on the floor of an open floating capture trap located off Norris Rock near Denman Island, British Columbia. Viral culture of the placenta demonstrated cytopathic effect. Although no specific signal was obtained in microarray experiments using RNA obtained from viral culture, elution and sequence analysis revealed the presence of a reovirus. Complete genome pyrosequencing led to the identification of an orthoreovirus that we have tentatively named Steller sea lion reovirus (SSRV). Phylogenetic analysis revealed similarity between SSRV and orthoreoviruses of birds, bats and other mammals that suggests potential for interspecies transmission.

Introduction

Steller sea lions (*Eumetopias jubatus*) are large otariid pinnipeds found in the northern Pacific. The genetically distinct eastern North American population of Steller sea lions is comprised of animals born on rookeries from central California to northern Southeast Alaska and is listed as a threatened species (Pitcher *et al.*, 2007)

Systematic studies of neonatal mortality of pinnipeds have focused on antarctic fur seals (*Arctocephalus gazella*), northern fur seals (*Callorhinus ursinus*), and harbor seals (*Phoca vitulina*) (Baker & Doidge, 1984; Keyes, 1965; Steiger *et al.*, 1989). Reovirus-like particles were observed in one sample from Smith Island in Puget Sound, Washington, from a harbor seal pup that was emaciated even though its stomach contained fresh milk (Steiger *et al.*, 1989). These particles were 80 nm in diameter and appeared identical to others observed in tissues of California sea lions (*Zalophus californianus*), Steller sea lions and northern fur seals in the northern Pacific (Steiger *et al.*, 1989).

The virus family *Reoviridae* includes 15 recognized genera of viruses (Mertens *et al.*, 2005), including the recently described *Mimoreovirus and Cardoreovirus,* and *Dinovernavirus*, the genomes of which comprise 9-12 linear segments of dsRNA (Attoui *et al.*, 2006; Attoui *et al.*, 2005; Day, 2009; Mohd Jaafar *et al.*, 2008). Reoviruses have been found in many organisms, including vertebrates, arthropods, protists, fungi and plants. Those that infect aquatic organisms include members of the genera *Aquareovirus* and *Mimoreovirus*, which have 11 segments, and *Cardoreovirus*, which have 12 segments. The International Committee on Virus Taxonomy (ICTV) recognizes five species in the genus *Orthoreovirus* (Chappell *et al.*, 2005). One species (*Mammalian orthoreovirus*) includes all the nonfusogenic mammalian orthoreoviruses; all other species induce syncytium formation. A second species comprises the avian reoviruses (ARV), including those from chicken, Muscovy duck, turkey and goose. The third species

is represented by Nelson Bay virus (NBV), an atypical syncytium-inducing mammalian reovirus, isolated from a grey-headed flying fox (*Pteropus poliocephalus*). Recently, viruses related to NBV were obtained from bats (Pulau virus, (Pritchard *et al.*, 2006)) and humans (Melaka virus, (Chua *et al.*, 2007); Kampar virus,(Chua *et al.*, 2008); and HK23629/07, (Cheng *et al.*, 2009)). Phylogenetic analyses of the few available genome segments demonstrated that although NBV were more closely related to ARV isolates than to other mammalian or reptilian orthoreoviruses, they represent a distinct species. The main arguments were: (1) the extent of sequence divergence in the sigma-class core and major outer capsid protein; (2) the absence of evidence for reassortment between the ARV and NBV isolates; and, (3) the classical notion that viruses in each orthoreovirus species correspond to a specific or related host type. The two remaining species of the genus are baboon orthoreovirus and reptilian reoviruses (Chappell *et al.*, 2005).

Here we report the isolation and characterization of an orthoreovirus recovered from the aborted fetus and associated placenta of a Steller sea lion at Norris Rock near Denman Island, British Columbia. Surprisingly, characterization of the full genome of this virus identified it as a member of a clade that includes ARV and NBV.

RESULTS

Pathologic studies

The fetus was a mid-gestation male, in good body and post mortem condition (code 2) with a total length of 50 cm, axillary girth of 29 cm with a mid-sternal blubber thickness of 0.4 cm. A moderate amount of meconium was interspersed within the chorioallantoic villi of the placenta and, microscopically, there was a necrosuppurative placentitis. There was extensive hemorrhage with variable edema throughout the fetal mediastinum, lung, hypodermis, heart, and nasal turbinates with mild, nonsuppurative inflammation of the heart, adrenal gland, and lungs. The hemorrhage was attributed to agonal or terminal trauma, presumably during or shortly after parturition. Moderate hemosiderosis with florid extramedullary hematopoiesis was noted throughout the liver. There were no apparent lesions within sections of the esophagus, larynx, trachea, peripheral nerve, rib, peripheral vasculature, large blood vessel, urinary bladder, tongue, umbilicus, salivary gland, urethra, small intestine, colon, bone, bone marrow, trachea, pancreas, peripheral ganglia, lymph node, thymus, testes, epididymis, kidney, adipose tissue, brain, spleen or thyroid gland.

Aerobic culture yielded mixed alpha hemolytic streptococci and actinobacilli from the lung, small intestine, and placenta with scant to light growth of alpha haemolytic streptococci from the liver, brain, kidney and stomach contents. No bacteria were recovered from the spleen or gastric mucosa. Enrichment cultures of the small intestine did not yield *Salmonellae* or *Brucellae*. Based on the nature of the microbial isolates, lack of attendant inflammatory infiltrate, and multiple percutaneous lacerations, the bacteria that were found were not considered pathologically significant. PCR analysis of pooled lung, spleen, lymph node, and brain did not detect influenza virus, *Toxoplasma gondii, Brucellae*, phocid distemper virus, or canine distemper virus. *Leptospirae*

sequences were detected by PCR in extracts of lung, spleen, lymph node and brain but not placenta.

Virus isolation

Syncytial formation and rounding of individual cells were noted in both Vero and Vero.DogSLAMtag flasks inoculated with clarified tissue homogenates 3-5 days after inoculation (**Figure 1**). Infected cell sheets rapidly deteriorated thereafter, with CPE detected in flasks from both cell lines from all the tissues sampled. Supernatant fluid from infected cells was aliquoted and stored at -80˚C for further testing. Typical sized reovirus particles were observed by EM (**Supplementary Figure 1**).

Molecular characterization

PCR amplification of the infected cell extract using the degenerate reovirus primer pair resulted in a 549 bp product, after editing out the primer sequences. BLASTN results showed the highest score with an ARV (strain 176, GenBank accession # EU707936.1: 73% nucleotide identity).

