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Selectively maintained paleoviruses in Holarctic water fleas reveal an ancient origin for phleboviruses

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ABSTRACT

The ecological model, *Daphnia pulex* (Cladocera: *Daphniidae*), is broadly distributed in Holarctic freshwater habitats and has been the subject of multidisciplinary study for over half a century, but never has a natural RNA virus infection been reported in daphnids. Here we report on a group of paleoviruses related to RNA dependent RNA polymerase in the genome of *D. pulex*. Phylogenetic analysis suggests that these paleoviruses are derived from a viral lineage within the genus *Phlebovirus*. Comparison of the genomic sequences flanking individual paleoviruses reveal that some are orthologous viral insertions having been present in the common ancestor of the *D. pulex* species complex, which is millions of years old. Still, we detected some sites that have the signature of purifying selection. In contrast, other paleoviruses in this group seem to be unique to specific host lineages and even contain undisrupted open reading frames, suggesting either more recent acquisition, or selective maintenance.

Introduction

Our understanding of the deep evolutionary history of viruses and their host interactions is restricted by the absence of fossil records for viruses and the limited evolutionary reach of molecular clock estimates when based on the rapid nucleotide substitution rates of viruses (Holmes, 2003). Paleovirology, the study of endogenous viral elements (EVEs), including endogenous retroviruses (ERVs) and non-retroviral integrated RNA viruses (NIRVs), offers a needed glimpse into the deeper history of virus–host interactions. Analyses of individual ERVs and NIRVs in fungal, arthropod and vertebrate genomes have extended the minimum age of RNA viruses and their host associations, e.g. Filoviruses (Taylor et al., 2010), *Bornaviruses* (Horie et al., 2010) and *Lentiviruses* (Gifford et al., 2008), revealed genes of viral origin that have been selectively maintained or co-opted in host genomes (Malik et al., 2000; Mi et al., 2000; Taylor and Bruenn, 2009; Katzourakis and Gifford, 2010; Taylor et al., 2011; Fort et al., 2012), explained how viruses lacking reverse-transcriptase have integrated into DNA genomes (Katzourakis and Gifford, 2010; Ballinger et al., 2012), and supported a case of coevolution between viruses and hosts with a modified nuclear genetic code (Taylor et al., 2013). Yet, few attempts have been made to unravel the evolutionary history of what appear to be virally-derived gene families in eukaryotic genomes. In CTG-clade yeast, a family of totivirus capsid-like NIRVs are tandemly structured, suggesting host duplication, and some copies are expressed as proteins (Taylor and Bruenn, 2009; Taylor et al., 2013). Several arthropod genomes harbor a dozen or more NIRVs showing sequence similarity to a single viral gene (Katzourakis and Gifford, 2010; Fort et al., 2012), but many of these sequences are divergent pseudogenes, and little evidence remains to differentiate between an origin as a single ancient integration followed by duplication within the host, or multiple integrations of relatively closely related exogenous viruses. The ability to support one hypothesis over the other with statistical bioinformatics methods is further hindered by the incomplete representation of the ancient virosphere.

Phleboviruses (Bunyaviridae) are segmented, single-stranded, negative and ambisense RNA viruses. Bunyavirids display an extensive host range across vertebrates, invertebrates and plants. However, the arthropod-borne members of the genus *Phlebovirus*, appear to be curiously limited to unrelated blood-sucking dipterans and ticks. It is presently unknown if this distribution in arthropods is a sampling bias, or a real association with hematophagous arthropods. The arthropod-borne phleboviruses are subdivided into the Sandfly group, vectored by sand flies of the genera *Phlebotomus* and *Lutzomyia,* and mosquitoes (Tesh, 1988), and the Uukuniemi group, vectored by ticks (Saikku and Brummerk, 1973; Eley and Nuttall, 1984; Palacios et al., 2013). Some viruses in the Sandfly group, e.g. Toscana virus, have been shown to establish persistent, vertically transmitted infection in their vectors (Tesh and Modi, 1987; Bilsel et al., 1988). The bunyavirus genome is distributed across three segments (L, M, and S) and codes for up to five genes: a nucleoprotein (*N*), a glycoprotein (Gn-Gc), an RNA-dependent RNA polymerase (RdRp) and one or two nonstructural proteins, NSs and NSm, named

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for the segment on which they are coded (Elliott, 1990). As with most RNA viruses, little is known about the age of the phleboviruses. Several authors have used molecular clock and coalescent dating methods calibrated with empirically determined mutation rates to estimate the ages of extant lineages, e.g. severe fever with thrombocytopenia syndrome virus (Lam et al., 2013) and Rift Valley fever virus (Bird et al., 2007), and they have concluded that these viruses diversified within the past 200 years. The age of the genus has not been estimated, though there is some evidence to suggest that it is indeed ancient. For example, a clade of BEL retroelements (Cer7, 13 and 14) in *Caenorhabditis elegans* contains an envelope glycoprotein gene with unique ancestry compared to related BEL elements; it seems to have been co-opted from a phlebovirus glycoprotein (Malik et al., 2000), though the date of its acquisition is not known. Uukuniemi virus-like nucleoprotein and RdRp sequences have also been identified in the genome of the tick, *Ixodes scapularis*, but their age also remains unresolved (Katzourakis and Gifford, 2010).

The ecological model species, *Daphnia pulex* (Crustacea: Cladocera: *Daphniidae*), is a key member of freshwater communities worldwide. Cladocerans occupy a crucial trophic position, and have been extensively studied in ecotoxicology (Baird et al., 1991; Barata et al., 1998). Fungal and bacterial parasites of *Daphnia* are known, and their effects and coevolution have been described in detail (Ebert, 2008), while examples of natural viral infection in *Daphnia* are conspicuously absent. Indeed, until recently there have been no well-described cases of any viral infection in daphnids; the newly discovered ssDNA virus infection in *Daphnia mendotae* and *Daphnia retrocurva* populations represents the only confirmed case (Hewson, et al. 2013), to our knowledge, since the description of Chloriridovirus-like (Iridoviridae) particles in one population of *Daphnia magna* (Bergoin, et al. 1984). The sparsity of reported *Daphnia*-virus associations is especially surprising in light of the habitat overlap between *Daphnia* and one of the most widely-studied arbovirus vectors, mosquitoes.

The genus *Daphnia* is believed to have emerged at least 145 Mya (Colbourne and Hebert, 1996; Kotov and Taylor, 2011). Here, we describe an unexpected association between phleboviruses and daphnids based on the presence of phlebovirus RdRp-like NIRVs (PRNs) in the *D. pulex* genome. The PRNs form a monophyletic clade sister to the Uukuniemi virus group, firmly establishing this crustacean-infecting virus as a phlebovirus. We set the minimum age of this virus-host association as at least as ancient as the *D. pulex* species complex. We also consider evidence for co-option of these sequences by the host and we discuss the possible implications for the evolutionary history of the PRNs as well as this virus-host association.

Results

We discovered and assembled a dataset of 21 PRNs (Supplementary Table S1) by performing BLAST (Altschul et al., 1990) tBLASTn searches to the Joint Genome Institute's *D. pulex* (strain: The Chosen One [TCO]) genome assembly (Colbourne et al., 2011) available on GenBank using phlebovirus RdRp amino acid sequences as queries. An amino acid alignment of the 21 PRNs and four representative phleboviruses is available as Supplementary Fig. S1. In this alignment, the conserved motifs of exogenous phlebovirus RdRps (Muller et al., 1994) are labeled and are present in many of the PRNs. We used MAFFT 7 (Katoh and Standley, 2013) to create a codon alignment of the PRNs alone, and screened them for evidence of recombination using the single break point (SBP) and Genetic Algorithm Recombination Detection (GARD) methods (Pond et al., 2006) on the Datamonkey webserver (Delport et al., 2010). A single recombination breakpoint was identified with high support by SBP, but the GARD analysis identified a second breakpoint. We also performed a similar analysis for the same RdRp region of exogenous viruses in the Uukuniemi virus group and found significant support for at least one breakpoint, though KH tests did not support topological incongruence at any specific position (Supplementary Fig. S2).

