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Discovery of parvovirus-related sequences in an unexpected broad range of animals

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Our knowledge of the genetic diversity and host ranges of viruses is fragmentary. This is particularly true for the *Parvoviridae* family. Genetic diversity studies of single stranded DNA viruses within this family have been largely focused on arthropod- and vertebrate-infecting species that cause diseases of humans and our domesticated animals: a focus that has biased our perception of parvovirus diversity. While metagenomics approaches could help rectify this bias, so too could transcriptomics studies. Large amounts of transcriptomic data are available for a diverse array of animal species and whenever this data has inadvertently been gathered from virus-infected individuals, it could contain detectable viral transcripts. We therefore performed a systematic search for parvovirus-related sequences (PRSs) within publicly available transcript, genome and protein databases and eleven new transcriptome datasets. This revealed 463 PRSs in the transcript databases of 118 animals. At least 41 of these PRSs are likely integrated within animal genomes in that they were also found within genomic sequence databases. Besides illuminating the ubiquity of parvoviruses, the number of parvoviral sequences discovered within public databases revealed numerous previously unknown parvovirus-host combinations; particularly in invertebrates. Our findings suggest that the host-ranges of extant parvoviruses might span the entire animal kingdom.

Recent studies have shown that viruses are the most numerous and diverse genetic entities on Earth: a discovery that has completely changed both our views on their prevalence, and our perception that they are primarily disease-causing agents[1]. Viruses have been discovered infecting organisms throughout the entire tree of life, using a wide array of strategies to move between and infect hosts belonging to either the same or different species. The genomes of many viruses can also ligate to, and become a heritable part of, the genetic material of their hosts[2].

Largely because of their obvious medical and economic importance, the vast majority of viruses that have so far been studied are those that cause recognizable diseases of humans and our domesticated plants (almost exclusively angiosperms) and animals (mainly mammals and birds)[3,4]. One of the greatest achievements of environmental metagenomics has been the discovery that unknown viral species vastly outnumber the known species, and that there probably also remain more unknown genera (and possibly also entire families) than those we have currently discovered[4,5]. For example, it is now estimated that the ~2800 virus species that are currently recognised by the International Committee on Taxonomy of Viruses[6] (ICTV probably account for less than 1% of all viral species on Earth[7]. Our rapidly expanding appreciation of the actual diversity of viruses on Earth is well illustrated in the recent discoveries of viruses with ssDNA genomes that likely belong to multitudes of novel genera/families which are both genetically highly divergent from species in the known ssDNA virus families (such as parvoviruses, circoviruses, microviruses and geminiviruses) and likely infect hosts that span the entire tree of life[8–14].

Parvoviruses illustrate the chasm that likely exists between the known diversity of species within particular virus families, and that which actually exists in all of their potential animal hosts. The linear ssDNA viruses belonging to the family *Parvoviridae* are presently divided into two sub-families: the *Parvovirinae*, which contains

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species infecting vertebrates (which have, to date, mostly been birds and mammals), and the *Densovirinae*, which contains species infecting arthropods[10] (which have, to date, mostly been crustaceans and insects). Parvovirus genomes are characteristically 4 to 6 kb long, with inverted terminal repeats (ITRs) that bracket two sets of genes encoding non-structural (Rep or NS) and structural (VP) proteins[15]. While degrees of sequence identity between viruses from different parvovirus genera are very low (e.g. some pairs of densoviruses share <15% VP sequence identity), most parvoviruses likely express both a NS1 protein with a super family 3 helicase (SF3) domain in the C terminus and a VP containing a unique phospholipase A2 (PLA2) motif in the N terminus[16,17]. These two proteins, even if the PLA2 motif is missing in some parvoviruses[16], are therefore useful for parvovirus phylogenetic inferences.

Our current appreciation of parvovirus diversity is limited to the 41 *Parvovirinae* and 15 *Densovirinae* species which are presently recognized by the ICTV[18]. We estimated that only a few hundred animal species have been reported as hosts of parvoviruses, representing only approximately 0.0001% of the 1.2 million animal species that have presently been described[19]. There is likely a particularly extreme imbalance of sampling between the vertebrate-infecting *Parvovirinae* and the arthropod-infecting *Densovirinae* in that there are likely almost 20 times more arthropod species on Earth than there are species of vertebrates[19]. Given the diversity in sequence and genome organisation displayed by parvoviruses, it is entirely plausible that parvoviruses are ubiquitous in the environment, and that there exist tens of thousands of undiscovered vertebrate parvoviruses, and hundreds of thousands of undiscovered arthropod parvoviruses. Indeed, high throughput sequencing technologies and the advent of routine whole genome sequencing of eukaryotes have revealed the occurrence of "fossil" parvovirus sequences integrated into the genomes of multiple animals including those of humans. While these integrated sequences reflect a history of parvovirus interactions with an unexpectedly broad range of animal hosts[20,21], the recent discovery of a densovirus associated with sea-star wasting disease supports the hypothesis that the host range of extant parvoviruses probably extends far beyond the major animal phyla in which they have already been detected[16]. This discovery also raises questions about whether densoviruses are predisposed to frequently shifting hosts, or whether they may have existed and co-evolved with their hosts during the early evolutionary radiation of multi-cellular animals[22,23].

Parvoviruses belonging to the *Dependovirus* genus may depend on another virus to complete their replication-deficient life cycle. These viruses can be persistent and include a host genome integration step that results in latent infections, as has been shown for *Adeno-Associated-Virus* (AAV) where integration requires the NS1 homologue, Rep[24]. Integration and persistence of densoviruses may in some cases even be beneficial for their hosts in that it could protect them against viral infections[25,26].

We therefore hypothesised that while transcriptome datasets might contain evidence of parvovirus sequences originating either from either *bona fide* transmissible episomal viruses, or from integrated (albeit possibly only transiently) viruses or viral sequence elements (from heritably integrated but still transcriptionally active parvovirus genes), animal genome sequences might contain evidence of heritably integrated, and possibly transcriptionally dormant, parvovirus sequence elements. Crucially, large volumes of transcriptomic and genomic sequence data for a wide array of animal species are currently available in public databases. Several studies have already revealed the presence of a variety of novel RNA and DNA virus sequences within transcriptome datasets[25–28] (including those of animal-model organisms and environmental transcriptomes). We opted to initially focus our search for novel parvoviruses within transcriptome datasets from animals covering vertebrate and invertebrate phyla, spanning the entire animal kingdom and representing an extend of in depth searches that has not been achieved so far for parvoviruses.

The results presented here highlight the extraordinary diversity, abundance and ubiquity of expressed parvoviral sequences in numerous animal phyla, revealing previously unknown parvovirus-host associations–particularly with invertebrates including arthropods, molluscs, annelids, nematodes, and cnidarians–and supporting the hypothesis that the collective host ranges of extant parvoviruses might indeed span the entire animal kingdom.

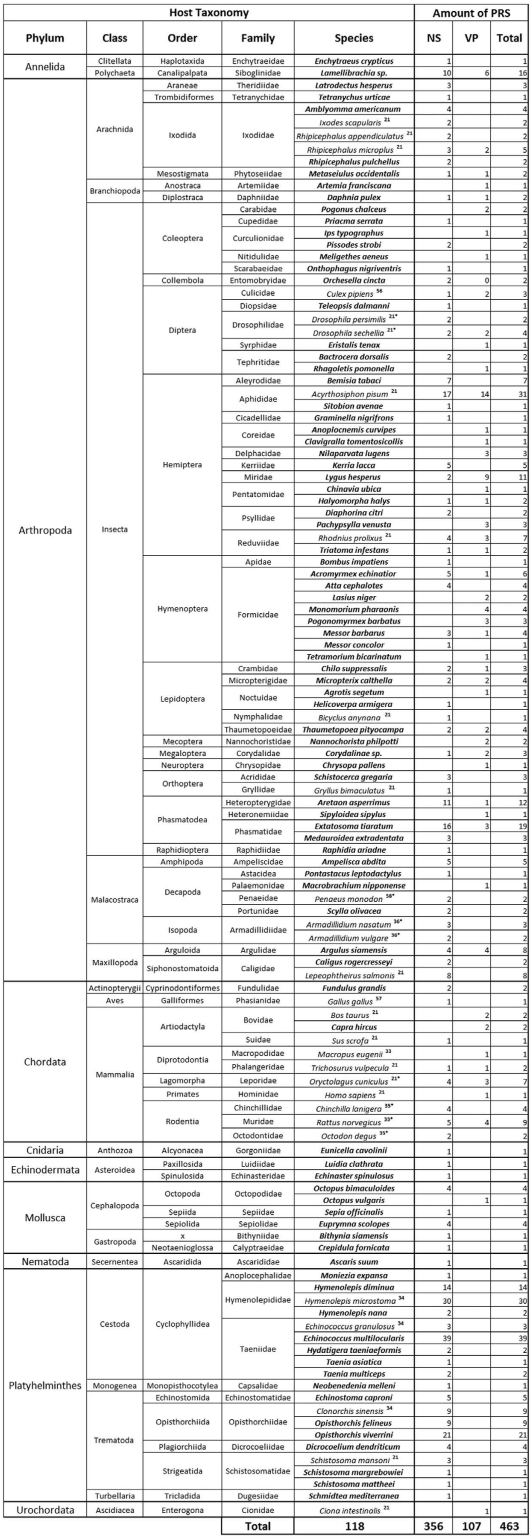
Results

Identification of parvovirus-related sequences in animal transcriptome datasets.

We used 74 representative parvovirus genomes as queries (Supplementary Table S1) to perform BLASTX searches against the National Centre for Biotechnological Information (NCBI) non-redundant (nr) cDNA expressed sequence tag (EST), transcriptome shotgun assembly (TSA) and protein (Uniprot) databases[29,30]. We also used these as queries to screen, eleven new transcriptomes generated from invertebrate datasets provided by N. Galtier (European Research Council advanced grant 232971 (PopPhyl)). All hits were next selected and used as queries to perform BLASTX or BLASTP reciprocal searches of the NCBI non-redundant sequence database (as described in the materials and methods).

Three hundred and fifty-six homologues of parvoviral non-structural protein (NS) sequences and 107 homologues of capsid protein (VP) sequences, from partial to near complete coding sequences (Fig. 1 and Supplementary Table S2), were recovered from the NCBI transcriptome (230 sequences) and genome (206 sequences) databases and from the 11 new invertebrate transcriptome datasets (27 sequences found in 8/11 datasets). These 463 PRSs were found from the transcriptomes and genomes of 118 animal species, including 74 arthropods, 19 platyhelminthes, 12 vertebrates, six molluscs, two echinoderms, two annelids, one tunicate, one nematode, and one cnidarian (Fig. 1).