Pyrosequencing libraries yielded approximately 60,233 reads. In concert these reads represented approximately 9.2 kilobases (kb) of sequence distributed along the reovirus genome scaffolds when aligned to the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) using the Basic Local Alignment Search Tool (BLASTN/BLASTX; (Altschul *et al.*, 1990)). The sequences comprised singleton and assembled contiguous fragments, representing approximately 39% of reoviral sequence. Gaps between fragments and the termini of gene segments were completed by PCR, cloning, and sequencing. The genomic sequence was verified by classical dideoxynucleotide sequencing using primers designed using the draft sequence.

Consistent with the genome organization characteristic for members of the genus *Orthoreovirus*, the genome of SSRV comprises 10 RNA segments (GenBank Accession numbers HM222971-HM222980).

Phylogenetic analysis of the polymerase in the context of representative members of the *Reoviridae* family (**Figure 2**) and analysis of all other segments in the context of representative members of the *Aquareovirus* and *Orthoreovirus* genera (**Figure 3, 4, 5 and Supplementary Figures 2, 3, 4 and 5**) indicated that SSRV showed consistent association with other ARV and with NBV. Interestingly, SSRV is most similar to an orthoreovirus detected in a captive psittacine bird in Germany (eastern rosella, *Platycercus eximius*, GenBank accession # EU252582 (S1), EU189200 (S2), EU189201 (S3), and EU189202 (S4)).

The genomic organizations of orthoreoviruses are disparate. Although homologues of the λA, λB, λC, μA, μB, μNS, σB and σNS sequences of SSRV are found in other orthoreoviruses, segments S1 and S2 differ (**Supplementary Figure 6 to 11**). Some orthoreoviruses have polycistronic segments in either S1 or S2. Members of the NBV, most ARV, and SSRV showed identical genomic organization in the S1 segment with 3 ORFs called σC, p10, and p17. The known exception is Muscovy duck ARV, which contains only one ORF, σC (25). In contrast, mammalian orthoreovirus presents two overlapping ORFs in S1 (called σ1 and σ1s); and reptilian orthoreovirus (RRV) and baboon orthoreovirus (BRV) two in the analog segment 4 (called p14 and σC in RRV; p16 and p15 for BRV).

Reovirus fusion-associated small transmembrane (FAST) proteins are nonstructural, single-pass membrane proteins that induce cell-cell fusion and syncytium formation (Shmulevitz & Duncan, 2000). With exception of the mammalian orthoreoviruses, all orthoreoviruses have a FAST protein (Clancy & Duncan, 2009).

There is evidence that FAST proteins are virulence factors (Brown *et al.*, 2009). The predicted structure of the SSRV p10 ORF is similar to FAST proteins of other reoviruses (**Figure 6**).

DISCUSSION

To our knowledge, this is the first characterization of an orthoreovirus in marine mammals. SSRV was isolated from all tissues submitted for isolation, indicating a pancytotropic infection within the developing fetus. Reovirus-like particles have previously been seen in pinnipeds by electron microscopy but were not further characterized (Steiger et al, 1989); a reovirus in the genus *Rotavirus* has been identified in Galapagos sea lions (*Zalophus wollebaeki*) (Coria-Galindo *et al.*, 2009).

Reoviruses have been demonstrated to cause abortion in mice (Hassan & Cochran, 1969) hamsters (Kilham & Margolis, 1974), rats (Priscott, 1983) and swine (Kirkbride & McAdaragh, 1978). Although this precedent suggests plausibility, and we recovered SSRV from multiple tissues, we have not proven a causal relationship between SSRV infection and abortion. *Leptospira sp.* was detected by PCR in pooled lung, spleen, lymph node and brain but not in placenta. Although the site of isolation seems not related with the abortion, *L. interrogans* has been associated with reproductive failure in California sea lions (Smith *et al.*, 1974). Lesions seen in an aborted California sea lion fetus with *L. interrogans* included a friable liver, subcapsular hemorrhage of the liver and both kidneys, and unclotted blood in the peritoneal cavity (Gilmartin *et al.*, 1976). *L. interrogans* is not prevalent in Steller sea lions (Burek *et al.*, 2005), although significant diversity exists amongst *L. interrogans* serotypes (He *et al.*, 2007). Given that our findings only relate to one case, the prevalence of SSRV and role as a potential cause of fetal loss and abortion in Steller sea lions and more broadly, marine mammals needs to be further explored.

Virus isolation attempts from animals presenting unusual clinical signs remains a powerful tool for the discovery of new and possibly emerging viruses of importance to both human and animal health. Cell culture propagated virus isolates also provide

abundant genetic material, thereby facilitating the identification and subsequent phylogenic relationship to other virus family members.

Our findings may impact the view of host/virus relationships of viruses in the family *Reoviridae*. It has previously been suggested that aquareoviruses and orthoreoviruses and their respective hosts have co-speciated (Attoui *et al.*, 2002), which implies significant host fidelity. ARV and NBV have already been shown to form a clade (Duncan, 1999; Wellehan *et al.*, 2009). SSRV appears to be an additional member of this clade, and represents the first complete genome available in this clade. Partial genomic information (the 4 small segments) indicates that a reovirus isolated from a psittacine bird in Germany (de Kloet, 2008) is very closely related to SSRV.

Viruses within the *Reoviridae* have been found to cause disease in hosts from diverse *taxa*, illustrating their ability to replicate in cells of diverse hosts (Attoui *et al.*, 2006; Wellehan *et al.*, 2009). The ability of SSRV to grow efficiently in cells from African green monkey (*Cercopithecus aethiops*) origin, albeit in an *in vitro* setting, also underscores its potential broad host range. One recent study scored the viruses infecting mammals for biological properties that were considered advantageous to host switching, and found that *Reoviridae* scored highest (Pulliam, 2008). The finding of closely related reoviruses in sea lions, bats, and psittacine birds implies host switching and lack of host fidelity.

According to the ICTV, conclusive species classification requires the direct demonstration [or lack] of exchange of genetic material via reassortment of genome segments (Chappell *et al.*, 2005). Reassortment between avian orthoreoviruses has been demonstrated (Liu *et al.*, 2003), and further experiments are indicated to look for genetic exchange between SSRV, ARV, and NBV. However, this criterion for species delineation may need to be reconsidered; evidence for genetic exchange between two

distinct reoviral genera, *Aquareovirus* and *Coltivirus*, has recently been published (Mohd Jaafar *et al.*, 2008).

Reassortment and reclassification pose challenges to viral classification; different regions of viral genomes may not share common lineages. The advent of high throughput sequencing technologies has facilitated full genome sequencing. Where feasible, complete genome information should be obtained to allow analysis of the evolution and relationships of all regions, providing greater understanding of virus ecology and behavior.

MATERIALS AND METHODS

Tissue sampling

As part of a study on foraging behavior, Steller sea lions were being captured using a floating trap anchored off Norris Rock, near Denman Island British Columbia (49°48′N 124°64′W). On January 25, 2005, a dead, freshly aborted mid-gestational male Steller sea lion fetus with an attached placenta was found on the floor of the capture trap prior to a capture event. An adult Steller sea lion assumed to be the mother of the aborted fetus was subsequently captured and restrained for handling and processing. Following being trapped the adult female attempted to eat or attack the dead fetus prior to its removal from the trap at which time the fetal abdomen and head were punctured and the placenta detached from the fetus. The aborted fetus and placenta were removed from the trap and examined.