Our phylogenetic analysis places all 21 PRNs in a well-supported monophyletic clade within the genus *Phlebovirus* (Fig. 1A). With regard to the evolutionary history of the PRNs, there are at least two interpretations of this tree topology. The first is that monophyly is the natural result of a single ancestral host integration event, which has subsequently undergone extensive duplication (illustrated by Fig. 1B), while the second is that this clade is the result of multiple, independent integrations of related, exogenous phleboviruses (Fig. 1C). We attempted to disqualify the multiple integration scenario by identifying homologous flanking sequences at the PRN sites in the *D. pulex* TCO genome, but the majority of such flanking regions lack evidence for homology.

To determine whether these *D. pulex* TCO PRNs are present in other species of *Daphnia*, we performed PCR on taxa throughout the genus. We consistently found taxa within the *D. pulex* species complex to be positive for PRNs, while those outside of this complex were universally negative (Fig. 2). We also blasted TCO PRNs to the *Daphnia pulicaria* hybrid genomic sequence database (dubbed the rejected one [TRO]) available at http://wfleabase.org/blast and found distinct matches for most PRNs that are present in *D. pulex* TCO (Supplementary Fig. S3). We tested whether individual PRNs are orthologous or lineage-specific by PCR amplifying from the PRN flanking regions. Importantly, some PRNs, e.g. PRN5 (Fig. 3), were successfully amplified from the flanking sequences across all *D. pulex* species complex taxa screened, indicating that such viral inserts were present in the common ancestor of the studied species. Other copies amplified only from reactions in which the primers targeted the interior PRN sequences. Still others, e.g. PRN1, could not be amplified in any lineage other than *D. pulex* TCO, regardless of the primer target.

We next performed tests for site-specific detection of selection in the *D. pulex* TCO PRN sequences using the Fast Unconstrained Bayesian Approximation (FUBAR) (Murrell et al., 2013) and the Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012) methods of the HyPhy package (Pond et al., 2005) available on the Datamonkey webserver (Delport et al., 2010). We removed PRN1 from the alignments prior to performing these analyses as our inability to amplify any orthologous copy of this PRN suggested a more recent, independent integration. Of the 855 codon positions in the alignment, FUBAR identified 367 positions under purifying selection (43% of sites) and none under diversifying selection (Fig. 4), though MEME did detect evidence of episodic diversifying selection at 25 sites (Supplementary Fig. S4). We also performed these analyses on orthologous and putatively orthologous (i.e. those that amplified from internally-primed sequences only, but showed very high sequence identity) interspecific PRNs and found reduced, though still significant evidence of purifying selection at specific sites in these sequences (Fig. 4). We found no statistical support for pervasive or episodic diversifying selection between interspecific PRN sequences.

Discussion

Our results support the hypothesis that the association of phleboviruses with blood-sucking arthropods is a sampling artifact. Daphniid crustaceans are neither bloodsucking nor closely related to dipterans or to ticks. Yet, we find evidence of prior association of a unique clade of phleboviruses with *Daphnia* in the form of at least 21 paleoviruses. It is unknown if the phleboviruses that infected *Daphnia* were transmitted to other animals—certainly

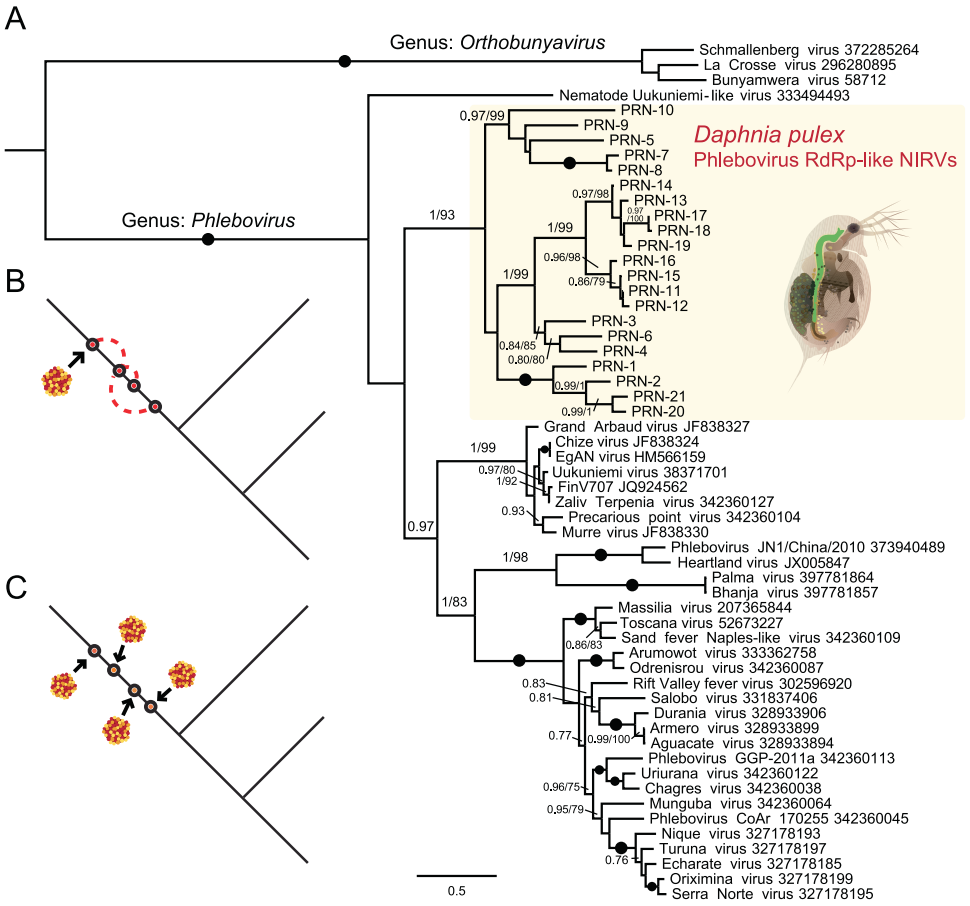

Fig. 1. Evolutionary relationship of phlebovirus RdRp-like paleoviruses (PRNs) to bunyavirus RdRp. (A) A midpoint-rooted, maximum likelihood phylogram of PRN and bunyavirus RdRp amino acid sequences showing the position of the PRNs as a monophyletic clade within the genus *Phlebovirus*. Branches are labeled with aLRT support values greater than 0.75 and bootstrap support values greater than 75. Branches marked with a solid black circle have maximum support (1 and 100, respectively). (B) and (C) Hypothetical host tree diagrams illustrating two hypotheses for the evolutionary origin of the PRN clade, where (B) represents a single viral integration followed by duplication within the host genome, and (C) represents multiple integrations of closely related viruses. Each multicolored sphere represents a single exogenous phlebovirus particle. The *D. pulex* image marking the PRN clade was created by Kim Kraeer and Lucy Van Essen-Fishman, and provided courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

viral infection does occur via ingestion and many types of animals ingest *Daphnia*. There is a need to expand discovery efforts of some RNA viruses beyond blood-sucking arthropods (Junglen and Drosten, 2013). Paleoviral knowledge of prior host associations can aid in targeting viral discovery (Taylor et al., 2013).