Overall, 89 potentially new parvovirus host species were identified, including species belonging to animal phyla with no previously known parvovirus host species: *Mollusca*, *Annelida*, *Nematoda* and *Cnidaria* (Fig. 1). Whereas the 88% of PRSs that were recovered from 95 invertebrates (including 69 arthropods) were more similar to viruses belonging to the arthropod-infecting *Densovirinae* subfamily, the remaining 12% of PRSs that were recovered from 12 vertebrate species and 4 molluscs were more similar to vertebrate-infecting viruses in the *Parvovirinae* subfamily (Supplementary Table S2). Among arthropods, PRSs were found in species within classes



Host Taxonomy					Amount of PRS		
Phylum	Class	Order	Family	Species	NS	VP	Total
Annelida	Clitellata	Haplotaxida	Enchytraeidae	***Enchytraeus crypticus***	1		1
	Polychaeta	Canalipalpata	Siboglinidae	***Lamellibrachia sp.***	10	6	16
Arthropoda	Arachnida	Araneae	Theridiidae	***Latrodectus hesperus***	3		3
		Trombidiformes	Tetranychidae	***Tetranychus urticae***	1		1
		Ixodida	Ixodidae	***Amblyomma americanum***	4		4
				Ixodes scapularis [21]	2		2
				Rhipicephalus appendiculatus [21]	2		2
				Rhipicephalus microplus [21]	3	2	5
				Rhipicephalus pulchellus	2		2
		Mesostigmata	Phytoseiidae	***Metaseiulus occidentalis***	1	1	2
	Branchiopoda	Anostraca	Artemiidae	***Artemia franciscana***		1	1
		Diplostraca	Daphniidae	***Daphnia pulex***	1	1	2
	Insecta	Coleoptera	Carabidae	***Pogonus chalceus***		2	2
			Cupedidae	***Priacma serrata***	1		1
			Curculionidae	***Ips typographus***		1	1
				Pissodes strobi	2		2
			Nitidulidae	***Meligethes aeneus***		1	1
			Scarabaeidae	***Onthophagus nigriventris***	1		1
		Collembola	Entomobryidae	***Orchesella cincta***	2	0	2
		Diptera	Culicidae	*Culex pipiens* [56]	1	2	3
			Diopsidae	***Teleopsis dalmanni***	1		1
			Drosophilidae	*Drosophila persimilis* [21*]	2		2
				Drosophila sechellia [21*]	2	2	4
			Syrphidae	***Eristalis tenax***		1	1
			Tephritidae	***Bactrocera dorsalis***	2		2
				Rhagoletis pomonella		1	1
		Hemiptera	Aleyrodidae	***Bemisia tabaci***	7		7
			Aphididae	*Acyrthosiphon pisum* [21]	17	14	31
				Sitobion avenae	1		1
			Cicadellidae	***Graminella nigrifrons***	1		1
			Coreidae	***Anoplocnemis curvipes***		1	1
				Clavigralla tomentosicollis		1	1
			Delphacidae	***Nilaparvata lugens***		3	3
			Kerriidae	***Kerria lacca***	5		5
			Miridae	***Lygus hesperus***	2	9	11
			Pentatomidae	***Chinavia ubica***		1	1
				Halyomorpha halys	1	1	2
			Psyllidae	***Diaphorina citri***	2		2
				Pachypsylla venusta		3	3
			Reduviidae	*Rhodnius prolixus* [21]	4	3	7
				Triatoma infestans	1	1	2
		Hymenoptera	Apidae	***Bombus impatiens***	1		1
			Formicidae	***Acromyrmex echinatior***	5	1	6
				Atta cephalotes	4		4
				Lasius niger		2	2
				Monomorium pharaonis		4	4
				Pogonomyrmex barbatus		3	3
				Messor barbarus	3	1	4
				Messor concolor	1		1
				Tetramorium bicarinatum		1	1
		Lepidoptera	Crambidae	***Chilo suppressalis***	2	1	3
			Micropterigidae	***Micropterix calthella***	2	2	4
			Noctuidae	***Agrotis segetum***		1	1
				Helicoverpa armigera	1		1
			Nymphalidae	*Bicyclus anynana* [21]	1		1
			Thaumetopoeidae	***Thaumetopoea pityocampa***	2	2	4
		Mecoptera	Nannochoristidae	***Nannochorista philpotti***		2	2
		Megaloptera	Corydalidae	***Corydalinae sp.***	1	2	3
		Neuroptera	Chrysopidae	***Chrysopa pallens***		1	1
		Orthoptera	Acrididae	***Schistocerca gregaria***	3		3
			Gryllidae	*Gryllus bimaculatus* [21]	1		1
		Phasmatodea	Heteropterygidae	***Aretaon asperrimus***	11	1	12
			Heteronemiidae	***Sipyloidea sipylus***		1	1
			Phasmatidae	***Extatosoma tiaratum***	16	3	19
				Medauroidea extradentata	3		3
		Raphidioptera	Raphidiidae	***Raphidia ariadne***	1		1
	Malacostraca	Amphipoda	Ampeliscidae	***Ampelisca abdita***	5		5
		Decapoda	Astacidea	***Pontastacus leptodactylus***	1		1
			Palaemonidae	***Macrobrachium nipponense***		1	1
			Penaeidae	*Penaeus monodon* [58*]	2		2
			Portunidae	***Scylla olivacea***	2		2
		Isopoda	Armadillidiidae	*Armadillidium nasatum* [36*]	3		3
				Armadillidium vulgare [36*]	2		2
	Maxillopoda	Arguloida	Argulidae	***Argulus siamensis***	4	4	8
		Siphonostomatoida	Caligidae	***Caligus rogercresseyi***	2		2
				Lepeophtheirus salmonis [21]	8		8
Chordata	Actinopterygii	Cyprinodontiformes	Fundulidae	***Fundulus grandis***	2		2
	Aves	Galliformes	Phasianidae	*Gallus gallus* [57]	1		1
	Mammalia	Artiodactyla	Bovidae	*Bos taurus* [21]		2	2
				Capra hircus		2	2
			Suidae	*Sus scrofa* [21]	1		1
		Diprotodontia	Macropodidae	*Macropus eugenii* [33]		1	1
			Phalangeridae	*Trichosurus vulpecula* [21]	1	1	2
		Lagomorpha	Leporidae	*Oryctolagus cuniculus* [21*]	4	3	7
		Primates	Hominidae	*Homo sapiens* [21]		1	1
		Rodentia	Chinchillidae	*Chinchilla lanigera* [35*]	4		4
			Muridae	*Rattus norvegicus* [33*]	5	4	9
			Octodontidae	*Octodon degus* [35*]	2		2
Cnidaria	Anthozoa	Alcyonacea	Gorgoniidae	***Eunicella cavolinii***	1		1
Echinodermata	Asteroidea	Paxillosida	Luidiidae	***Luidia clathrata***	1		1
		Spinulosida	Echinasteridae	***Echinaster spinulosus***	1		1
Mollusca	Cephalopoda	Octopoda	Octopodidae	***Octopus bimaculoides***	4		4
				Octopus vulgaris		1	1
		Sepiida	Sepiidae	***Sepia officinalis***	1		1
		Sepiolida	Sepiolidae	***Euprymna scolopes***	4		4
	Gastropoda	x	Bithyniidae	***Bithynia siamensis***	1		1
		Neotaenioglossa	Calyptraeidae	***Crepidula fornicata***	1		1
Nematoda	Secernentea	Ascaridida	Ascarididae	***Ascaris suum***	1		1
Platyhelminthes	Cestoda	Cyclophyllidea	Anoplocephalidae	***Moniezia expansa***	1		1
			Hymenolepididae	***Hymenolepis diminua***	14		14
				Hymenolepis microstoma [34]	30		30
				Hymenolepis nana	2		2
			Taeniidae	*Echinococcus granulosus* [34]	3		3
				Echinococcus multilocularis	39		39
				Hydatigera taeniaeformis	2		2
				Taenia asiatica	1		1
				Taenia multiceps	2		2
	Monogenea	Monopisthocotylea	Capsalidae	***Neobenedenia melleni***	1		1
	Trematoda	Echinostomida	Echinostomatidae	***Echinostoma caproni***	5		5
		Opisthorchiida	Opisthorchiidae	*Clonorchis sinensis* [34]	9		9
				Opisthorchis felineus	9		9
				Opisthorchis viverrini	21		21
		Plagiorchiida	Dicrocoeliidae	***Dicrocoelium dendriticum***	4		4
		Strigeatida	Schistosomatidae	*Schistosoma mansoni* [21]	3		3
				Schistosoma margrebowiei	1		1
				Schistosoma mattheei	1		1
	Turbellaria	Tricladida	Dugesiidae	***Schmidtea mediterranea***	1		1
Urochordata	Ascidiacea	Enterogona	Cionidae	*Ciona intestinalis* [21]		1	1
			Total	**118**	**356**	**107**	**463**

Figure 1. Distribution of PRSs among animals. VP and NS refer to viral structural and non-structural proteins respectively. PRSs were identified using the full sequences of 74 representative parvovirus genomes (provided in Table S1) as queries to search the EST (Expressed Sequence Tags), TSA (Transcriptome Shotgun Assembly), nr Nucleotide collection, and Uniprot databases as well as search in 11 un-deposited transcriptomes either produced for this study (*Lamellibrachia spp.*) or already published in another context (*Artemia franciscana*, *Culex pipiens*, *Crepidula fornicata*, *Eunicella cavolinii* and *Messor barbarus and Messor concolor*)[40,41]. Animal species wherein PRSs were first identified in this study are represented in bold, while PRSs that have already been identified are associated with numbers corresponding to the respective references. (*) PRS endogenization has been confirmed by PCRs.

(e.g. *Branchiopoda* and *Arachnida*), orders (*Phasmatodea* and *Coleoptera*) and families (e.g. *Formicidae*) that contained no previously identified parvovirus hosts (PRSs are summarized in Table S2).

It is also noteworthy that several copies of PRSs homologous to both NS and VP encoding genes were found in 72/118 of the animal transcriptomes (Table S2).

Most of the 463 PRSs (77%, corresponding to 79 animal species) potentially encoded proteins sharing between 30–85% aa identity to the NS or VP proteins of a known extant parvovirus, while 17% (derived from 30 animal species) potentially encoded proteins sharing less than 30% identity to any known extant parvoviruses. This degree of similarity is below the parvovirus genus demarcation threshold recommended by the ICTV (i.e. >30% amino acid identity in NS1), suggesting that, if these divergent PRSs are derived from extant viruses, these likely belong to species within as yet uncharacterized parvovirus genera[14]. Finally, 6% of the PRSs found within the transcriptome datasets of nine animal species potentially encoded proteins with more than 85% identity to those expressed by known extant parvoviruses (Supplementary Table S2).

Altogether, we concluded that, while parvoviruses are probably associated with a wider variety of animals than has previously been thought, the PRSs we found were mostly associated with invertebrate species within phyla, classes, orders and families containing no previously known parvovirus host species (Fig. 2).

PRSs likely originate from both fossil viral sequences and extant viruses. The PRSs that we detected could have had a number of different origins including: (1) endogenous "fossil" viral sequences resulting from ancient integration events; (2) endogenous viruses constituting latent infections resulting from recent integration events; or (3) exogenous viruses. Further, it was possible that all three types of PRS elements could have been present either within the cells of the species from which the transcriptome datasets were derived, or from (likely eukaryotic) species either parasitizing, or in some other way associated with, the species from which the transcriptome datasets were derived.