A full necropsy of the fetus was conducted in the field and portions of placenta and of each major organ were preserved in 10% neutral buffered formalin for histopathology examination. Representative samples of placenta, brain, lung, liver, kidney, spleen, gastric mucosa and small intestine were cultured for aerobic bacteria. Tissue homogenate of pooled brain, lung, spleen and lymph node were processed for *Toxoplasma gondii,* generic *Brucella* spp., *Leptospira sp.,* phocid distemper virus, and canine distemper virus by polymerase chain reaction (PCR), and for virus isolation. Additional samples of lung, spleen and mesenteric lymph node were also available for virus isolation.

Cells and virus isolation

Tissues were homogenized in a MiniMix bag system homogenizer (Interscience, Topac, Hingham, Massachusetts, USA) to give a 10% w/v suspension in Hanks Balanced Salt Solution (HBSS) containing antibiotics (penicillin 200 International

Units/ml, streptomycin 200 µg/ml and gentamicin 50 µg/ml). Suspensions were centrifuged at low speed (2060*g* or 700 rcf) for 15 min to remove cell debris. Inocula consisted of 250 µl of each cell free suspension added onto drained, 80% confluent cultures of African green monkey kidney cells Vero C1008 (American Type Culture Collection, Manassas, Virginia, USA) and Vero.DogSLAMtag cells (Vero cells stably expressing canine SLAM; donated by Dr. Yasuke Yanagi, Kyushu University, Fukuoka, Japan), all grown in 25 cm^2 flasks (Corning Inc., Corning, New York, USA). Adsorption was allowed to continue for one hour at 37^0C before the medium was removed and 5 ml of fresh media (Dulbecco's Modified Eagle's Medium/Ham's F-12, DMEM/F-12, respectively; with antibiotics and 2% Cosmic calf serum (HyClone Inc., Logan, Utah, USA)) was added to each flask. Flasks were incubated at 37^0C and observed daily for signs of cytopathic effects (CPE). Flasks were subcultured at a ratio of 1:2 every week.

Molecular virus characterization

Using an RNeasy Tissue Kit (Qiagen, Valencia, California), RNA was extracted from vero cells displaying CPE. The extracted RNA was analysed using GreeneChip Vr1.5 (Palacios *et al.*, 2007; Quan *et al.*, 2007). Probe intensities were background corrected, log_2-transformed, Z-score converted, and their corresponding p-values calculated. Positive hybridization events were selected as those spots with log_2-fluorescence values greater than two standard deviations above the mean signal. Candidate viruses (defined by their TaxID identifier (Genbank, NCBI)) were ranked by combining the p-values for the positive probes within that TaxID using the QFAST method of Bailey and Gribskov (Bailey & Gribskov, 1998).

Microarray analysis using GreeneLAMP yielded no statistically significant viral signal. Nonetheless, nucleic acid bound to the array was eluted with the intent of enriching for cryptic hybridized viral sequences. One hundred µL of water at 90ºC were

added to the array and mixed 10 times. Eluate was recovered and re-amplified by PCR. The library of DNA obtained was cloned into a plasmid vector (TOPO TA, Invitrogen, Carlsbad, California). After transformation into *Escherichia coli*, colonies were screened by sequencing, revealing the presence of reovirus nucleic acid. This finding was subsequently confirmed by RT-PCR of tissue culture extracts using consensus primers for orthoreoviruses and aquareoviruses (Wellehan *et al.*, 2009). The protocol was modified to use the primer 1607F as a forward primer and 2200R as a reverse primer in the second round (Landolfi *et al.*, 2010), thus yielding a 549bp product.

Viral genome sequencing and analysis of Open Reading Frames (ORFs)

RNA extracts from virus supernatant were amplified and prepared for unbiased high-throughput pyrosequencing. Total RNA extracts were treated with DNase I (DNA-free, Ambion, Austin, Texas) and cDNA generated by using the Superscript II system (Invitrogen) for reverse transcription primed by random octamers that were linked to an defined 17-mer primer sequence (Palacios *et al.*, 2007). The resulting cDNA was treated with ribonuclease H and then randomly amplified by PCR (Palacios *et al.*, 2008). Products of >70 base pairs (bp) were selected by column purification (MinElute, Qiagen, Hilden, Germany) and ligated to specific linkers for sequencing using the 454 Genome Sequencer FLX (454 Life Sciences, Branford, Connecticut, USA) without fragmentation of the cDNA (Cox-Foster *et al.*, 2007; Margulies *et al.*, 2005; Palacios *et al.*, 2008). Removal of primer sequences, redundancy filtering, and sequence assembly were performed with software programs accessible through the analysis applications at the GreenePortal website.

Sequence gaps between the aligned fragments were filled by specific PCR amplification with primers designed using the data from pyrosequencing. Terminal sequences were generated by ligation (Potgieter *et al.*, 2009). Sequence was verified by

311 classical dideoxynucleotide sequencing, using primers designed based on the draft
312 sequence.
313
314 **Phylogenetic analysis**
315 The initial sequences were compared to those in GenBank (National Center for
316 Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, England), and
317 Data Bank of Japan (Mishima, Shizuoka, Japan) databases using BLASTN (Altschul *et*
318 *al.*, 1990).
319 A set of sequences of viruses of the family *Reoviridae* was used to assess the
320 phylogenetic history of the Steller sea lion reovirus (SSRV). Polymerase amino acid
321 sequences of reoviruses were aligned using the programs PROMALS3D (Pei *et al.*,
322 2008) and 3DCoffee (O'Sullivan *et al.*, 2004) with the purpose of obtaining an alignment
323 that not only considered primary sequence data but also the secondary structure of the
324 protein. To evaluate the robustness of the approach, the ability to find and align motifs
325 previously identified as conserved amongst *Reoviridae* was used as a marker.
326 Phylogenetic analysis was performed using p-distance as model of aminoacid
327 substitution as accepted by ICTV for analysis of the *Reoviridae* family (Attoui *et al.*,
328 2006; Mertens *et al.*, 2005). MEGA (Kumar *et al.*, 2004) was used to produce
329 phylogenetic trees, reconstructed through the Neighbor Joining (NJ) method (Saitou &
330 Nei, 1987). The statistical significance of a particular tree topology was evaluated by
331 bootstrap re-sampling of the sequences 1000 times. Identical results were obtained by
332 Bayesian phylogenetic analyses using the BEAST, BEAUti and Tracer analysis software
333 packages (Drummond & Rambaut, 2007) (data not shown). All other orthoreovirus and
334 aquareovirus segment sequences were aligned using a similar approach. The
335 evolutionary distances were computed using the Poisson correction method and are in
336 the units of number of amino acid substitutions per site. All positions containing

alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

Sequence analysis

Programs of the Geneious package (Biomatters, Auckland, New Zealand) were used for sequence assembly and analysis; p-distances were calculated using MEGA3. Topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (http://www.cbs.dtu.dk/services), the web-based version of TopPred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), and Phobius (http://phobius.cgb.ki.se/index.html) (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Käll *et al.*, 2004; Krogh *et al.*, 2001).