Our phylogenetic and flanking region analysis reveal that the association of phleboviruses with *Daphnia* is at least as old as the common ancestor of the species in the complex. Clear resolution of the relationships within the *D. pulex* species complex is an ongoing effort, but work to the present has been substantial and supports at least three major clades within the complex, one containing the North American (NA) *D. pulex* and *D. pulicaria* lineages, a second containing *Daphnia tenebrosa* and the Eurasian (EU) *D. pulicaria* lineage, and a third, well-supported as the most basal lineage of the complex, containing only the EU *D. pulex* lineage (Colbourne and Hebert, 1996; Colbourne et al., 1998; Crease et al., 2012). As shown in Fig. 2, there are other accepted members of the complex, e.g. *Daphnia melanica*, which consistently group within the NA *D. pulex/pulicaria* clade. Our PCR screens included taxa from each of these major clades and, for those reactions that targeted PRN flanking regions, establish conclusively that the oldest PRNs predate the divergence of the *D. pulex* species complex. Well-calibrated divergence dates for the entire *D. pulex* species complex have not been estimated, though estimations have been made for some of the younger clades. For example, the ages of the NA *D. pulex/D. pulicaria* and the *D. tenebrosa*/EU *D. pulicaria* clades are estimated at 2.2 and 3.2 Myr old, respectively, but the divergence of EU *D. pulex* from these clades is outside the reach of the calibration used for these estimates (see Colbourne et al., 1998 for further reading). Based on these estimations, other authors have proposed that extension of the divergence patterns suggests that the age of the entire species complex is likely 8–10 Myr old (Ambrose and Crease, 2011). Our results, therefore, highlight a surprising phlebovirus–crustacean relationship that is ancient.

The exact number of independent insertions for the phlebovirus-*Daphnia* association is unknown. Several PRNs were amplified successfully from internal sequences but not from flanking sequences. A lack of flanking sequence similarity can result from the PRN duplication process (e.g. inserted expressed NIRVs) and from independent viral insertion. From a parsimony perspective, we do not expect independent insertion of viral genome fragments to involve the same section of viral genome on multiple occasions. Indeed, eukaryotic genomes often contain NIRVs of differing sections of viral genomes. Also, the nucleotide sequence divergence of these PRNs is consistent with divergence from a common host at the root of the *D. pulex* species complex ($>90\%$ sequence identity) (Ambrose and Crease, 2011). This point is illustrated in Fig. S3, in which phylogenetic analysis of several of the internally-amplified PRNs results in distinct clades of interspecific PRNs. Multiple, independent and lineage-specific integrations would be

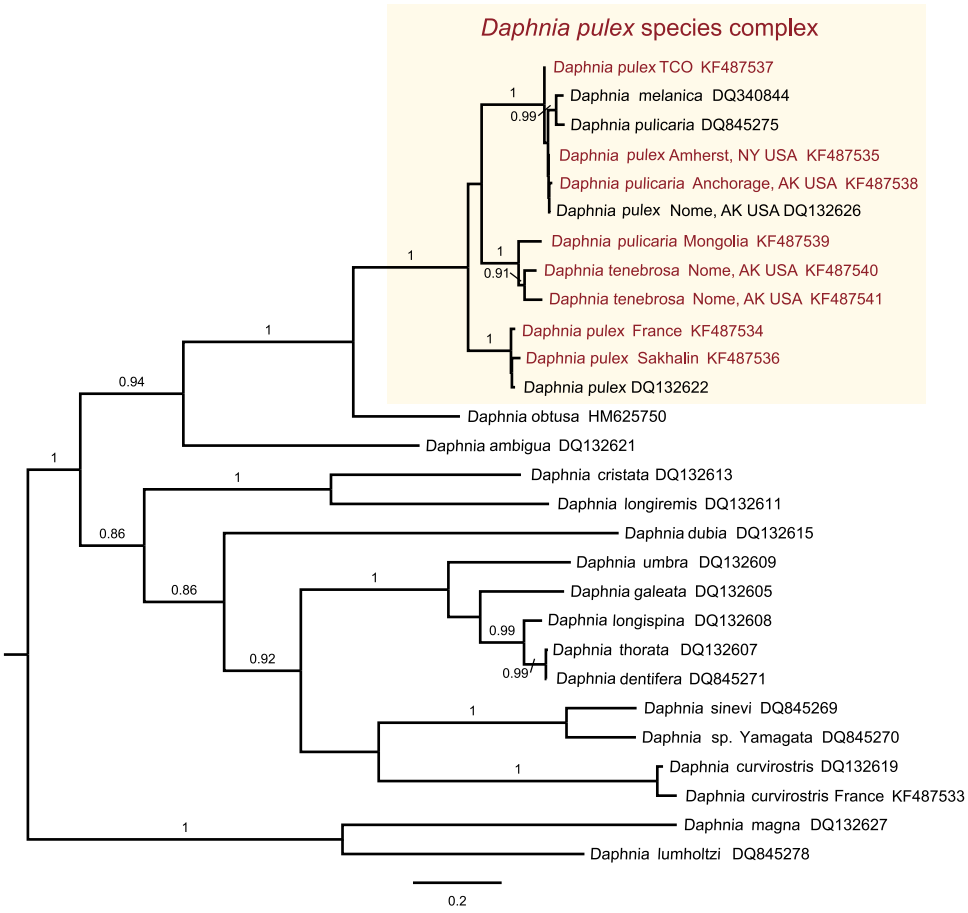

Fig. 2. Amplification of phlebovirus RdRp-like paleoviruses (PRNs) in the *D. pulex* species complex. A midpoint-rooted, maximum likelihood phylogram of ND2 mtDNA sequences of daphnids. The *D. pulex* species complex is indicated by a shaded box. Branches are labeled with aLRT support values greater than 0.85. Red taxa indicate *D. pulex* complex species that were screened for PRNs in this study. All taxa in red were positive for PRNs. *D. pulex* TCO refers to the chosen one strain. Black taxa within the *D. pulex* species complex are present for reference purposes and were not screened for PRNs. Number and letter codes following taxa are GenBank accession numbers. Some black taxa outside of the *D. pulex* complex were screened for PRNs (e.g. *Daphnia obtusa*, *Drosophila ambigua*, *Daphnia curvirostris*, *Daphnia galeata*) but these screens were universally negative. A phylogram showing the relationships between the internally-amplified PRN sequences and those identified in *D. pulex* TCO, and *D. pulicaria*, the rejected one strain, is available in Supplementary Fig. S3.

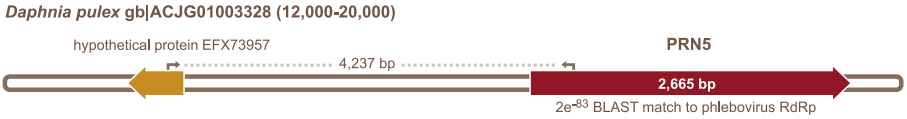

Fig. 3. Structure of the orthologous phlebovirus RdRp-like paleovirus in *Daphnia* (PRN5). Approximately 8000 base pairs of the *D. pulex* genomic sequence (GenBank ID: ACJG01003328) structure surrounding PRN5 is represented by a cartoon map. The yellow arrow indicates the nearby hypothetical protein (GenBank ID: EFX73957) targeted by one PRN5 primer (right-facing brown arrow), while the second primer (left-facing brown arrow) targeted the interior of PRN5 (red arrow). The BLAST match expected value refers to a BLASTx of PRN5 nucleotides against the non-redundant protein sequence database (nr). The direction of the red and yellow arrows indicate the orientation of their respective reading frames. The 4237 base pair product, indicated by dashed lines, was amplified in representatives of each major clade of the *D. pulex* species complex (see Fig. 2).

expected to produce a pattern of PRNs grouping by host as a result of virus–host co-divergence (assumed to be the case for a scenario in which independent integrations repeatedly occur in each host lineage through evolutionary time). The observed topology is quite different; the putatively orthologous PRN sets are nested with one or two PRNs per host in each clade, suggesting descent from a common ancestral PRN rather than several ancestral exogenous viruses. Although the balance of the evidence seems to favor single viral insertion, we cannot presently rule out additional insertions.