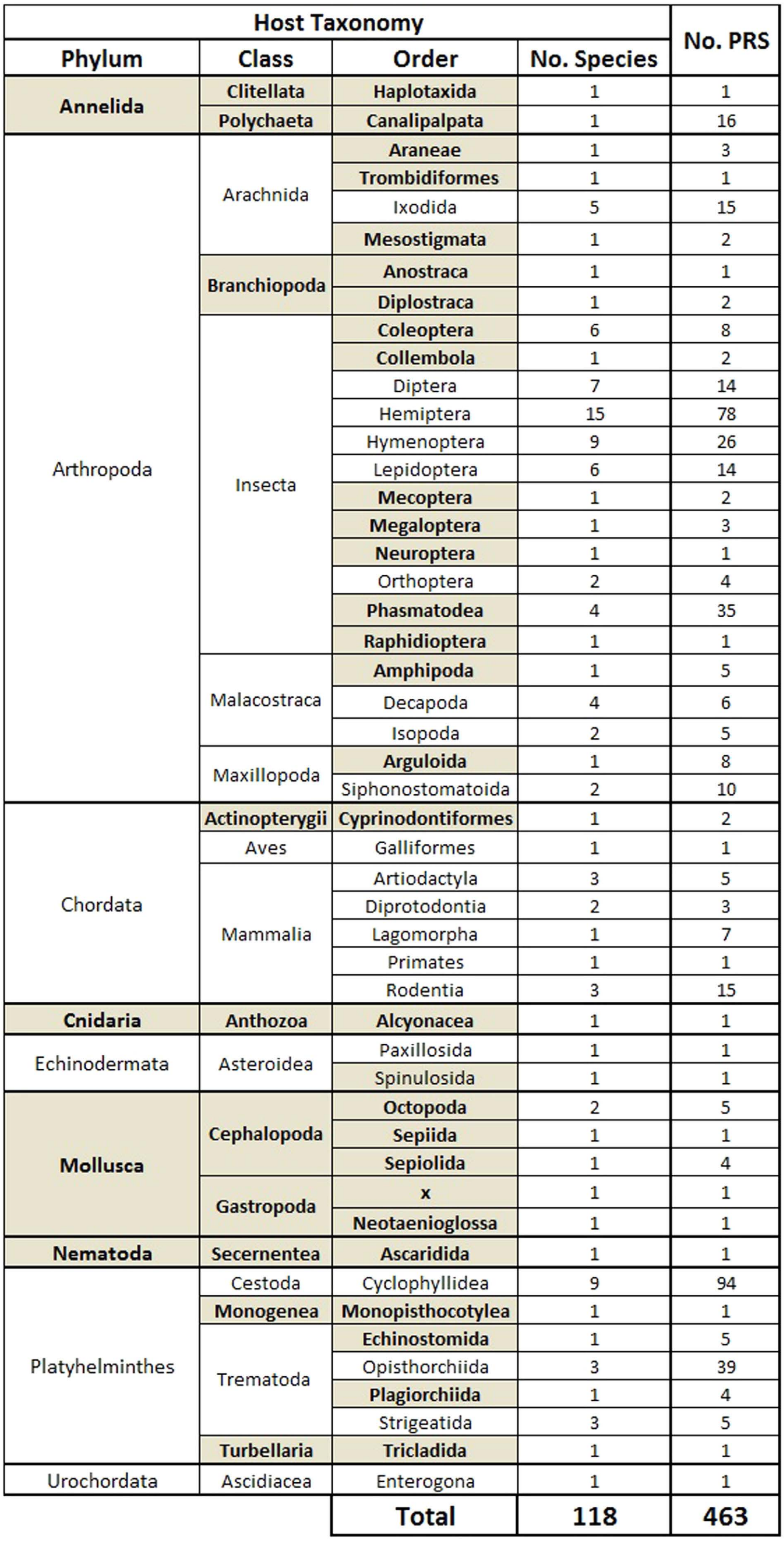
To identify PRSs potentially corresponding to endogenized parvoviral fragments, we used each of the 463 PRSs as queries to screen the publically available eukaryotic genome datasets (both assembled and unassembled) within the NCBI genomic database. This search identified 76 genomic sequences (summarized in Supplementary Table S3) of various sizes (0.05–8 kb) displaying significant matches (cutoff 95% identity, e-value $<10^{-5}$) to PRSs within the genomes of 31 invertebrates from six phyla including 16 arthropods (33 PRSs), 13 platyhelminthes (36 PRSs), one mollusc (2 PRSs) and one nematode (4 PRS), for which endogenization of parvoviruses has never been found before (Table S3). In addition to the possibly endogenized PRSs identified in these 31 invertebrate species, PRSs were also identified in the genomes of 16 animal species for which potential parvovirus endogenization has been previously reported (including nine arthropods, six chordates and one platyhelminthes; Fig. 3)[20,21,31–34].

Ancient integration events are often characterised by degraded integrons with the extent of degradation varying depending on whether the integrated sequences were selectively disadvantageous, beneficial or neutral[2]. We thus searched animal genome sequence databases for degraded PRSs including those containing potential transposable elements (TE) or repeated sequence insertions within the vicinity of the integration site, which may have contributed to their integration. We found 31 PRSs displaying truncations due to the accumulation of internal stop codons and/or adjacent transposable elements in the genomes of fifteen arthropod and seven platyhelminthes species (Table S3): a finding strongly supporting the hypothesis that these PRSs were the product of ancient endogenisation events in these phyla. In arthropods, whereas endogenization has been proposed previously for one of these PRSs–found within the genome of *A. pisum*[21,25]–we detected various other putative PRS endogenization events in a number of other Arthropods, i.e. six in Hymenoptera (*Formicidae*), three in Hemiptera (*Pachypsylla venusta*; family *Psyllidae*, *Halyomorpha halys*; family *Pentatomida*e, *Nilaparvata lugens*; family *Delphacidae*), one in Araneae (*Latrodectus hesperus*; family *Theridiidae*), one in Coleoptera (*Priacma serrata*; family *Cupedidae*) one in Mesostigmata (*Metaseiulus occidentalis*; family *Phytoseiidae*), one in Diplostraca (*Daphnia pulex*; family *Daphniidae*) and one in a Siphonostomatoida (*Caligus rogercresseyi*; family *Caligidae*), (Supplementary Tables S2 and S3); all these PRSs displayed internal stop codons and/or adjacent TE elements.

In platyhelminthes, potential densovirus endogenization has already been reported in one cestode (*Echinococcus granulosis*) and one trematode (*Schistosoma mansoni*), and all platyhelminthes associated PRSs recovered here share >95% nt identity with known platyhelminthes genome sequences[18]. The PRSs were detected in the genomes of 15 species (Fig. 3); mostly cestodes (e.g. *Taenia* sp. and *Echinococcus* sp.) and trematodes (e.g. *Schistosoma* sp.). All the PRSs detected in this phylum displayed ≥30% aa identity corresponding to the same domain of the NS1 protein although the inferred encoded amino acid sequences of this domain contained internal stop codons. For example, 39 PRSs were detected in the genome of the cestode, *Echinococcus multilocularis* that, when translated, displayed approximately 30% aa similarity with the NS1 of the shrimp parvovirus, Decapod penstyldensovirus 1 (PstDV1). These results suggest the endogenization of the NS1-like domain in the genome of several platyhelminthes species. The phylogenetic relationship between these PRSs will be addressed below.

Most genomic PRSs that we detected were, however, located at the extremities of genomic scaffolds that were less than 10 kb in length, and there was therefore limited information regarding their possible genomic context and flanking sequences: a factor which impaired our ability to definitively determine whether these sequences too were ancient degraded integrons associated with transposable elements or repeat-sequence insertions (Supplementary Table S3).

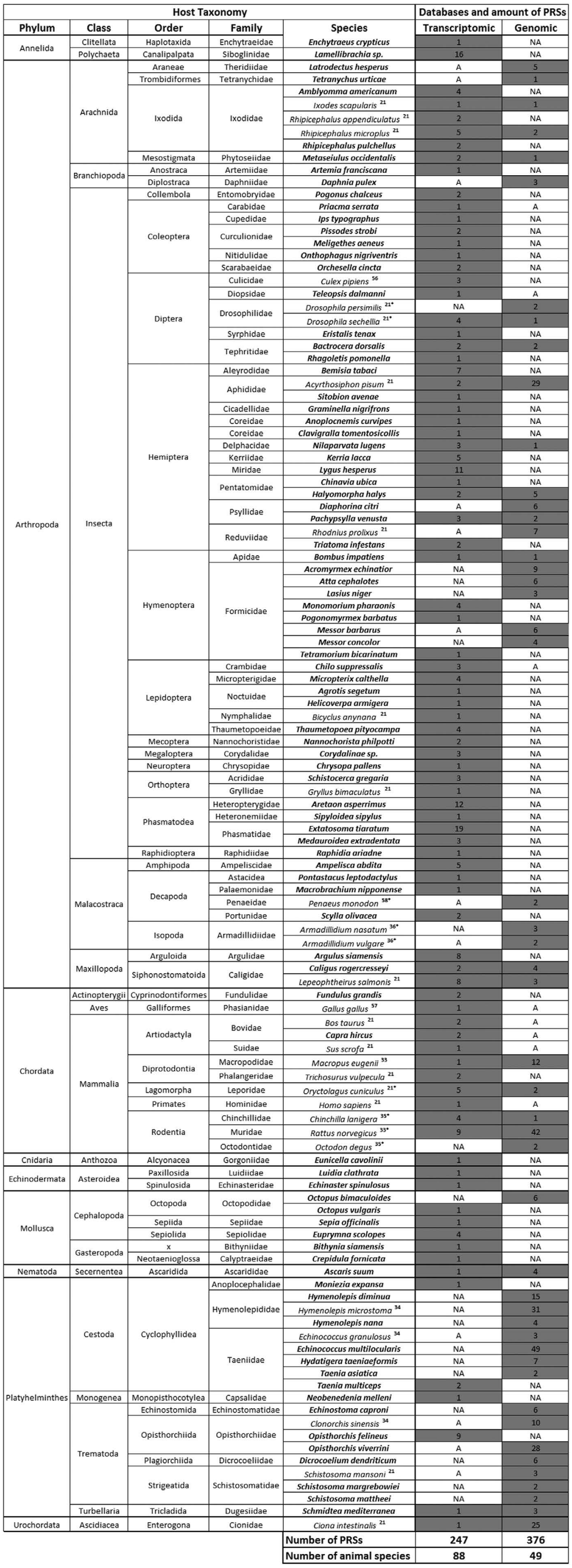
We next searched for PRSs corresponding to non-degraded viral ORFs as these might correspond to extant viruses. Four large PRSs covering both the NS and VP ORFs were found in both the genomes and transcriptomes of four arthropods (mentioned as NS-VP in Supplementary Table S2). One of these large PRS corresponded with the genomic sequence integrated in the pea aphid genome (*A. pisum*) that has been previously characterized by Liu *et al.*[35]. This PRS shares 52% aa identity with both NS and VP of the ambidensovirus infecting the aphid *Dysaphis plantaginea* (DpDV). A similar PRS (sharing >95% identity) was also detected in the genome of the peach-potato aphid (*Myzus persicae*)[23]. The transcription of both the NS and VP encoding genes was also