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Figure legends

Figure 1

Syncytia formation in SSRV-infected Vero cells.

Figure 2

Phylogenetic analysis of the RNA-dependent RNA-polymerase of *Reoviridae*. Full length amino acid sequences were aligned using the ClustalX (Thompson *et al.*, 2002) implementation on the MEGA software (Tamura *et al.*, 2007) and further refined using T-Coffee (Notredame *et al.*, 2000) to incorporate protein structure data on the alignment. Phylogenetic analysis was performed using p-distance as model of amino acid substitution as implemented by ICTV for analysis of the Reoviridae family (Mertens *et al.*, 2005). MEGA was used to produce phylogenetic trees, reconstructed through the Neighbor Joining (NJ) method. The statistical significance of a particular tree topology was evaluated by bootstrap re-sampling of the sequences 1000 times. Gaps in the alignment were treated as unknown characters.

Figure 3-5. Phylogenetic analysis of the (3) Lambda-1, (4) Mu-1, and (5) Sigma-A ORFs of the Aquareovirus and Orthoreovirus. Neighbor-joining phylogenetic analyses of nucleotide sequence differences among λ1, μ1, and σA, were conducted using MEGA 3.0. The black dot indicates SSRV and the white dots indicate the closely related psittacine virus.

Figure 6. p10 ORF in S1 of SSRV has characteristics similar to FAST proteins. Hydrophobicity plot of ARV, NBRV, and SSRV obtained using the Kyle-Doolittle algorithm implemented in the program TopPred (available at http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred). Sequence analysis show that SSRV contains the components for a FAST protein: hydrophobic region (HP), transmembrane domain (TM), and basic region (BR).

Supplementary Figure 1. Electron micrograph of negative stained SSRV. Scale bar= 100 nm.

Supplementary Figure 2-4. Phylogenetic analysis of the (2) Lambda-2, Lambda-3 (3) Mu-2, Mu-3 and (4) Sigma-B and Sigma-NS ORFs of the Aquareovirus and Orthoreovirus. Neighbor-joining phylogenetic analyses of nucleotide sequence differences were conducted using MEGA 3.0. The red dot indicates SSRV and the white dots indicate the closely related psittacine virus.

Supplementary Figure 5. Phylogenetic analysis of the Sigma-C, p10, and p17 ORFs of the Avian and Nelson Bay Reovirus. Neighbor-joining phylogenetic analyses of the nucleotide sequence differences among the σC, p10, and p17 were performed using MEGA 3.0. The red dot indicates SSRV and the white dots indicate the closely related psittacine virus.

Supplementary Figure 6

Protein λA: The 1303 aa λA protein (143.4 kDa, pI=6.70) is the only protein product from the L1 gene, which functions as the core shell scaffold. The hydrophilic amino-terminal region is distinct from all other ARVs and is clearly divergent when compared with the λ1 from MRVs (Xu & Coombs, 2009). Unfortunately, no Nelson Bay virus is available for comparison. Xu and Coombs identify many conserved regions between MRV and ARV, all of which are conserved in SSRV (Xu & Coombs, 2009). The hypervariable region of the N-terminal domain (orange box), its hypervariable region, the six previously identified helicase domains (green box) (Bisaillon & Lemay, 1999), the potential 5' RNA triphosphate domains regions (Bisaillon & Lemay, 1999), the characteristic C_2H_2 zinc-binding motif and other areas of high conservation (but without assigned biological function; light blue) (Xu & Coombs, 2009), among ARV, MRV, and AqRV were recognizable. In all cases, SSRV domains were closely related with the ARVs.

Supplementary Figure 7

Protein λB: The 1259 aa RNA dependent RNA polymerase λB protein (140 kDa, pI=8.37) is encoded by the L2 segment. The aa sequence is least conserved in the N-terminal and C-terminal bracelet domains whereas most conserved aa were found within canonical fingers, palm, and thumb polymerase motifs. The polymerase motifs I, II and III, the classical $G_{997}DD$ polymerase motif (Bisaillon & Lemay, 1999) (not shown), and additional residues shown to be important for a variety of polymerase functions (Arg_{522}, Arg_{523}, Arg_{525}, $Ala_{587,}$ needed to properly position the incoming NTP triphosphate [red arrow]; Ile_{527} and Pro_{529}, needed to help position template nucleosides [green arrow]; and Thr_{557}, Ser_{558}, Gly_{559}, Ser_{560}, and Val_{562}, portion of a loop that maintains priming NTP; [blue arrow] and Asp_{589}, Ser_{681}, and Gln_{731} (Kim *et al.*, 2004; Xu & Coombs, 2008)) are all conserved.

Supplementary Figure 8

Protein λC: The L3 segment encodes the λC protein (142.7 kDa, pI=5.92), which extends from the inner core to the outer capsid of the avian reovirion (Martinez-Costas *et al.*, 1997; Zhang *et al.*, 2005) and is comparable to the λ2 protein of MRV. Although the ATP/GTP-binding site motif A in ARV (residues 379 to 386) or the equivalent in MRV (residues 893 to 900) (Hsiao *et al.*, 2002) are not conserved in SSRV (data not shown), Lys_{170} and Lys_{189}, [black arrows] which are believed to be important contributors for the guanylyltransferase activity by acting as the GMP-acceptor the of λC capping enzyme (Hsiao *et al.*, 2002; Luongo *et al.*, 2000), the *S*-adenosyl-$_L$-methionine (SAM) binding pocket [green box], and the K_{189}DAT surrounding the putative GMP-acceptor site (Hsiao *et al.*, 2002; Luongo *et al.*, 2000) are all recognizable in SSRV.

Supplementary Figure 9

Protein μA: The 737 aa M1-encoded μA protein (83.3 kDa, pI=8.68) is a minor component of the inner capsid (Martinez-Costas *et al.*, 1997). Computer searches by Su et al. revealed that the μA possesses a sequence motif LLALDPPF (aa 458–464)

characteristics for N-6 adenine-specific DNA methylases (Timinskas *et al.*, 1995); however, such a sequence is only conserved in SSRV in three amino acids, shown in bold (V_{459}TK**L**S**P**D**F**). Fifteen cysteines are conserved in all ARVs (Su *et al.*, 2006); SSRV shows only 13 (purple marks), although 10 are conserved, suggesting similar topology.