With this in mind, it is difficult to justify attributing the extensive purifying selection detected throughout the *D. pulex* TCO PRNs to the host rather than multiple viral ancestors. We did, however, detect significant evidence of purifying selection between interspecific PRN5 sequences, which we have shown to be orthologous, as well as between interspecific PRN2, PRN3 and PRN7 sequences, for which we have successfully amplified only internal sequences, indicating that some PRNs may have been co-opted by the host during their history. With the exception of PRN1, all of the PRNs we identified and tested for selection have acquired at least one ORF disruption, therefore it is likely that the detectable selection is a remnant of past function rather than ongoing selective maintenance in *Daphnia*. We can only speculate at this point as to what role these PRN elements might have played, but the combination of phlebovirus RdRp motif conservation and purifying selection does hint at the possibility that they were being maintained to interact with viral components.

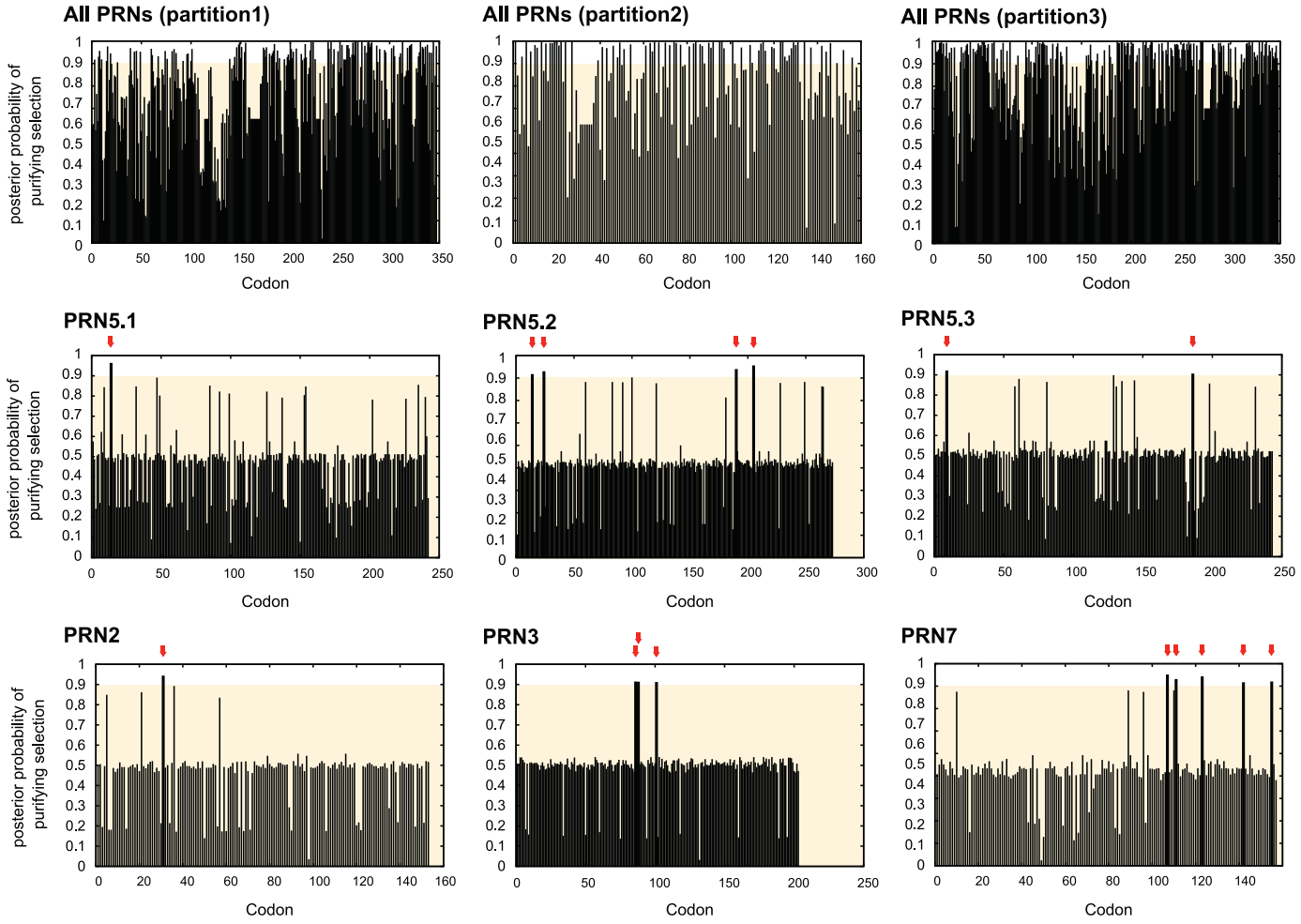

Fig. 4. Site-specific detection of purifying selection in phlebovirus RdRp-like paleoviruses (PRNs) by FUBAR. Posterior probability of purifying selection (*y* axis) is plotted against each site (*x* axis) in alignments of all the *D. pulex* TCO PRNs as well as individual orthologous (PRN5) and putatively orthologous (PRN2, 3 and 7) PRNs. The recommended significance cutoff of >0.9 is indicated by the unshaded region in the uppermost portion of each plot. For PRN2, 3, 5 and 7, in which fewer sites were identified, those positions are indicated by a heavier line weight and a downward-pointing red arrow. The partitions of the 'All PRN' tests are divided based on the recombination breakpoints identified by GARD. The three partitions of PRN5 correspond to three distinct regions of this PRN that were targeted by three unique primer sets.

The apparent absence of PRN1 from all lineages except *D. pulex* TCO is interesting in that this PRN is also the only one present as an undisrupted ORF. In the *D. pulex* TCO genome assembly, PRN1 is divided between the termini of two separate contigs that map side-by-side on the same scaffold with a predicted gap of 166 bp between them. Aligning these ends with phlebovirus RdRp sequences predicted about 810 bp of missing phlebovirus-like sequence. We performed PCR across the gap to confirm that the intervening sequence was phlebovirus-like and maintained the reading frame. Amplification of the anticipated 800 base pairs was successful in *D. pulex* TCO, but failed in all other lineages screened. Primer mismatch is a possibility (we attempted several unique primer sets), but the high nucleotide similarity between many members of this species complex coupled with the success of the other PRN amplifications suggests that PRN1 is simply not present, either by loss or by unique integration in *D. pulex* TCO. Supporting the latter is the fact that PRN1 is present as an uninterrupted 2985 bp open reading frame, yet has no identifiable RNA transcript in the *D. pulex* expressed sequence tags (EST) database or through our own RT-PCR experiments. If PRN1 were ancient and at one time present throughout the *D. pulex* species complex, then it would likely have been selectively maintained to preserve the ORF for millions of years until the present. Under this scenario, it should be one of the PRNs for which expressed transcripts and orthologous genomic copies could be easily identified. It seems more likely that exclusivity of PRN1 to *D. pulex* TCO and its uninterrupted ORF are side effects of a younger integration. The only piece of evidence to support an older PRN1 integration is the incomplete fragment of PRN1-like sequence we identified in the *D. pulicaria* TRO genome (Fig. S3).

Conclusions

We have presented paleovirological evidence of a virus–host association between *Daphnia* and a virus in the genus *Phlebovirus*, which represents the first confirmed case of a natural RNA virus infection in *Daphnia*, to our knowledge. This historical association expands the known host range of phleboviruses to include non-hematophagous arthropods, and establishes a minimum age for the genus as millions of years old. While we are uncertain of the role these paleoviruses may have played, if any, in this virus–host association, our data suggests that for some period of time during their evolutionary history they were co-opted by the host. Though little is yet known about the origins and scope of virally-derived gene families in eukaryotic genomes, our results contribute to a growing body of evidence that exogenous non-retroviral RNA viruses have served as novel genetic material in eukaryotic genomes throughout evolutionary time.