Host Taxonomy				**No. PRS**
Phylum	**Class**	**Order**	**No. Species**	
Annelida	**Clitellata**	**Haplotaxida**	1	1
	Polychaeta	**Canalipalpata**	1	16
Arthropoda	Arachnida	**Araneae**	1	3
		Trombidiformes	1	1
		Ixodida	5	15
		Mesostigmata	1	2
	Branchiopoda	**Anostraca**	1	1
		Diplostraca	1	2
	Insecta	**Coleoptera**	6	8
		Collembola	1	2
		Diptera	7	14
		Hemiptera	15	78
		Hymenoptera	9	26
		Lepidoptera	6	14
		Mecoptera	1	2
		Megaloptera	1	3
		Neuroptera	1	1
		Orthoptera	2	4
		Phasmatodea	4	35
		Raphidioptera	1	1
	Malacostraca	**Amphipoda**	1	5
		Decapoda	4	6
		Isopoda	2	5
	Maxillopoda	**Arguloida**	1	8
		Siphonostomatoida	2	10
Chordata	**Actinopterygii**	**Cyprinodontiformes**	1	2
	Aves	Galliformes	1	1
	Mammalia	Artiodactyla	3	5
		Diprotodontia	2	3
		Lagomorpha	1	7
		Primates	1	1
		Rodentia	3	15
Cnidaria	**Anthozoa**	**Alcyonacea**	1	1
Echinodermata	Asteroidea	Paxillosida	1	1
		Spinulosida	1	1
Mollusca	**Cephalopoda**	**Octopoda**	2	5
		Sepiida	1	1
		Sepiolida	1	4
	Gastropoda	**x**	1	1
		Neotaenioglossa	1	1
Nematoda	**Secernentea**	**Ascaridida**	1	1
Platyhelminthes	Cestoda	Cyclophyllidea	9	94
	Monogenea	**Monopisthocotylea**	1	1
	Trematoda	**Echinostomida**	1	5
		Opisthorchiida	3	39
		Plagiorchiida	1	4
		Strigeatida	3	5
	Turbellaria	**Tricladida**	1	1
Urochordata	Ascidiacea	Enterogona	1	1
		Total	**118**	**463**

Figure 2. Summary of the distribution of PRSs among animal transcriptomes and genomes. Animal orders wherein PRSs were first identified in this study are represented in bold.

demonstrated by these authors, suggesting that aphid endogenous parvoviral sequences represent recently integrated persistent viruses that could potentially become exogenous[25]. Remarkably, two other large PRSs (4.2 kb and 5.6 kb covering almost complete viral ORFs) were respectively recovered from the stick insect *Aretaon*



Host Taxonomy					Databases and amount of PRSs	
Phylum	Class	Order	Family	Species	Transcriptomic	Genomic
Annelida	Clitellata	Haplotaxida	Enchytraeidae	***Enchytraeus crypticus***	1	NA
	Polychaeta	Canalipalpata	Siboglinidae	***Lamellibrachia sp.***	16	NA
Arthropoda	Arachnida	Araneae	Theridiidae	***Latrodectus hesperus***	A	5
		Trombidiformes	Tetranychidae	***Tetranychus urticae***	A	1
		Ixodida	Ixodidae	***Amblyomma americanum***	4	NA
				Ixodes scapularis [21]	1	1
				Rhipicephalus appendiculatus [21]	2	NA
				Rhipicephalus microplus [21]	5	2
				Rhipicephalus pulchellus	2	NA
		Mesostigmata	Phytoseiidae	***Metaseiulus occidentalis***	2	1
	Branchiopoda	Anostraca	Artemiidae	***Artemia franciscana***	1	NA
		Diplostraca	Daphniidae	***Daphnia pulex***	A	3
	Insecta	Collembola	Entomobryidae	***Pogonus chalceus***	2	NA
		Coleoptera	Carabidae	***Priacma serrata***	1	A
			Cupedidae	***Ips typographus***	1	NA
			Curculionidae	***Pissodes strobi***	2	NA
				Meligethes aeneus	1	NA
			Nitidulidae	***Onthophagus nigriventris***	1	NA
			Scarabaeidae	***Orchesella cincta***	2	NA
		Diptera	Culicidae	*Culex pipiens* [56]	3	NA
			Diopsidae	***Teleopsis dalmanni***	1	A
			Drosophilidae	*Drosophila persimilis* [21*]	NA	2
				Drosophila sechellia [21*]	4	1
			Syrphidae	***Eristalis tenax***	1	NA
			Tephritidae	***Bactrocera dorsalis***	2	2
				Rhagoletis pomonella	1	NA
		Hemiptera	Aleyrodidae	***Bemisia tabaci***	7	NA
			Aphididae	*Acyrthosiphon pisum* [21]	2	29
				Sitobion avenae	1	NA
			Cicadellidae	***Graminella nigrifrons***	1	NA
			Coreidae	***Anoplocnemis curvipes***	1	NA
			Coreidae	***Clavigralla tomentosicollis***	1	NA
			Delphacidae	***Nilaparvata lugens***	3	1
			Kerriidae	***Kerria lacca***	5	NA
			Miridae	***Lygus hesperus***	11	NA
			Pentatomidae	***Chinavia ubica***	1	NA
				Halyomorpha halys	2	5
			Psyllidae	***Diaphorina citri***	A	6
				Pachypsylla venusta	3	2
			Reduviidae	*Rhodnius prolixus* [21]	A	7
				Triatoma infestans	2	NA
		Hymenoptera	Apidae	***Bombus impatiens***	1	1
			Formicidae	***Acromyrmex echinatior***	NA	9
				Atta cephalotes	NA	6
				Lasius niger	NA	3
				Monomorium pharaonis	4	NA
				Pogonomyrmex barbatus	1	NA
				Messor barbarus	A	6
				Messor concolor	NA	4
				Tetramorium bicarinatum	1	NA
		Lepidoptera	Crambidae	***Chilo suppressalis***	3	A
			Micropterigidae	***Micropterix calthella***	4	NA
			Noctuidae	***Agrotis segetum***	1	NA
				Helicoverpa armigera	1	NA
			Nymphalidae	*Bicyclus anynana* [21]	1	NA
			Thaumetopoeidae	***Thaumetopoea pityocampa***	4	NA
		Mecoptera	Nannochoristidae	***Nannochorista philpotti***	2	NA
		Megaloptera	Corydalidae	***Corydalinae sp.***	3	NA
		Neuroptera	Chrysopidae	***Chrysopa pallens***	1	NA
		Orthoptera	Acrididae	***Schistocerca gregaria***	3	NA
			Gryllidae	*Gryllus bimaculatus* [21]	1	NA
		Phasmatodea	Heteropterygidae	***Aretaon asperrimus***	12	NA
			Heteronemiidae	***Sipyloidea sipylus***	1	NA
			Phasmatidae	***Extatosoma tiaratum***	19	NA
				Medauroidea extradentata	3	NA
		Raphidioptera	Raphidiidae	***Raphidia ariadne***	1	NA
	Malacostraca	Amphipoda	Ampeliscidae	***Ampelisca abdita***	5	NA
		Decapoda	Astacidea	***Pontastacus leptodactylus***	1	NA
			Palaemonidae	***Macrobrachium nipponense***	1	NA
			Penaeidae	*Penaeus monodon* [58*]	A	2
			Portunidae	***Scylla olivacea***	2	NA
		Isopoda	Armadillidiidae	*Armadillidium nasatum* [36*]	NA	3
				Armadillidium vulgare [36*]	A	2
	Maxillopoda	Arguloida	Argulidae	***Argulus siamensis***	8	NA
		Siphonostomatoida	Caligidae	***Caligus rogercresseyi***	2	4
				Lepeophtheirus salmonis [21]	8	3
Chordata	Actinopterygii	Cyprinodontiformes	Fundulidae	***Fundulus grandis***	2	NA
	Aves	Galliformes	Phasianidae	*Gallus gallus* [57]	1	A
	Mammalia	Artiodactyla	Bovidae	*Bos taurus* [21]	2	A
				Capra hircus	2	A
			Suidae	*Sus scrofa* [21]	1	A
		Diprotodontia	Macropodidae	*Macropus eugenii* [33]	1	12
			Phalangeridae	*Trichosurus vulpecula* [21]	2	NA
		Lagomorpha	Leporidae	*Oryctolagus cuniculus* [21*]	5	2
		Primates	Hominidae	*Homo sapiens* [21]	1	A
		Rodentia	Chinchillidae	*Chinchilla lanigera* [35*]	4	1
			Muridae	*Rattus norvegicus* [33*]	9	42
			Octodontidae	*Octodon degus* [35*]	NA	2
Cnidaria	Anthozoa	Alcyonacea	Gorgoniidae	***Eunicella cavolinii***	1	NA
Echinodermata	Asteroidea	Paxillosida	Luidiidae	***Luidia clathrata***	1	NA
		Spinulosida	Echinasteridae	***Echinaster spinulosus***	1	NA
Mollusca	Cephalopoda	Octopoda	Octopodidae	***Octopus bimaculoides***	NA	6
				Octopus vulgaris	1	NA
		Sepiida	Sepiidae	***Sepia officinalis***	1	NA
		Sepiolida	Sepiolidae	***Euprymna scolopes***	4	NA
	Gasteropoda	x	Bithyniidae	***Bithynia siamensis***	1	NA
		Neotaenioglossa	Calyptraeidae	***Crepidula fornicata***	1	NA
Nematoda	Secernentea	Ascaridida	Ascarididae	***Ascaris suum***	1	4
Platyhelminthes	Cestoda	Cyclophyllidea	Anoplocephalidae	***Moniezia expansa***	1	NA
			Hymenolepididae	***Hymenolepis diminua***	NA	15
				Hymenolepis microstoma [34]	NA	31
				Hymenolepis nana	NA	4
			Taeniidae	*Echinococcus granulosus* [34]	A	3
				Echinococcus multilocularis	NA	49
				Hydatigera taeniaeformis	NA	7
				Taenia asiatica	NA	2
				Taenia multiceps	2	NA
	Monogenea	Monopisthocotylea	Capsalidae	***Neobenedenia melleni***	1	NA
	Trematoda	Echinostomida	Echinostomatidae	***Echinostoma caproni***	NA	6
		Opisthorchiida	Opisthorchiidae	*Clonorchis sinensis* [34]	A	10
				Opisthorchis felineus	9	NA
				Opisthorchis viverrini	A	28
		Plagiorchiida	Dicrocoeliidae	***Dicrocoelium dendriticum***	NA	6
		Strigeatida	Schistosomatidae	*Schistosoma mansoni* [21]	A	3
				Schistosoma margrebowiei	NA	2
				Schistosoma mattheei	NA	2
	Turbellaria	Tricladida	Dugesiidae	***Schmidtea mediterranea***	1	3
Urochordata	Ascidiacea	Enterogona	Cionidae	*Ciona intestinalis* [21]	1	25
				Number of PRSs	**247**	**376**
				Number of animal species	**88**	**49**

Figure 3. Distribution of PRSs in animal transcriptomes and genomes (in grey). Animal species wherein PRSs were first identified in this study are represented in bold while numbers correspond to references where PRSs were previously described. (*) PRS endogenization proved by PCRs. A: transcriptomic/genomic data (EST and TSA databases) available at NCBI. NA: Not Available, i.e. transcriptomic/genomic data (gss, WGS, chromosome and refseq_genomic databases) are not available at NCBI.