Protein μB: The primary translation product of the ARV M2 is the μB protein (73.5 kDa, pI=5.09) (Varela & Benavente, 1994). A large proportion of the μB molecules synthesized are cleaved to form a myristoylated amino terminal peptide, termed μBN, and a large carboxy-terminal protein, termed μBC (Benavente & Martinez-Costas, 2007; Su *et al.*, 2006; Varela *et al.*, 1996). The cleavage site in SSRV is conserved and predicted to occur between N_{42} and P_{43}. The site of trypsin cleavage between Arg_{581} and Gly_{582}, identified in other ARVs is also conserved (Nibert & Fields, 1992; Su *et al.*, 2006). Similar topology for SSRV μB to the AVR counterparts is suggested by conservation of all cysteine residues (purple marks).

Protein μC: Encoded by the M3 segment of the ARV, the μC (71.9 kDa, pI=5.43) is a nonstructural protein that is predicted to be cleaved into a small ~15 kDa N terminal peptide and larger ~55 kDa carboxy terminal peptide (Benavente & Martinez-Costas, 2007). Although these products have been detected *in vitro*, the position of the cleavage and the protease responsible has yet to be identified. There are two coiled-coil regions identified in ARVs from positions 451-472 and 540-599 (Touris-Otero *et al.*, 2004). These two coiled-coil regions are present in SSRV from positions 455-484 and 559-611, but a third regions is predicted, using the program COILS (Lupas *et al.*, 1991), from positions 422 to 449. This third coil is located in a similar position in MRV.

Supplementary Figure 10

Protein σC: Encoded by the 3'-proximal cistron of the S1 gene, the σC (34.5 kDa, pI=4.74) is the viral cell attachment protein. Several conserved aromatic amino acids in

716 the C-terminal portion were previously identified (Day *et al.*, 2007); all are conserved in
717 SSRV (black arrows).
718 *Protein p17*: This protein (10.3 kDa, pI=9.10), encoded by the second ORF of the S1
719 gene, has no sequence homology with any other viral or cellular proteins. The only
720 functional domain identified in p17 (Costas *et al.*, 2005), a nuclear localization signal
721 (NLS), is conserved in SSRV (position 119-128).
722 **Supplementary Figure 11**
723 *Protein σB*: Encoded by the S3 segment, σB (40.8 kDa, pI=6.75) is a major component
724 of the reovirion outer capsid. The transmembrance predictions graphs show the
725 probability of a region being inside of the cell (red), outside the cell (blue) or a
726 transmembrane region (green).The hydrophobic region predicted for duck reovirus
727 (Zhang *et al.*, 2007) and the conserved zinc finger domain (position 51-77) (Kapczynski
728 *et al.*, 2002; Le Gall-Recule *et al.*, 1999; Mabrouk & Lemay, 1994; Schiff, 1998) are both
729 conserved in SSRV. However, the basic stretch from aa 287-293 in DRV is not present
730 in SSRV.
731 *Protein σNS*: The nonstructural protein σNS (40.4 kDa, pI=7.06) is encoded by the avian
732 reovirus S4 segment. The 12 aa conserved region at the N terminus of this protein has
733 been implicated in the ssRNA binding and aa 135-270 is a central region of conserved
734 secondary structure in addition to highly conserved hydropathy profiles (Duncan, 1999).