Materials and methods

PRN identification and sequence alignment

An initial dataset of phlebovirus RdRp-like NIRVs was identified by BLAST (Altschul et al., 1990) tBLASTn searches of the *D. pulex* JGI draft genome assembly v1.0 (GenBank ID: PRJNA12756) using phlebovirus amino acid sequences as queries (Supplementary Table S1). We also used the translated PRN-1 sequence to identify PRNs that match to more divergent regions of phlebovirus RdRp. We reversed the query process for every PRN in our final dataset to confirm that the best viral match was within the genus *Phlebovirus*. To produce codon alignments, PRN nucleotide sequences were blasted against phlebovirus RdRp amino acid sequences and high-scoring segment pairs (HSPs) for each PRN were concatenated and codon aligned to phlebovirus RdRps using the translational alignment tool in Geneious 5.6.5 (created by Biomatters, available from http://www.geneious.com) with the MAFFT 7 alignment algorithm (Katoh and Standley, 2013). When necessary, Ns were inserted between HSPs to match the phlebovirus reading frame.

Flanking sequence comparison

We used the GEvo tool on the Comparitive Genomics (CoGe) (Lyons et al., 2008) webserver to perform nucleotide sequence alignments under the BlastZ algorithm (Schwartz et al., 2003) for large region alignments. We used a minimum cutoff size of 1 kb per HSP. We deemed flanking sequences to be homologous if >4 HSPs were identified directly flanking the PRN locations (or fewer HSPs of >4 kb).

Phylogenetic analyses

To demonstrate the position of the PRNs within bunyaviruses, PRN and viral nucleotide sequences were translated and aligned with the MAFFT 7 plugin in Geneious. Maximum likelihood analyses were carried out with PhyML 3.0 as implemented by SeaView 4.3.5 (Gouy et al., 2010) for SH-like approximate likelihood ratio tests and RAxML (Stamatakis, 2006) implemented by RaxML GUI (Silvestro and Michalak, 2012) for 1000 bootstrapping replicates. Both methods used the LG substitution model with the gamma parameter for among site rate variation (+G). The subtree pruning and regrafting (SPR) tree-searching algorithm was used with 5 random starts. *Daphnia* ND2 sequences were codon aligned with MAFFT 7.0 in Geneious and tree-building was done with PhyML 3.0 in SeaView under the GTR+I+G model and SPR.

Selection analyses

Our partitioned PRN alignments were screened for evidence of selection with FUBAR and MEME. MEME was run under the HKY rate substitution parameters with the recommended posterior probability cutoff of 0.1 for MEME and 0.9 for FUBAR. Prior to submitting the PRN sequences for these analyses, they were partitioned according to the predicted recombination breakpoints.

Nucleotide extraction and PCR amplification

Specimens of *Daphnia* were collected (collection sites available in Table S2) and identified based on morphological characteristics. Specimens were crushed in 50 ul of Epicentre QuickExtract DNA extraction solution and incubated at 62 °C for 1–2 h then for 10 min at 95° C to denature proteinase K. A quantity of 50–100 ng of nucleic acid template was used per PCR reaction. A complete list of the primer sequences used in this study is available in Supplementary Table S3. For the PRN screening reactions, thermal cycling was performed with an initial Taq polymerase activation heat step at 95 °C for 2 min followed by 40 cycles of 95 °C for 30° s, 53 °C for 30 s, and 72 °C for 1 min. The gap in the PRN-1 region of the genome assembly was amplified from *D. pulex* TCO nucleic acids extracted as above. Thermal cycling was performed with an initial Taq polymerase activation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s (a 0.5 °C reduction was done for each cycle until 55 °C was reached and used for the remaining cycles), and 72 °C for 2 min 30 s. Thermal cycling for internal PRN5 targets was performed with an initial Taq polymerase activation step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 57 °C (for PRN5.2 and 5.3) or 54 °C (for PRN5.1) for 30 s (a 0.5 °C reduction was done per cycle until 54° [5.2 and 5.3] or 51 °C [5.1] was reached and used for the remaining cycles), and 72 °C for 1 min. Thermal cycling for ND2 reactions was performed with an initial Taq polymerase activation step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min 15 s. A final extension step of 72 °C for 10 min was done for all PCR reactions.

Long PCR was performed to demonstrate orthology for PRN5. TaKaRa LA Taq was used to amplify the 4237 bp product across species within the *D. pulex* species complex. Thermal cycling was performed with an initial denaturation step of 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 68 °C for 4 min 30 s. A final extension step of 72 °C for 10 min was also done for this reaction.

An RT-PCR screen for an RNA transcript of PRN-1 was also carried out, with negative results. RNA was extracted from *D. pulex* TCO using the Qiagen RNeasy Mini Kit and treated with Promega RQ1 DNase I. We used the Qiagen OneStep RT-PCR kit to carry out cDNA synthesis and amplification. The primers were successfully used to amplify genomic DNA but failed to amplify from an RNA template. A control for RNA amplification (targeting 16S rRNA) was positive. Thermal cycling included a cDNA synthesis step at 51/47 °C for 45 min, a reverse transcriptase deactivation/Taq activation heat step of 94 °C for 15 min, followed 35 cycles of 94 °C for 30 s, 51/47 °C for 30 s, and 72 °C for 2 min. All PCR and RTPCR products were separated by DNA agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.

Author contributions

All authors contributed to the experimental design, analyses and manuscript preparation. DJT, AAK and MJB collected and identified specimens of *Daphnia*. MJB carried out the bioinformatics analyses and PCR experiments.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.07.032. Sequence data are available on GenBank under accession numbers KF487515-KF487541.

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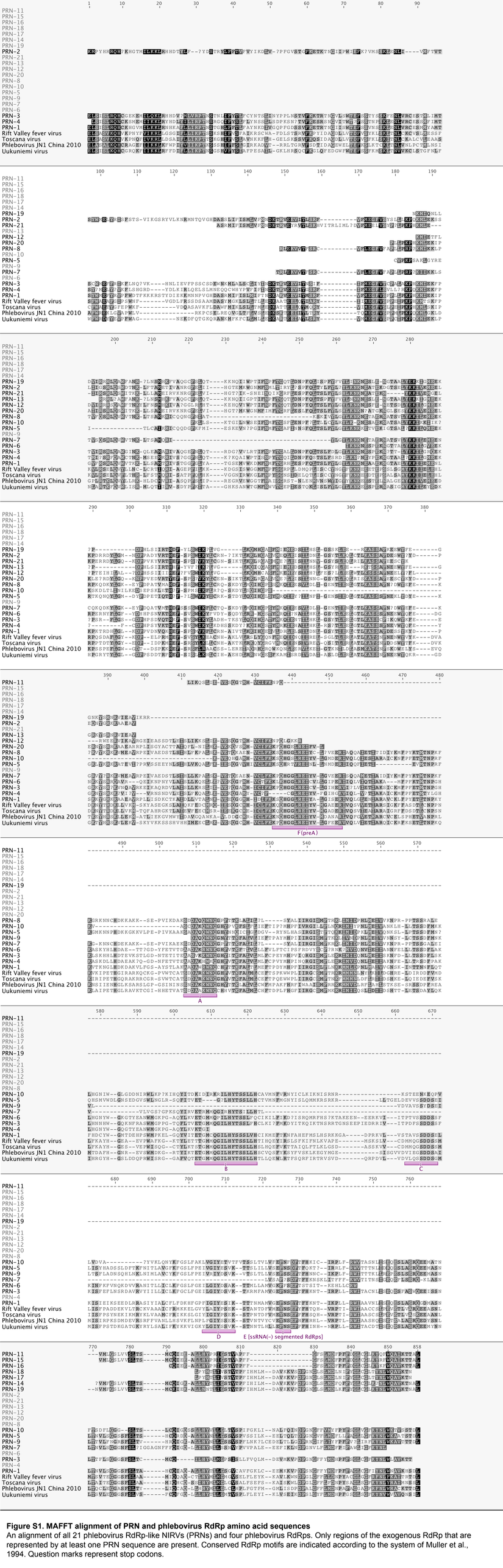


Figure S1. MAFFT alignment of PRN and phlebovirus RdRp amino acid sequences
An alignment of all 21 phlebovirus RdRp-like NIRVs (PRNs) and four phlebovirus RdRps. Only regions of the exogenous RdRp that are represented by at least one PRN sequence are present. Conserved RdRp motifs are indicated according to the system of Muller et al., 1994. Question marks represent stop codons.