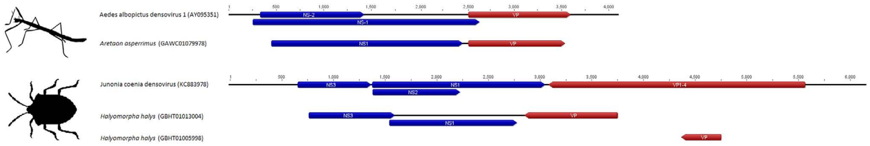

Aedes albopictus densovirus 1 (AY095351)
NS-2
NS-1
VP
Aretaon asperrimus (GAWC01079978)
NS1
VP
Junonia coenia densovirus (KC883978)
NS3
NS1
NS2
VP1-4
Halyomorpha halys (GBHT01013004)
NS3
NS1
VP
Halyomorpha halys (GBHT01005998)
VP
1
500
1,000
1,500
2,000
2,500
3,000
3,500
4,000
4,500
5,000
5,500
6,000

Figure 4. Organization of open reading frames of three PRSs (GAWC01079978 from *Aretaon asperrimus*, GBHT01013004 and GBHT01005998 from *Halyomorpha halys*). Arrowhead boxes indicate viral and predicted viral genes (NS are in blue and VP in red).

asperrimus, belonging to the order *Phasmatodea*, and from the stink-bug, *Halyomorpha halys*, belonging to the order *Hemiptera*: neither species had previously been identified as densovirus hosts. These two large PRSs encompass two ORFs (NS1 and VP; GAWC1079978 recovered from *A. asperrimus*) and three ORFs (NS1, NS3 and VP; GBHT01013004 from *H. halys*; Fig. 4). The arrangement of these ORFs is similar to Brevi- and Ambidensoviruses that respectively infect mosquitoes and caterpillars (Fig. 4). Since only a few reads of the genomes of these potentially new insect hosts are available (for example we only found one contig containing the stink-bug PRS), it is not possible to conclude whether these PRSs are involved in latent infections by undescribed viruses (as is possibly the case for the integrated densovirus in aphids that is described above), or are derived from previously unknown extant exogenous *A. asperrimus* and/or *H. halys* infecting densoviruses[18].

Last, we must emphasize that contamination of genomic datasets may come either from animals that are infected by parvoviruses or from animals that are infected by parasites that are themselves infected by parvoviruses. Intriguingly, we found one ~0.8 kb PRS in the genome sequence dataset of *Gregarina niphandrodes*; a protozoan Apicomplexa that infects a number of invertebrates and which was isolated from the coleopteran, *Tenebrio molitor* according to Genebank data. This PRS shared 100% identity with the VP sequence of a yet undescribed *Blatella germanica* densovirus-like virus found in the metagenome of insectivorous bat faeces[36] (Table S2). We could not find any detectably homologous sequence within either the genome or the transcriptome of its potential host, *T. molitor*. Although we cannot rule out the possibility that the densovirus from which this PRS was derived was able to infect *G. niphandrodes*, we concluded that the PRS probably originated from a contaminant.

In total we discarded 16 PRSs from further analysis due to contamination concerns. Among these were three found in the transcript datasets of plant species (Table S2): all of these plant PRSs had high degrees of identity with extant insect-infecting densoviruses and were therefore likely derived from insects associated with the plants (Table S2). Similarly, PRSs found in the transcriptomes of three vertebrates (all amphibian species), one echinoderm (*Amphiura filiformis*) and one insect (*Drosophila ananassae*) were >85% identical to known mammal-infecting parvoviruses and were thus assumed to be contaminants.

Altogether these results highlight the large diversity of PRSs that can be found by screening publically available transcriptomes and genomes. In total, 623 PRSs were found when adding up all PRSs found in transcriptomic (247) and genomic databases (376) including the newly found and already reported sequences (Fig. 3). Our search identified that parvoviruses are/were associated with a large number of animal species that have never previously been identified as parvovirus host species (Summarized in Fig. 2).

Phylogenetic analyses of PRSs. We next attempted to evaluate the genetic relationships of the PRSs with known exogenous parvoviruses. While the parvovirus protein NS1 contains a SF3 helicase domain that is highly conserved in all known parvoviruses (it can also be found in proteins of viruses in other families)[37], the most conserved domain of parvoviral VPs, PLA2, is missing in certain parvoviruses: a factor which may explain why our search identified more sequences related to NS1 than to VP. The SF3 domain is thus typically used for phylogenetic analyses of divergent parvoviruses[18].

As is shown in the *Parvoviridae* maximum likelihood trees (Figs 2 and 3), all exogenous known parvovirus species (in black text) were clearly placed within the 13 genera recognized by ICTV with bootstrap values >80%. While validating the use of the small SF3 domain of NS1 to study relationships amongst PRSs, it is apparent from the trees that most of the PRSs are situated on long branches that connect to the tree with low degrees of bootstrap support (<70%), basal to clusters of sequences from the established parvovirus genera. This phylogenetic placement is consistent with PRSs belonging to currently undescribed genus-level parvovirus lineages; although we cannot exclude ambiguous alignment of divergent sequences as the cause of low degrees of bootstrap support for the clustering of these PRSs with viruses from the known parvovirus genera.

Nevertheless, the PRSs drawn from the transcriptome datasets of animals belonging to particular genera were frequently monophyletic within clades supported by bootstrap values >50%: consistent with the hypothesis that these PRSs are derived from viruses belonging to undiscovered parvovirus lineages with genus-specific host-ranges. This situation is exemplified with PRSs found within transcriptome datasets of ticks, stick insects and stink-bugs (Fig. 5). Interestingly, the two large transcripts that correspond to almost complete densovirus genomes that were detected in the stink-bug and stick insect transcriptome datasets cluster together with PRSs found in related triatomine insects in either the *Reduviidae* family (for the stink bug PRSs) or the *Phasmatodea* order (for the stick insect PRSs). These large PRSs might correspond to exogenous or persistent viruses belonging to densovirus lineages that infect these insects (Fig. 4). Interestingly, PRSs found in four classes of marine molluscs and more related to vertebrate parvoviruses according to results above, branched out of all known parvovirus genera (represented by the light blue branches in Fig. 5), although we cannot exclude some contamination of these animals. These results suggest that these PRSs belong to new parvovirus lineages yet to be characterized in this phylum.