Figure 1

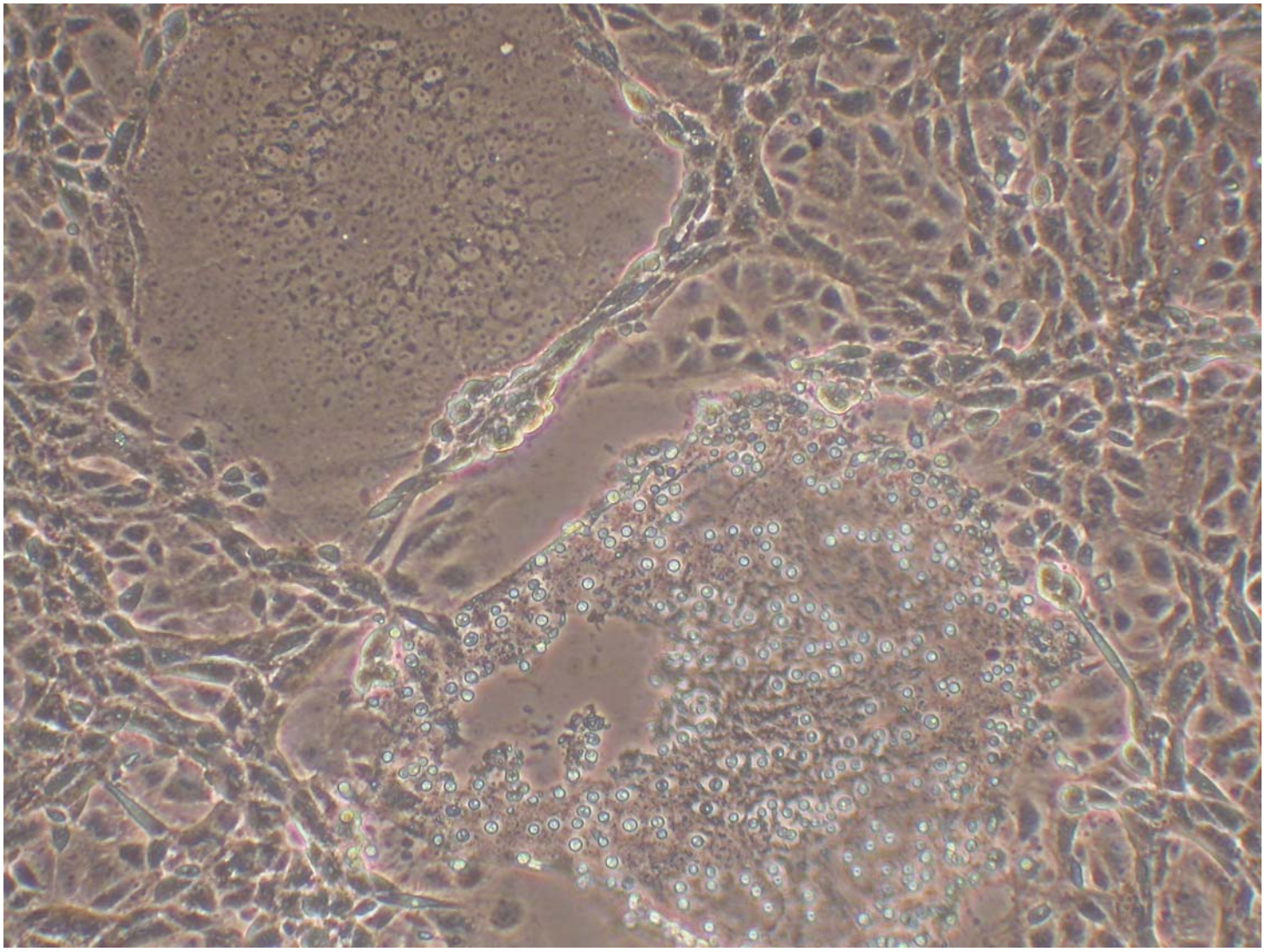
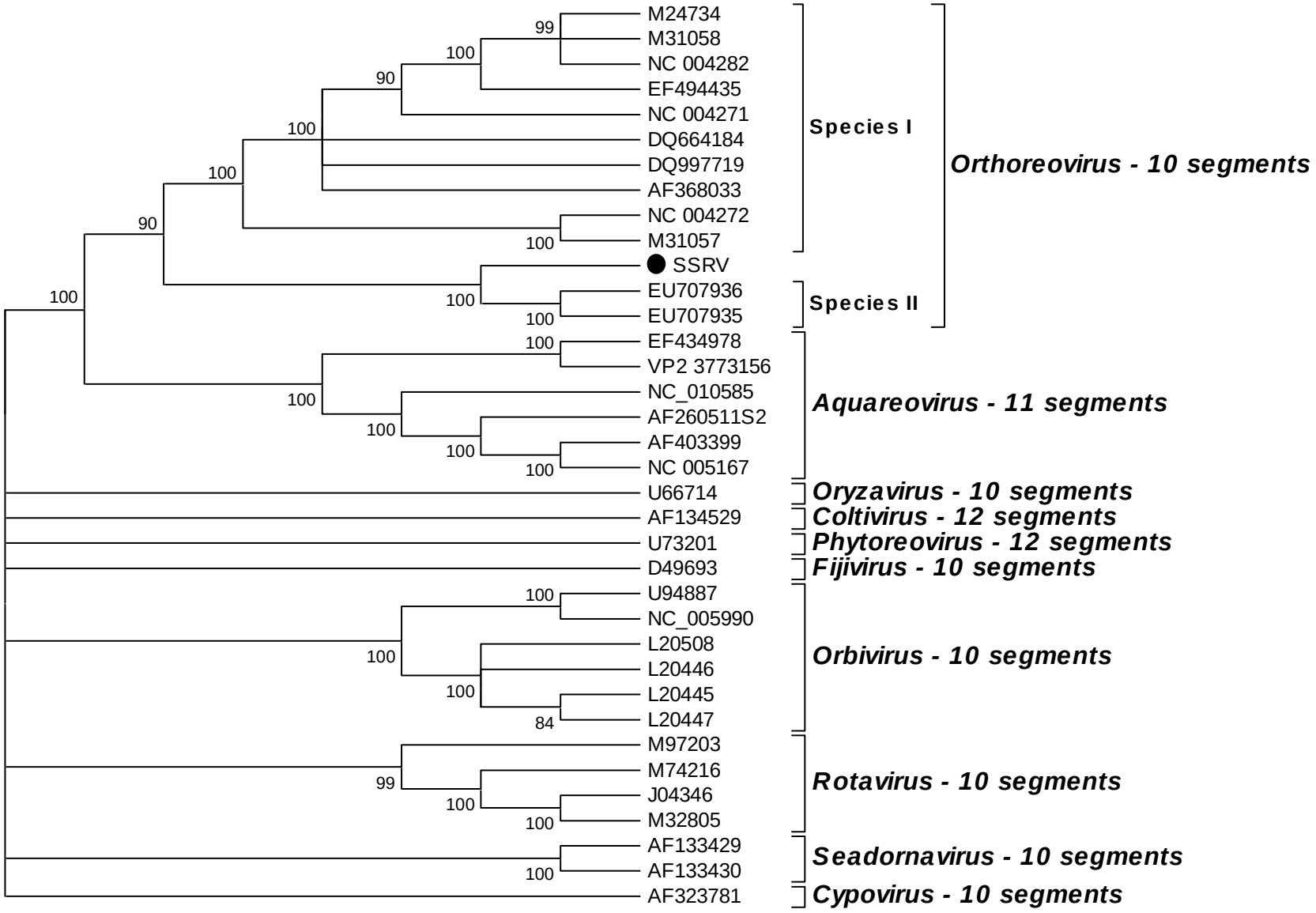


Figure 2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84
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Species I
Species II
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Aquareovirus - 11 segments
Oryzavirus - 10 segments
Coltivirus - 12 segments
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Seadornavirus - 10 segments
Cypovirus - 10 segments