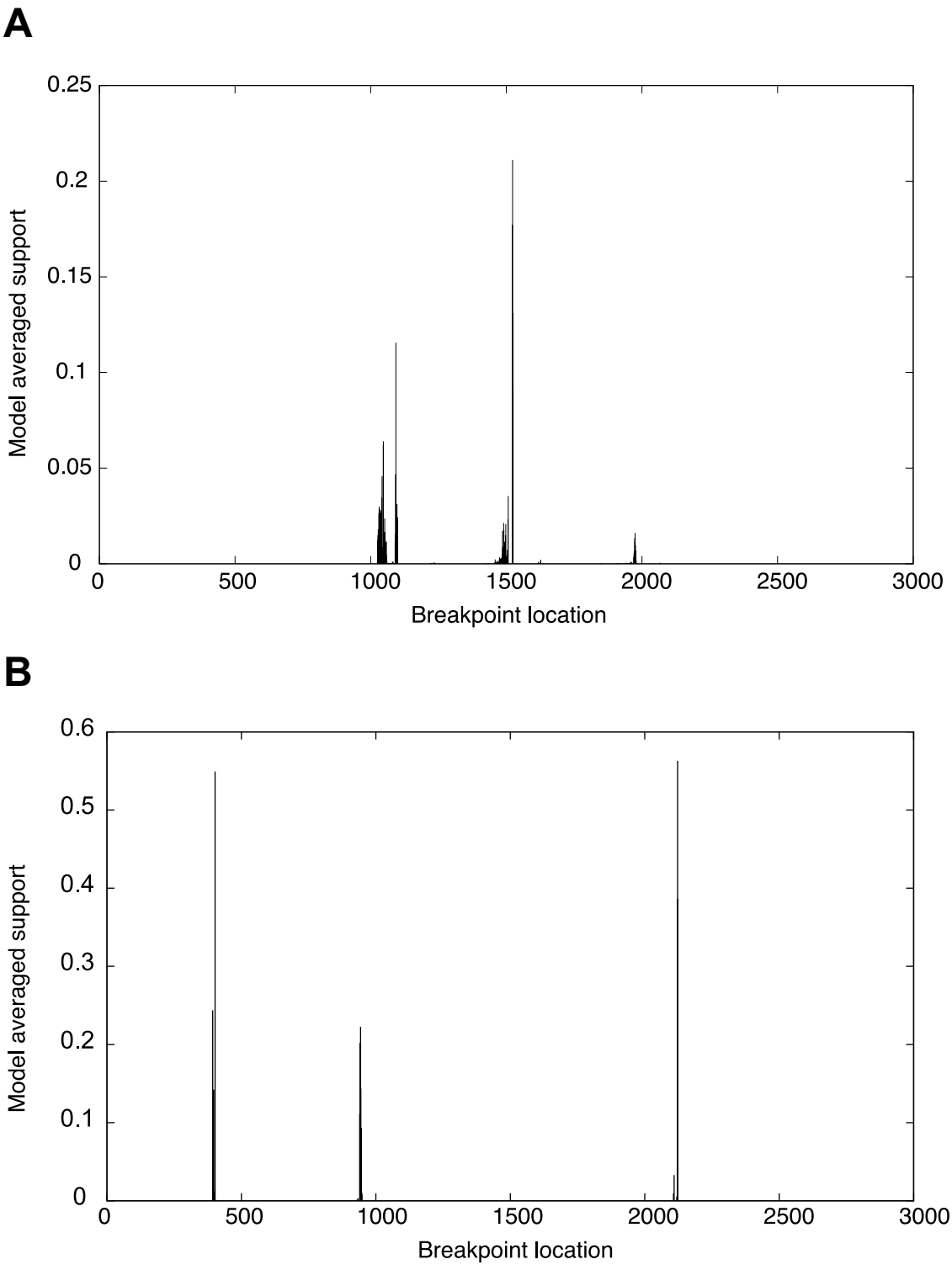


Figure S2. GARD analysis for detection of recombination in PRN and phlebovirus sequences

Detection of putative recombination breakpoints in A) PRN sequences and B) exogenous Uukuniemi group phleboviruses identifed evidence for multiple breakpoints in each. Akaike information criterion support values (y axis) are plotted against nucleotide positions (x axis). KH tests supported the PRN breakpoints at $p<0.001$, while they failed to support the phlebovirus breakpoints at $p<0.05$.

!