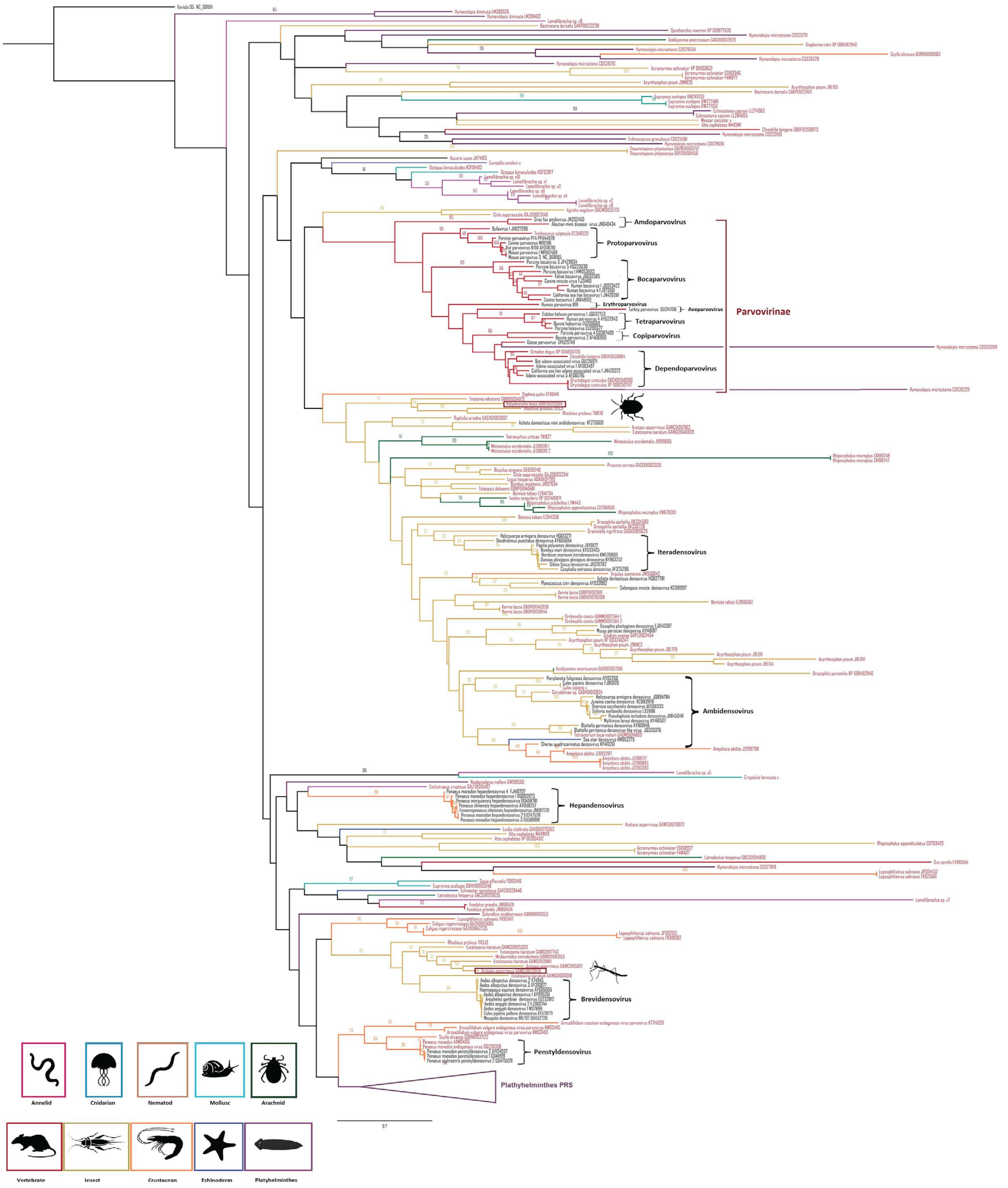
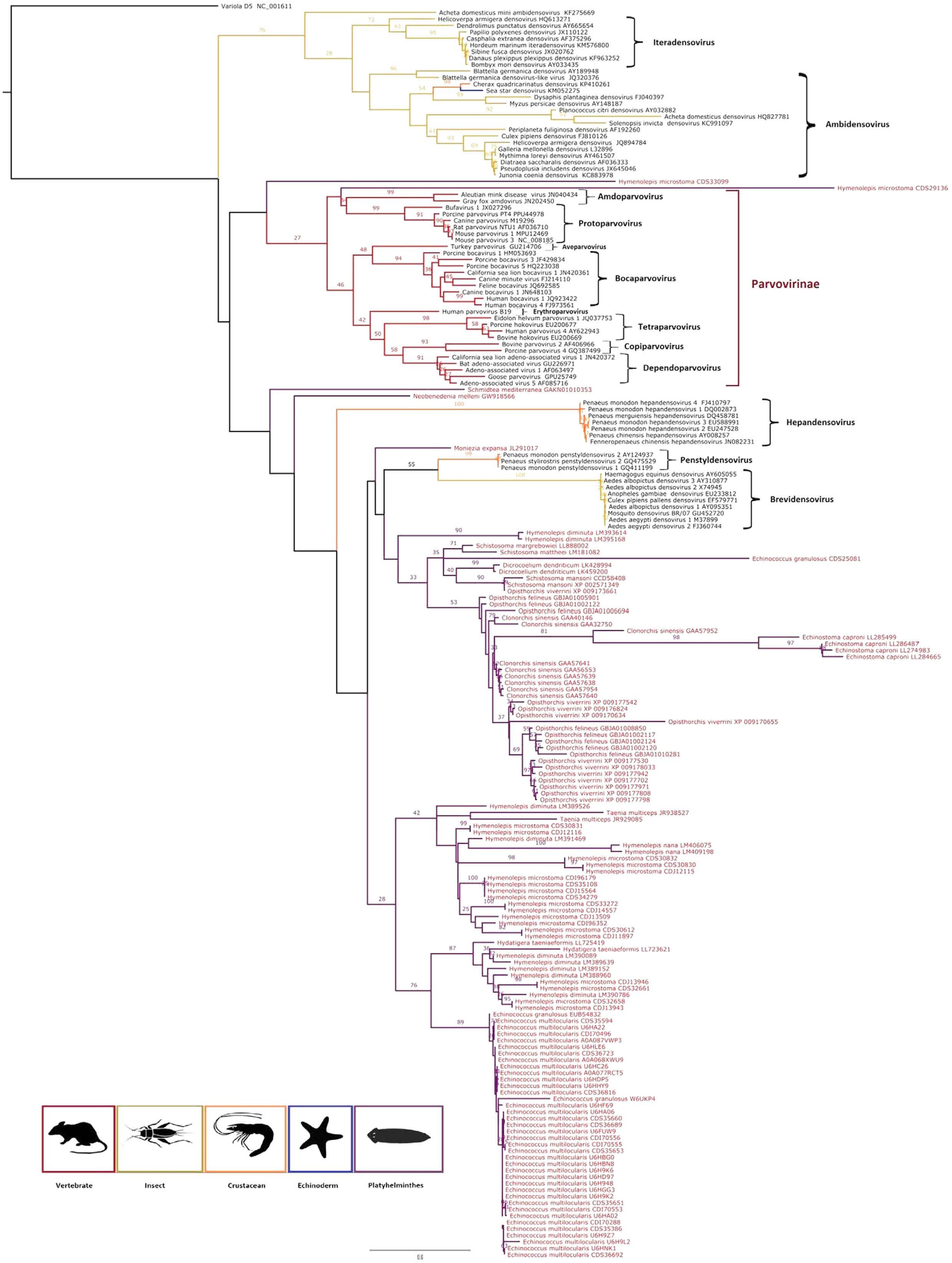


Figure 5. Maximum likelihood phylogenetic tree based on partial SF3 domains of the NS1 protein, including 74 parvovirus species (in black) and 264 PRSs (in red). The alignment was produced using MUSCLE 3.7 with default settings. The tree was rooted with the SF3 domain of the variola virus D5 protein. Bootstrap values >25% are indicated at each node. Scale bars correspond to amino acid substitutions per site. Genera of the *Parvoviridae* family are indicated in brackets. Associated hosts are indicated in the tree by different branch colours and silhouettes at the bottom of the figure.

Such clustering of PRSs according to the evolutionary relationships of the animal species they are associated with is particularly apparent for PRSs found within the platyhelminthes transcriptome datasets (Fig. 6). PRSs found within these datasets form two clades supported by >70% bootstrap values (Fig. 6). The inferred SF3 amino acid sequences encoded by these PRSs share identities of <30% with those of previously known parvoviruses, suggesting that these flatworm-associated PRSs may represent new genera (Fig. 6) of either extant but undiscovered circulating parvoviruses, or integrated (and possibly extinct) parvoviruses.

The data presented here indicate that parvoviruses are likely widespread among multicellular animals. If we consider all clusters with bootstrap values >70%, then we can tentatively estimate that these phylogenetic analyses indicate the existence of around 20 currently undescribed genus-level densovirinae lineages.


Variola D5 NC_001611
Acheta domesticus mini ambidensovirus KF275669
Helicoverpa armigera densovirus HQ613271
Dendrolimus punctatus densovirus AY665654
Papilio polyxenes densovirus JX110122
Casphalia extranea densovirus AF375296
Hordeum marinum iteradensovirus KM576800
Sibine fusca densovirus JX020762
Danaus plexippus plexippus densovirus KF963252
Bombyx mori densovirus AY033435
Iteradensovirus
Blattella germanica densovirus AY189948
Blattella germanica densovirus-like virus JQ320376
Cherax quadricarinatus densovirus KP410261
Sea star densovirus KM052275
Dysaphis plantaginea densovirus FJ040397
Myzus persicae densovirus AY148187
Planococcus citri densovirus AY032882
Acheta domesticus densovirus HQ827781
Solenopsis invicta densovirus KC991097
Periplaneta fuliginosa densovirus AF192260
Culex pipiens densovirus FJ810126
Helicoverpa armigera densovirus JQ894784
Galleria mellonella densovirus L32896
Mythimna loreyi densovirus AY461507
Diatraea saccharalis densovirus AF036333
Pseudoplusia includens densovirus JX645046
Junonia coenia densovirus KC883978
Ambidensovirus
Hymenolepis microstoma CDS33099
Hymenolepis microstoma CDS29136
Aleutian mink disease virus JN040434
Gray fox amdovirus JN202450
Amdoparvovirus
Bufavirus 1 JX027296
Porcine parvovirus PT4 PPU44978
Canine parvovirus M19296
Rat parvovirus NTU1 AF036710
Mouse parvovirus 1 MPU12469
Mouse parvovirus 3 NC_008185
Protoparvovirus
Turkey parvovirus GU214706
Aveparvovirus
Porcine bocavirus 1 HM053693
Porcine bocavirus 3 JF429834
Porcine bocavirus 5 HQ223038
California sea lion bocavirus 1 JN420361
Canine minute virus FJ214110
Feline bocavirus JQ692585
Canine bocavirus 1 JN648103
Human bocavirus 1 JQ923422
Human bocavirus 4 FJ973561
Bocaparvovirus
Human parvovirus B19
Erythroparvovirus
Eidolon helvum parvovirus 1 JQ037753
Porcine hokovirus EU200677
Human parvovirus 4 AY622943
Bovine hokovirus EU200669
Tetraparvovirus
Bovine parvovirus 2 AF406966
Porcine parvovirus 4 GQ387499
Copiparvovirus
California sea lion adeno-associated virus 1 JN420372
Bat adeno-associated virus GU226971
Adeno-associated virus 1 AF063497
Goose parvovirus GPU25749
Adeno-associated virus 5 AF085716
Dependoparvovirus
Parvovirinae
Schmidtea mediterranea GAKN01010353
Neobenedenia melleni GW918566
Penaeus monodon hepandensovirus 4 FJ410797
Penaeus monodon hepandensovirus 1 DQ002873
Penaeus merguiensis hepandensovirus DQ458781
Penaeus monodon hepandensovirus 3 EU588991
Penaeus monodon hepandensovirus 2 EU247528
Penaeus chinensis hepandensovirus AY008257
Fenneropenaeus chinensis hepandensovirus JN082231
Hepandensovirus
Moniezia expansa JL291017
Penaeus monodon penstyldensovirus 2 AY124937
Penaeus stylirostris penstyldensovirus 2 GQ475529
Penaeus monodon penstyldensovirus 1 GQ411199
Penstyldensovirus
Haemagogus equinus densovirus AY605055
Aedes albopictus densovirus 3 AY310877
Aedes albopictus densovirus 2 X74945
Anopheles gambiae densovirus EU233812
Culex pipiens pallens densovirus EF579771
Aedes albopictus densovirus 1 AY095351
Mosquito densovirus BR/07 GU452720
Aedes aegypti densovirus 1 M37899
Aedes aegypti densovirus 2 FJ360744
Brevidensovirus
Hymenolepis diminuta LM393614
Hymenolepis diminuta LM395168
Schistosoma margrebowiei LL888002
Schistosoma mattheei LM181082
Dicrocoelium dendriticum LK428994
Dicrocoelium dendriticum LK459200
Echinococcus granulosus CDS25081
Schistosoma mansoni CCD58408
Schistosoma mansoni XP 002571349
Opisthorchis viverrini XP 009173661
Opisthorchis felineus GBJA01005901
Opisthorchis felineus GBJA01002122
Opisthorchis felineus GBJA01006694
Clonorchis sinensis GAA40146
Clonorchis sinensis GAA32750
Clonorchis sinensis GAA57952
Echinostoma caproni LL285499
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Echinostoma caproni LL274983
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Opisthorchis viverrini XP 009170655
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Opisthorchis felineus GBJA01002117
Opisthorchis felineus GBJA01002124
Opisthorchis felineus GBJA01002120
Opisthorchis felineus GBJA01010281
Opisthorchis viverrini XP 009177530
Opisthorchis viverrini XP 009178033
Opisthorchis viverrini XP 009177942
Opisthorchis viverrini XP 009177702
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Opisthorchis viverrini XP 009177808
Opisthorchis viverrini XP 009177798
Hymenolepis diminuta LM389526
Taenia multiceps JR938527
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Hymenolepis microstoma CDS30831
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Hymenolepis microstoma CDS35108
Hymenolepis microstoma CDJ15564
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Hymenolepis microstoma CDJ14557
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Hymenolepis microstoma CDS32658
Hymenolepis microstoma CDJ13943
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Echinococcus multilocularis CDS35594
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Echinococcus multilocularis A0A068XWU9
Echinococcus multilocularis U6HC26
Echinococcus multilocularis A0A077RCT5
Echinococcus multilocularis U6HDP5
Echinococcus multilocularis U6HHY9
Echinococcus multilocularis CDS36816
Echinococcus granulosus W6UKP4
Echinococcus multilocularis U6HF69
Echinococcus multilocularis U6HA06
Echinococcus multilocularis CDS35660
Echinococcus multilocularis CDS36689
Echinococcus multilocularis U6FUW9
Echinococcus multilocularis CDI70556
Echinococcus multilocularis CDI70555
Echinococcus multilocularis CDS35653
Echinococcus multilocularis U6HBG0
Echinococcus multilocularis U6HBN8
Echinococcus multilocularis U6H9K6
Echinococcus multilocularis U6HD97
Echinococcus multilocularis U6H948
Echinococcus multilocularis U6HGG3
Echinococcus multilocularis U6H9K2
Echinococcus multilocularis CDS35651
Echinococcus multilocularis CDI70553
Echinococcus multilocularis U6HA02
Echinococcus multilocularis CDI70288
Echinococcus multilocularis CDS35386
Echinococcus multilocularis U6H9Z7
Echinococcus multilocularis U6H9L2
Echinococcus multilocularis U6HNK1
Echinococcus multilocularis CDS36692
Vertebrate
Insect
Crustacean
Echinoderm
Platyhelminthes
0.6