Figure 3

Lambda-A

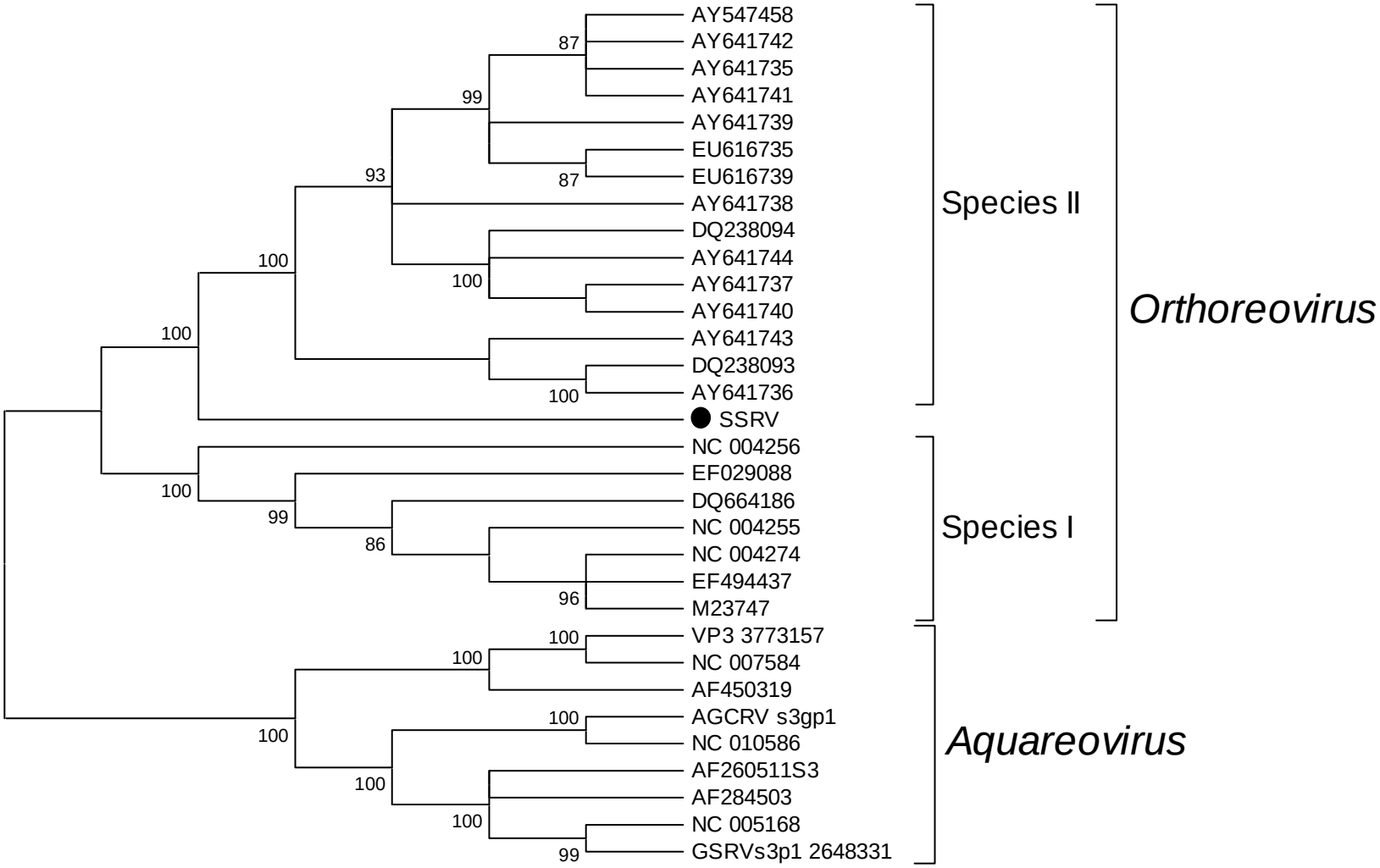
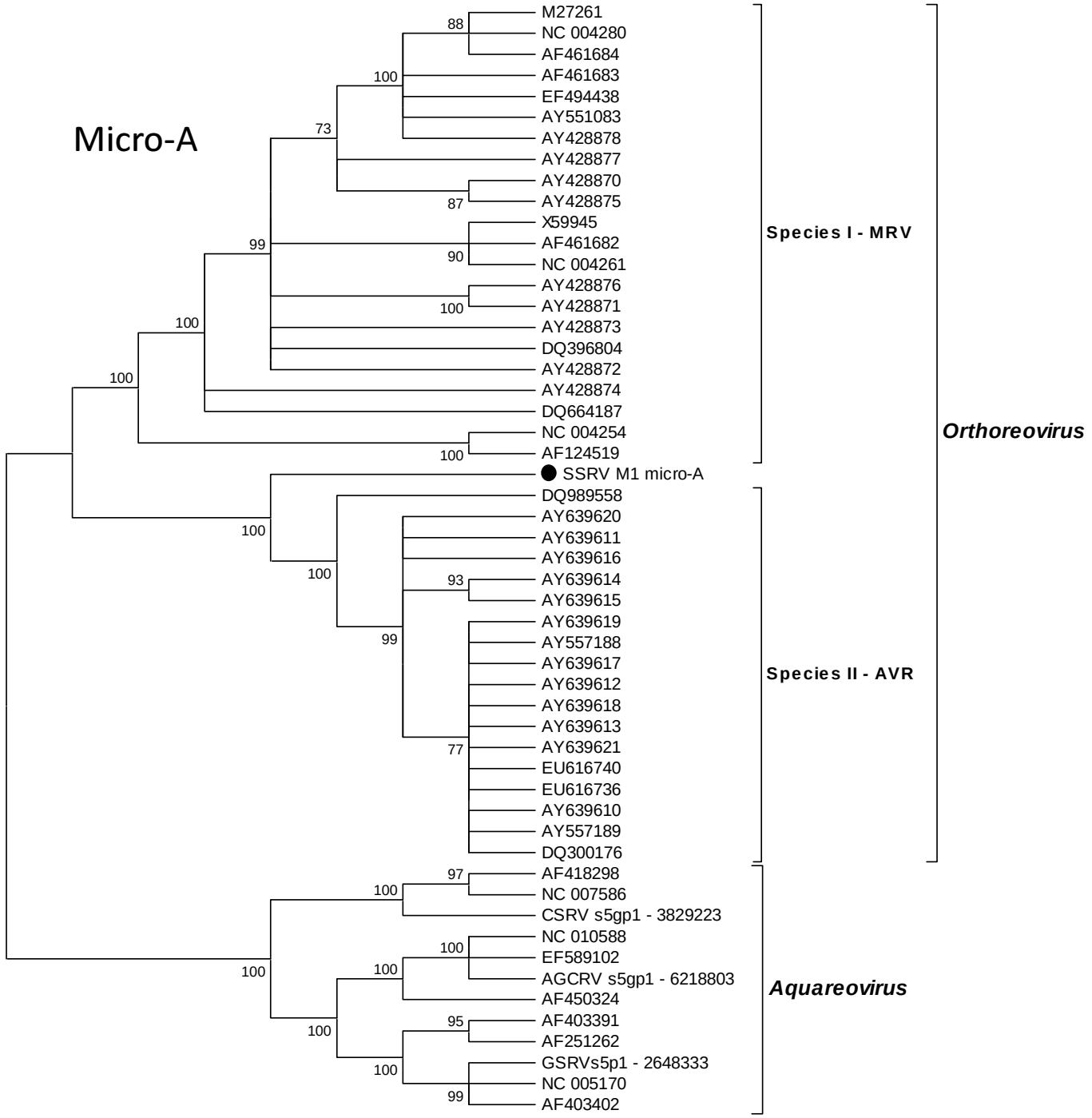