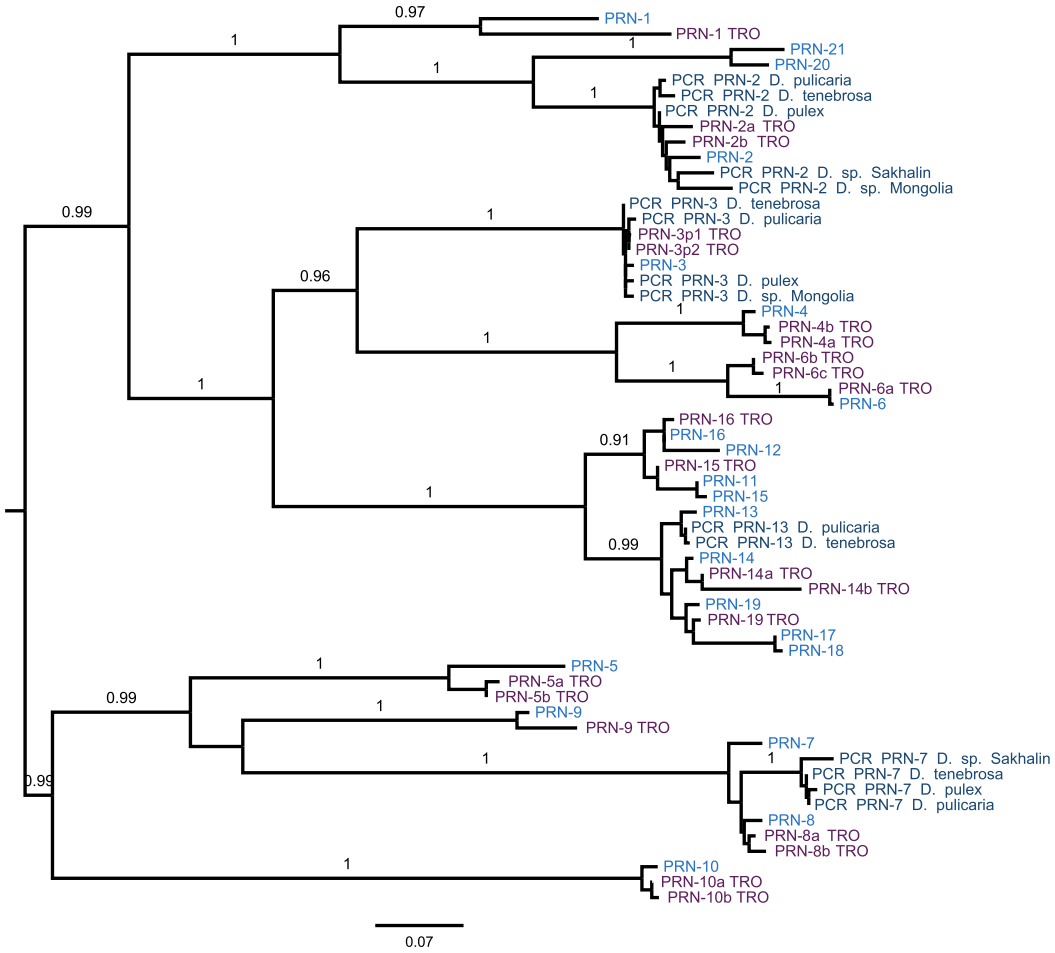
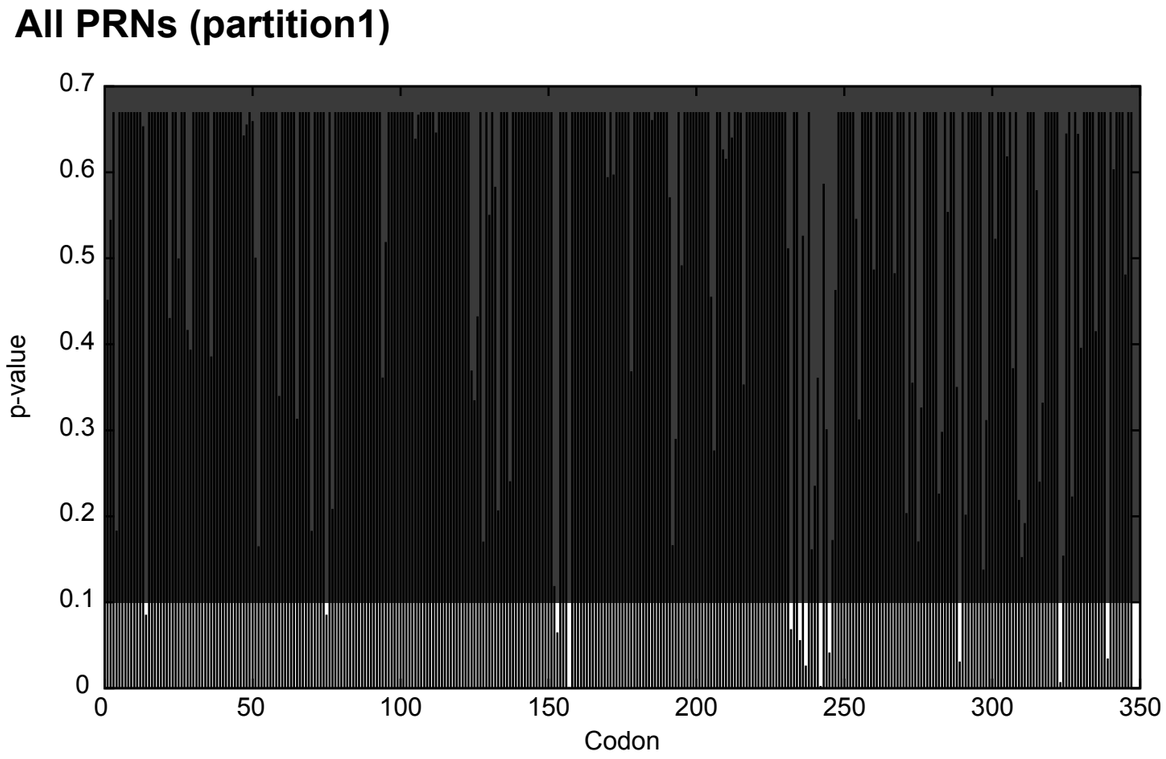
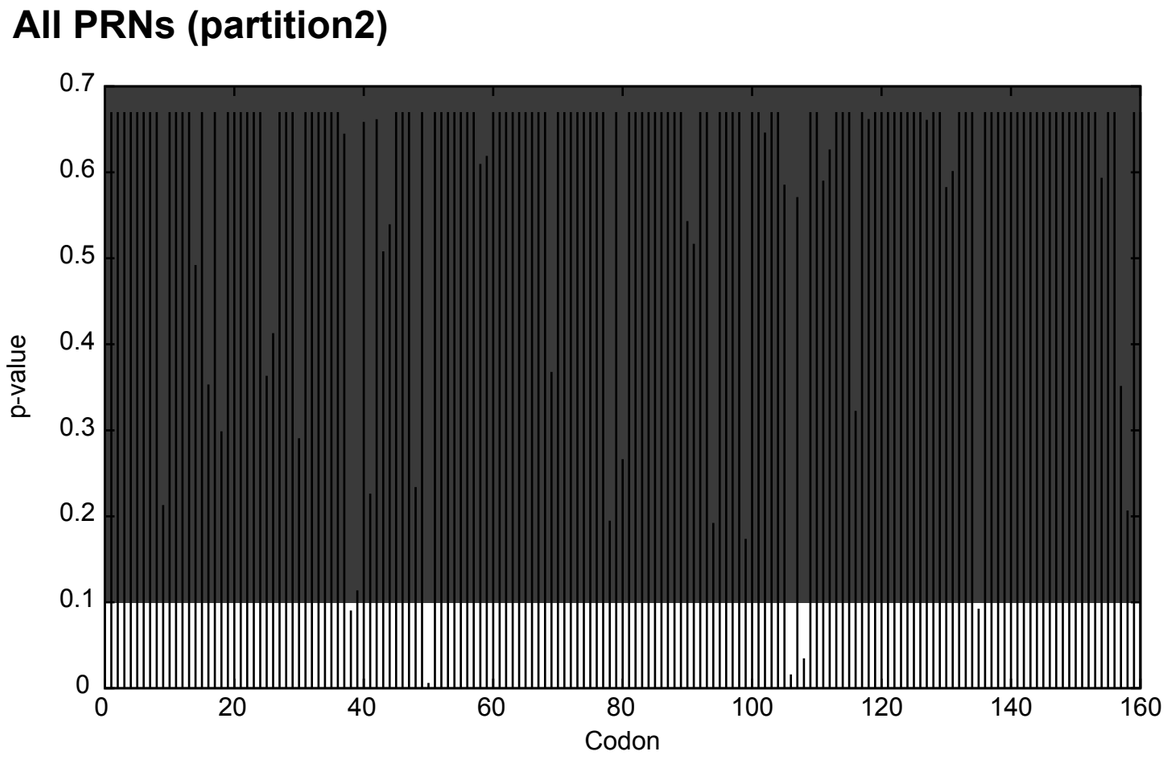


Figure S3. Evolutionary relationships of PRNs in the *Daphnia pulex* species complex

Midpoint-rooted maximum likelihood phylogram of phlebovirus RdRp-like NIRVs (PRNs) in the *Daphnia pulex* TCO genome (light blue), the *Daphnia pulicaria* hybrid TRO genome (purple), and amplified by PCR in related members of the *Daphnia pulex* species complex (dark blue). Branches are labeled with aLRT support values ≥0.9.