Figure 6. Maximum likelihood phylogenetic tree of platyhelminthes PRSs based on partial SF3 domains of the NS1 protein, including 74 parvovirus species (in black) and 118 platyhelminthes PRSs (in red). The alignment was produced using MUSCLE 3.7 with default settings. The tree was rooted with the variola virus D5 protein SF3 domain. Bootstrap values >25% are indicated at each node. Scale bars correspond to amino acid substitutions per site. The genera of the *Parvoviridae* family are indicated in brackets. Host phyla are represented by different colours and silhouettes at the bottom of the figure.

Discussion

Since the discovery of parvoviruses four decades ago, our perception of the species diversity within this family has been strongly biased by an overwhelming focus on discovering viruses of health and economic interest. While a high diversity of viral genome organisations and sequences has been revealed, few genomes were characterized: a factor which has limited our understanding of the global diversity, prevalence and host-associations of these viruses. The advent of the genomic era has now provided an alternative way to explore virus diversity in a slightly less biased way via the computational screening of large transcriptome and whole genome sequence datasets[18,21,22].

By scanning public genomic and transcriptomic resources, we have found that parvoviruses likely infect a larger number and wider diversity of animal-hosts, particularly among invertebrate phyla, than has previously been appreciated. These newly discovered potential parvovirus hosts include molluscs, annelids, nematodes, cnidarians and arthropods.

Although these results suggest widespread parvovirus integration into the genomes of diverse invertebrates, the limited number of complete invertebrate genome sequences that are currently available hinders both the definitive identification of these PRSs as integrated sequences, and an accurate estimation of the time-scale of individual PRS integration events. Data summarized in Fig. 3 and Supplementary Table S2 highlight the fact that 60% (376/623) of PRSs were found in animal genomes, among which 10% (37/367) are likely integrated. Integration was thus uncertain for 90% of the PRSs, mostly due to either unavailable or incomplete genomes for most of the invertebrate phyla for which transcriptome data was available. However, it is plausible that some of the endogenous PRSs were integrated millions of years ago since the presence of what appear to be closely related PRSs in different mammalian species have previously suggested that parvoviruses have likely coexisted with mammals for at least 98 million years[21].

It is particularly clear that most of the PRSs identified in this study were more similar to members of the *Densovirinae* sub-family than they were to members of the *Parvovirinae* sub-family. Considering that arthropod species vastly outnumber all other animal species, such over-representation of densoviruses, although unsurprising, contrasts with the similar numbers of described species in both subfamilies in the current ICTV report[18,19].

We cannot exclude the possibility that contamination of transcriptome and genome sequence datasets with unaccounted for eukaryote and/or viral DNA could yield spurious host-virus associations in studies such as that carried out here: as is apparently exemplified by the presence of PRSs in a small number of plant datasets. It is noteworthy, however, that densovirus-plant associations actually do occur in nature. For example, aphid-infecting densoviruses can be injected into, and circulate within, plants[38]. It has, in fact, been speculated that plants might be stationary vectors of some densoviruses that infect plant-feeding insects[38].

It has been proposed that all viruses in a parvovirus genus should be monophyletic and encode NS1 proteins that share $>30\%$ amino acid sequence identity to each other[18]. The phylogeny that we have produced for the SF3 helicase domain of NS1 indicated that 15% of the PRSs that contain this domain are highly divergent. The low degrees of similarity shared between these PRSs and both known parvoviruses and the other PRSs meant that they could not be reliably aligned: a factor that could have contributed to these divergent sequences falling on long isolated branches of the phylogenetic tree. While the intermingling of these divergent PRS lineages amongst known members of the *Parvovirinae* and *Densovirinae* genera, suggests that numerous genus-level parvovirus lineages are presently undescribed, we can also not exclude the possibility that some of these PRSs may be derived from virus families, such as *Bidnaviridae*, that are related to the parvoviruses. Like parvoviruses, the bidnaviruses are also ssDNA viruses that express a SF3 domain containing protein[18,39]. Unlike parvoviruses, however, they have two-component genomes and encode DNA polymerases. Although all of the PRSs discovered here were more closely related to known parvovirus sequences than to known bidnaviruses, the possibility remains that some of these PRSs are potentially derived from viruses belonging to currently undescribed families.

Here we have highlighted the extraordinary diversity of PRSs that can be found in public databases. As more animal sequences will be released we can anticipate that our knowledge of the diversity of parvoviruses will also keep improving. Combining such database searches with more directed viral metagenomics approaches and classical etiological survey, will be of great value both for discovering new parvoviruses and, as more endogenous PRSs are discovered within eukaryote genomes, for illuminating the deep evolutionary history of this family in relation to that of the host species that they infect.

Methods

Biological samples and transcriptome datasets.

Lamellibrachia sp. (marine polychaete annelid) samples were collected in 2007 in the Gulf of Mexico at 1250 m depth for individual, GA27M, and in the Gulf of Guinea at 580–670 m depth for individuals, GA27P, GA27S and GA27U. Vestimentum tissue was dissected and stored immediately in liquid nitrogen. Due to poor yield with standard total RNA isolation methods, we used a modified protocol based on a Trizol-Chloroform method combined with a QIAshredder column (Qiagen) purification step[40] involving the addition of 4 μl of glycogen (Ambion, final concentration = 0.04 mg/μL) to increase RNA yield and a further polyvinylpolypyrrolidone (PVPP) purification step. Five μg of total RNA was reverse-transcribed using the SMART cDNA library construction kit (Clontech, Mountain View, USA). Libraries were sequenced to produce 100 bp paired-end reads on a Genome Analyzer II or Hiseq 2000 (Illumina, Inc.). Low-quality read extremities were trimmed using the SeqClean program (http://compbio.dfci.harvard.edu/tgi/). Reads were deposited in the Sequence Read Archive (SRA) NCBI database under bioproject PRJNA302863, accession numbers SRX1440230, SRX1447229, SRX1447303 and SRX1447300. *Lamellibrachia* transcriptomes produced in this study, as well as individual previously published transcriptomes of *Artemia franciscana* (individual GA17B; SRX565006), *Crepidula fornicata* (individual GA22E; SRX565072), *Eunicella cavolinii* (individual GA31L; SRX565138) and *Messor barbarus* (individual GA40E; SRX565206) were successively assembled using ABYSS V 1.2.0[41] and CAP3[42]. This assembly method was previously found to be suitable for other mollusc and animal transcriptomes[43,44]. Three supplementary transcriptomes of whole individual adults of *Messor barbarus*, *Messor concolor* and *Culex pipiens* were assembled as above and were added to this dataset (N. Galtier, unpublished data). In total eleven transcriptomes have been used in this study, four of which were generated for this study (*Lamellibrachia*) and seven of which were previously used for animal genomics studies that did not involve virus detection (*Artemia franciscana*, *Culex pipiens*, *Crepidula fornicata*, *Eunicella cavolinii*, two *Messor barbarus* and *Messor concolor*).

Homology searches for parvovirus-related sequences (PRSs). We assembled a dataset of NS and VP amino acid sequences derived from each of the 74 parvovirus species, recognized and yet to be approved by the ICTV (all obtained from GenBank; genomes listed in Table S1). These sequences were used as queries to perform BLASTX searches for PRSs within all the non-redundant (nr) nucleotide and protein sequences at the NCBI, including the cDNA EST (http://www.ncbi.nlm.nih.gov/nucest/), TSA (http://www.ncbi.nlm.nih.gov/genbank/tsa), and Uniprot databases[29]. All sequences from these databases that matched parvovirus sequences (E-value $< 10^{-3}$) were selected and used as queries to perform BLASTX or BLASTP reciprocal searches of the cDNA EST, TSA and Uniprot databases. Sequences were considered PRSs when they matched known parvovirus sequences with associated BLASTX or BLASTP E-values $< 10^{-3}$. The eleven new transcriptomes were also screened for the presence of PRSs as described above. Complete and 5′- or 3′-truncated ORFs were detected using Prodigal V2_60[45,46] using the standard genetic code. ORFs displaying internal stretches of undetermined nucleotides (N) were also considered. Putative protein sequences were first annotated by detecting protein homology using the HHblits component of the HHSuite package[47,48] of nr protein sequences of the NCBI database. ORFs matching parvoviral proteins (E-values $< 10^{-3}$) were selected and used as query for reciprocal BLASTP searches of the cDNA EST, TSA and Uniprot databases as described above.

Detection of endogenous parvovirus-related sequences. The 463 PRSs found from BLASTX searches described above were used as queries against the reference genomic sequences (Refseq_genomic, http://www.ncbi.nlm.nih.gov/refseq/), chromosome (http://www.ncbi.nlm.nih.gov/genome/), GSS (Genomic survey sequences) (http://www.ncbi.nlm.nih.gov/nucgss/) and WGS (Whole-Genome Shotgun contigs) (http://www.ncbi.nlm.nih.gov/genbank/wgs) databases using BLASTN and tBLASTN, with a minimum percentage similarity cutoff of 95% and an E-value cutoff of 10^{-5}. Five hundred nucleotide long genomic fragments located up- and down-stream of each PRS were scanned for transposable elements (TE) or repetitive sequences using WSCensor (http://www.girinst.org/censor/).