Figure 4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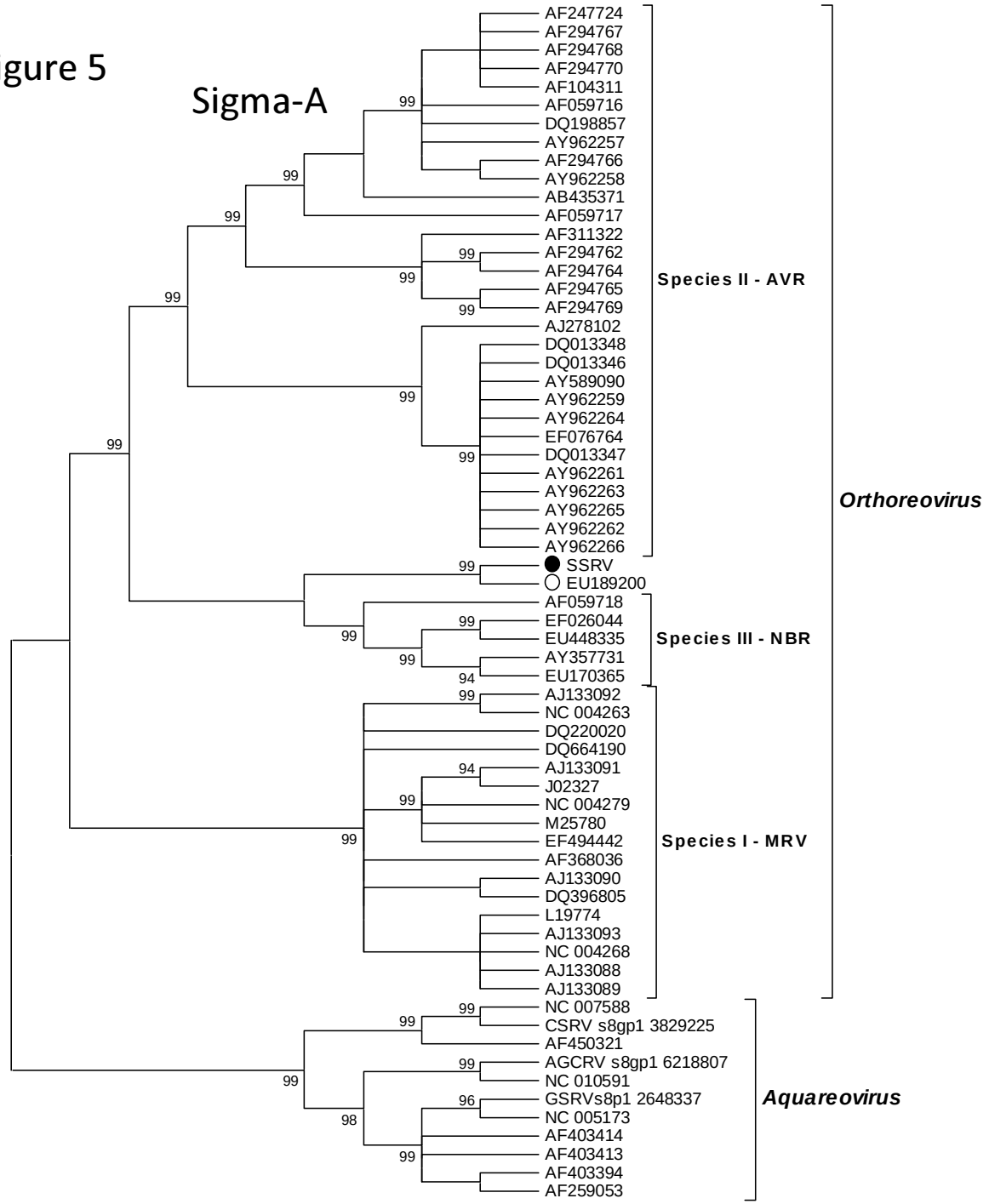
Species I - MRV
Species II - AVR
Aquareovirus
Orthoreovirus

Figure 5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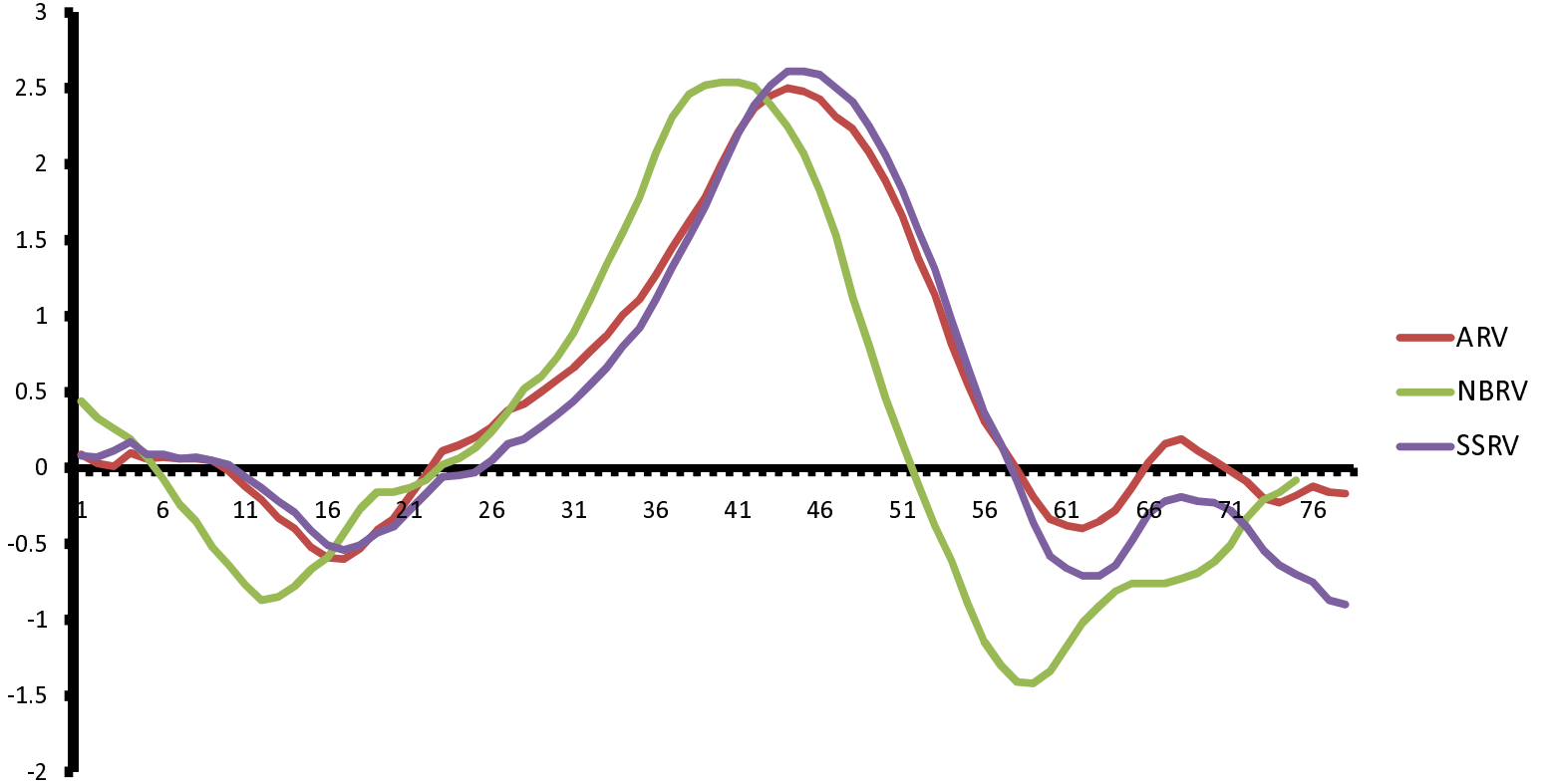
AF403413
AF403394
AF259053
Species II - AVR
Species III - NBR
Species I - MRV
Orthoreovirus
Aquareovirus

Figure 6


3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
ARV
NBRV
SSRV


ARV P10
HP
TM
BR
98
NBRV P10
HP
TM
BR
95
SSRV P10
HP
TM
BR
98