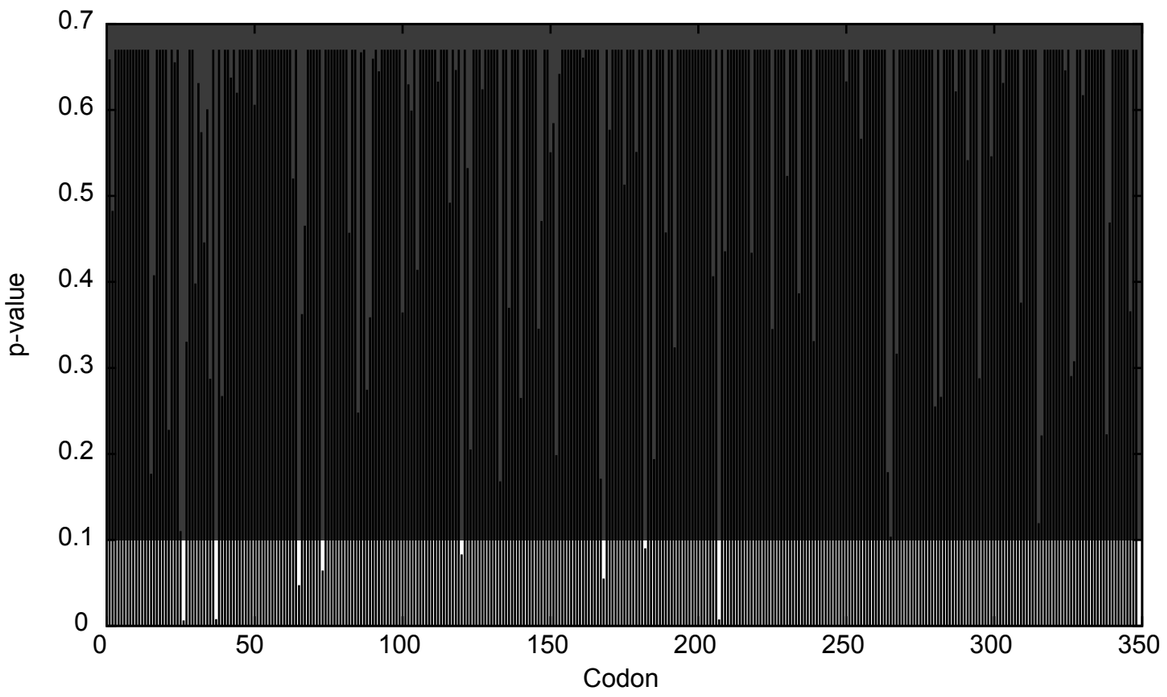


Figure S4. Site-specific detection of episodic diversifying selection in PRNs by MEME.
Posterior probability of episodic diversifying selection (y axis) is plotted against each site (x axis) in alignments of all the *Daphnia pulex* TCO PRNs. The recommended significance cutoff of <0.1 is indicated by the unshaded region at the base of each plot. Significant evidence of episodic diversifying selection was detected at 25 sites across all three alignment partitions.

Table S1. Phlebovirus RdRp-like sequences identified by tBlastn searches of the Daphnia pulex genome

PRN ID	Contig Accession #	Position	Strand	tBLASTn E value	Query	Corresponding EST
PRN-1	ACJG01002368 - 69	24394-25953; 1-620	+	1 E-78; 8 E-47	HM566159	None detected
PRN-2	ACJG01001533	16086-18594	-	2 E-37	HM566159	None detected
PRN-3	ACJG01004899	31195-33601	+	3 E-132	HM566159	None detected
PRN-4	ACJG01004740	12769-14779	+	4 E-103	HM566159	None detected
PRN-5	ACJG01003328	16846-19277	+	7 E-76	HM566159	None detected
PRN-6	ACJG01001371	18420-20123	-	3 E-51	HM566159	None detected
PRN-7	ACJG01006381	54924-56994	-	1 E-59	HM566159	None detected
PRN-8	ACJG01000925	19739-21820	+	1 E-44	HM566159	None detected
PRN-9	ACJG01002210	42617-43644	+	5 E-37	HM566159	None detected
PRN-10	ACJG01001622	62176-64191	-	5 E-42	HM566159	None detected
PRN-11	ACJG01006902	3110-6764	-	4 E-8	HM566159	None detected
PRN-12	ACJG01005787	359-1048	-	5 E-14	HM566159	None detected
PRN-13	ACJG01005795	1256-1788	+	6 E-8	HM566159	None detected
PRN-14	ACJG01017696	159-392	-	2 E-09	HM566159	None detected
PRN-15	ACJG01012275	1626-1851	-	2 E-11	PRN-1 (this study)	None detected
PRN-16	ACJG01007866	7420-7615	-	1 E-9	PRN-1 (this study)	None detected
PRN-17	ACJG01003720	354-595	-	5 E-10	PRN-1 (this study)	None detected
PRN-18	ACJG01006910	1310-1551	+	5 E-9	PRN-1 (this study)	None detected
PRN-19	ACJG01015620	1903-3285	+	5 E-12	HM566159	None detected
PRN-20	ACJG01000925	22160-22974	-	6 E-32	HM566159	FE406379, E = 0
PRN-21	ACJG01006381	53637-54578	+	9 E-32	HM566159	FE406378, E = E-131

21 phlebovirus RdRp-like sequences were identified in the *Daphnia pulex* genome by tBlastn searches using a phlebovirus RdRp sequence (accession number provided) and the amino acid translation of PRN-1. PRN number designations are arbitrary. Subject accession numbers refer to D. pulex contigs. tBlastn E value refers to the blast expected value score; matches greater than E-05 were not retained.

Table S2. Species of Daphnia that were PCR-screened for PRNs

Daphnia sp.	Location name	Country	Coordinates
D. pulicaria	Birch Lake	AK, USA	61.14558, -149.9384
D. tenebrosa	Glacial-08N	AK, USA	64.82526, -165.7465
D. pulex	Nome-04-2012	AK, USA	64.4777198, -165.2344387
D. tenebrosa	Taylor-12-2011	AK, USA	65.384906, -164.660289
D. pulex	Amherst	NY, USA	43.028756, -78.754544
D. curvirostris	Argeles plage (Pyrénées Orientales)	France	42.57499, 3.044131
D. pulicaria	A small puddle near Bayan nuur (AAKM-0761)	Mongolia	48.45144, 95.17455
D. pulex	A small forest pool (AAK M-0873)	Sakhalin, RU	47.31642, 142.7005
D. pulex	Marnay-sur-Seine (AAK M-1421)	France	48.53444, 3.574445
D. catawba	Laurel Lake	NY, USA	40.978736, -72.557425
D. obtusa	BDW-1	Unavailable	Unavailable
D. galeata	Laurel Lake	NY, USA	40.978889, 23.209167
D. cf. pulex	Pond near Hibara Park	Tokoname, Japan	34.857815, 136.888565
D. curvirostris	Pilgrim700	AK, USA	65.08552, -164.9267
D. dentifera	Teller-08-2012	AK, USA	65.0374609, -166.1610238

Taxonomic names, geographic locations and GPS coordinates are listed for each species of *Daphnia* we screened for phlebovirus RdRp-like paleoviruses in this study.

S3. PCR primer sequences

Target	Primer 1	Primer 2
PRN2 internal	GTGACAAGGATCAATCCAAATCCG	GGTGTGCTTTGTTTCACAATCCAG
PRN3 internal	GGAAGAATTGGGAACCCTAAAAGCC	GCCCCAGTGATTGTTATGTAGTTTTGA
PRN5.1 internal	CTGTTCCAAGTCTCAATCTCTGTTCTGG	CTGCTTAGTCCTCTTTTCTTCCATCTGC
PRN5.2 internal	TGCATTCATCAACAACTCCTTCCAACTGACC	GTGAACGTTGAAATCCCTCGTATGGATGC
PRN5.3 internal	TCAAGGCTGCTAAGTCAGCAGATGC	AGGTTGGAGGCCATTTCCTCTTGCC
PRN7 internal	CAGTGTCCCAAAGCAGACTACAG	GCTTCCTGTGTCTCCACTTTCG
PRN5 flanking	GTCTTCTCCCGAGTAGTAATTGTAGACG	CATTCATAAAAATAAGCAAGCTGACGGC
PRN1 transcript screen	AAGCATGGTTAAGTTCTTATGCCG	GCTTGTGCAATCTGACAATGAGC
PRN1 assembly gap	AGTGTTCAAGCCAGAAGATTACTGC	GCTTGTGCAATCTGACAATGAGC
PRN1 assembly gap	"	GCCTAGCTCTTTGGGCATCCAGC
ND2	GTTCATGCCCCATTTATAGGTTA	GAAGGTTTTTAGTTTAGTTAACTTAAA

The sequences of PCR primer pairs used to target and amplify phlebovirus RdRp-like paleoviruses in species of *Daphnia* in this study are listed. All sequences are in 5’ to 3’ orientation.