Phylogenetic analyses. The putative amino acid sequences of the SF3 helicase domains of parvoviral NS1and PRS NS1-like proteins were used for phylogenetic analyses. All PRSs were translated *in silico* using ORF finder (cut off >300 bp) (http://www.ncbi.nlm.nih.gov/projects/gorf/). Putative domains of the resulting proteins were predicted using Interproscan5 [49]. Among the 463 PRSs, 264 contained a SF3 helicase domain sequence from which 191 aa-long fragments were aligned with the corresponding SF3 fragments from the 74 known parvoviruses (Table S1) using MUSCLE 3.7 (16 iterations) with default settings[50]. Aligned sequences were manually edited (full alignment of the 191 aa-long partial SF3 helicase domains, is provided in FASTA format, and is presented in Supplementary Figure S1). Maximum likelihood phylogenetic trees were produced from this alignment using PhyML 3.1 [51] with a Blossum + G + F + I amino acid substitution model chosen as the best-fit using ProtTest[52]. Five hundred bootstrap replicates were used to test the support of branches. Trees were visualized with FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). In addition, a second tree focusing on the evolutionary relationships of 118 PRSs derived from platyhelminthes together with the 74 representative parvovirus SF3 domain sequences (Table S1) was constructed using the same approaches described above (full alignment of the 156 aa-long partial SF3 helicase domains is provided in FASTA format and in Supplementary Figure S2). The variola D5 protein (Genbank accession number: P33069) was used in both cases as an outgroup to root the trees[53–58].

References

1. Rosario, K. & Breitbart, M. Exploring the viral world through metagenomics. *Curr Opin Virol* **1,** 289–297 (2011).
2. Feschotte, C. & Gilbert, C. Endogenous viruses: insights into viral evolution and impact on host biology. *Nat Rev Genet* **13,** 283–296 (2012).
3. Wren, J. D. *et al.* Plant virus biodiversity and ecology. *PLoS Biol* **4,** e80 (2006).
4. Temmam, S., Davoust, B., Berenger, J. M., Raoult, D. & Desnues, C. Viral metagenomics on animals as a tool for the detection of zoonoses prior to human infection? *Int J Mol Sci* **15,** 10377–10397 (2014).
5. Roossinck, M. J., Martin, D. P. & Roumagnac, P. 2015. Plant Virus Metagenomics: Advances in Virus Discovery. *Phytopathology* **105(6),** 716–727 (2015).
6. King A. M. Q., Lefkowitz A., Adams M. J., Carstens E. B. (eds). *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier/Academic Press, San Diego, pp 353–369 (2011).
7. Mokili, J. L., Rohwer, F. & Dutilh, B. E. Metagenomics and future perspectives in virus discovery. *Curr Opin Virol* **2,** 63–77 (2012).
8. Bernardo, P. *et al.* Molecular characterization and prevalence of two capulaviruses: Alfalfa leaf curl virus from France and Euphorbia caput-medusae latent virus from South Africa. *Virology* **493,** 142–153 (2016).
9. Rosario, K., Duffy, S. & Breitbart, M. A field guide to eukaryotic circular single-stranded DNA viruses: insights gained from metagenomics. *Arch Virol* **157,** 1851–1871 (2012).
10. Roux, S. *et al.* Analysis of metagenomic data reveals common features of halophilic viral communities across continents. *Environ Microbiol* Mar **18(3),** 889–903 (2016).
11. Labonté, J. M. & Suttle, C. A. Previously unknown and highly divergent ssDNA viruses populate the oceans. *ISME J* **7,** 2169–2177 (2013).
12. Ng, T. F. F. *et al.* High variety of known and new RNA and DNA viruses of diverse origins in untreated sewage. *J Virol* **86,** 12161–12175 (2012).
13. Rosario, K. *et al.* Diverse circular ssDNA viruses discovered in dragonflies (Odonata: Epiprocta). *J Gen Virol* **93,** 2668–2681 (2012).
14. Hopkins, M. *et al.* Diversity of environmental single-stranded DNA phages revealed by PCR amplification of the partial major capsid protein. *ISME J* **8,** 2093–2103 (2014).
15. Bergoin, M. & Tijssen, P. *Densoviruses: a Highly Diverse Group of Arthropod Parvoviruses, In Insect Virology*, (eds Asgari, S. & Johnson, K. N.) 59–72 (Caister Academic Press, 2010).
16. Cotmore, S. F. & Tattersall, P. Parvoviruses: Small Does Not Mean Simple. *Annu Rev Virol* **1,** 517–537 (2014).
17. Zádori, Z. *et al.* A viral phospholipase A2 is required for parvovirus infectivity. *Dev Cell* **1,** 291–302 (2001).
18. Cotmore, S. F. *et al.* The family Parvoviridae. *Arch Virol* **159,** 1239–1247 (2014).
19. Chapman, A. D. Numbers of Living Species in Australia and the World. Second edition. Report for the Australian Biological Resources Study (2009).

20. Belyi, V. A., Levine, A. J. & Skalka, A. M. Sequences from ancestral single-stranded DNA viruses in vertebrate genomes: the parvoviridae and circoviridae are more than 40 to 50 million years old. *J Virol* **84,** 12458–12462 (2010).
21. Liu, H. *et al.* Widespread endogenization of densoviruses and parvoviruses in animal and human genomes. *J Virol* **85,** 9863–9876 (2011).
22. Gudenkauf, B. M., Eaglesham, J. B., Aragundi, W. M. & Hewson, I. Discovery of urchin-associated densoviruses (Parvoviridae) in coastal waters of the Big Island, Hawaii. *J Gen Virol.* Mar **95** (Pt 3), 652–658 (2014).
23. Hewson, I. *et al.* Densovirus associated with sea-star wasting disease and mass mortality. *Proc Natl Acad Sci USA* Dec 2 **111(48),** 17278–17283 (2014).
24. Bowles, D., Rabinowitz, J. & Samulski, R. *In Parvoviruses*. (eds Kerr, J. *et al.*) 15–23 (Hodder Arnold, London, 2006).
25. Clavijo, G., van Munster, M., Monsion, B., Bochet, N. & Brault, V. Transcription of densovirus endogenous sequences in Myzus persicae genome. *J Gen Virol. Apr* **97(4),** 1000–1009 (2016).
26. Flegel, T. W. Hypothesis for heritable, anti-viral immunity in crustaceans and insects. *Biol Direct* **4,** 32 (2009)
27. Liu, S., Vijayendran, D. & Bonning, B. C. Next generation sequencing technologies for insect virus discovery. *Viruses* **3,** 1849–1869 (2011).
28. DeBoever, C. *et al.* Whole transcriptome sequencing enables discovery and analysis of viruses in archived primary central nervous system lymphomas. *PLoS One* **8,** e73956 (2013).
29. Consortium, T. U. UniProt: a hub for protein information. *Nucleic Acids Res* **43,** 204–212 (2014).
30. Pruitt, K. D., Tatusova, T. & Maglott, D. R. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **33,** D501–D504 (2005).
31. Kapoor, A., Simmonds, P. & Lipkin, W. I. Discovery and characterization of mammalian endogenous parvoviruses. *J Virol* **84,** 12628–12635 (2010).
32. Thézé, J., Leclercq, S., Moumen, B., Cordaux, R. & Gilbert, C. Remarkable diversity of endogenous viruses in a crustacean genome. *Genome Biol Evol* **6,** 2129–2140 (2014).
33. Arriagada, G. & Gifford, R. J. Parvovirus-derived endogenous viral elements in two South American rodent genomes. *J Virol* **88,** 12158–12162 (2014).
34. Metegnier, G. *et al.* Comparative paleovirological analysis of crustaceans identifies multiple widespread viral groups. *Mob DNA 16* **6,** 16 (2015).
35. Liu, H. *et al.* Widespread endogenization of densoviruses and parvoviruses in animal and human genomes. *J Virol* **85,** 9863–9876 (2011).
36. Ge, X. *et al.* Metagenomic analysis of viruses from bat fecal samples reveals many novel viruses in insectivorous bats in China. *J Virol* **86,** 4620–4630 (2012).
37. Iyer, L. M., Koonin, E. V., Leipe, D. D. & Aravind, L. Origin and evolution of the archaeo-eukaryotic primase superfamily and related palm-domain proteins: structural insights and new members. *Nucleic Acids Res* **33,** 3875–3896 (2005).
38. Van Munster, M., Janssen, A., Clérivet, A. & van den Heuvel, J. Can plants use an entomopathogenic virus as a defense against herbivores? *Oecologia* **143,** 396–401 (2005).
39. Hu, Z., Li, G., Li, G., Yao, Q. & Chen, K. Bombyx mori bidensovirus: The type species of the new genus Bidensovirus in the new family Bidnaviridae. *Chinese Sci Bull* **58,** 4528–4532 (2013).
40. Gayral, P. *et al.* Next-generation sequencing of transcriptomes: a guide to RNA isolation in nonmodel animals. *Mol Ecol Resour* **11,** 650–661 (2011).
41. Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J. M. & Birol, I. ABySS: a parallel assembler for short read sequence data. *Genome Res* **19,** 1117–1123 (2009).
42. Huang, X. CAP3: A DNA Sequence Assembly Program. *Genome Res* **9,** 868–877 (1999).
43. Gayral, P. *et al.* Reference-free population genomics from next-generation transcriptome data and the vertebrate-invertebrate gap. *PLoS Genet* **9,** e1003457 (2013).
44. Romiguier, J. *et al.* Comparative population genomics in animals uncovers the determinants of genetic diversity. *Nature* **515,** 261–263 (2014).
45. Hyatt, D. *et al.* Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC *Bioinformatics 8* **11,** 119 (2010).
46. Hyatt, D., Locascio, P. F., Hauser, L. J. & Uberbacher, E. C. Gene and translation initiation site prediction in metagenomic sequences. *Bioinformatics 1* **28(17),** 2223–2230 (2012).
47. Remmert, M., Biegert, A., Hauser, A. & Söding, J. HHblits: lightning-fast iterative protein sequence searching by HMM-HMM alignment. *Nat Methods* **9,** 173–175 (2011).
48. Söding, J. Protein homology detection by HMM-HMM comparison. *Bioinformatics 1* **21(7),** 951–960 (2005).
49. Jones, P. *et al.* InterProScan 5: genome-scale protein function classification. *Bioinformatics 1* **30,** 1236–1240 (2014).
50. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res 19* **32,** 1792–1797 (2004).
51. Guindon, S. *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59(3),** 307–321 (2010).
52. Abascal, F., Zardoya, R. & Posada, D. ProtTest: selection of best-fit models of protein evolution. *Bioinformatics 1* **21(9),** 2104–2105 (2005).
53. Chandler, J. A., Liu, R. M. & Bennett, S. N. RNA shotgun metagenomic sequencing of northern California (USA) mosquitoes uncovers viruses, bacteria, and fungi. *Front Microbiol* **24(6),** 185 (2015).
54. Liu, S., Chen, Y. & Bonning, B. C. RNA virus discovery in insects. *Curr Opin Insect Sci* **8,** 54–61 (2015).
55. François, S. *et al.* A Novel Itera-Like Densovirus Isolated by Viral Metagenomics from the Sea Barley Hordeum marinum. *Genome Announce*. Dec 4, **2(6)** (2014).
56. Jousset, F. X., Baquerizo, E. & Bergoin, M. *A new densovirus isolated from the mosquito Culex pipiens (Diptera: Culicidae)* **67(1),** 11–16 (2000).
57. Kisary, J., Avalosse, B., Miller-Faures, A. & Rommelaere, J. The Genome Structure of a New Chicken Virus Identifies It as a Parvovirus. *J Gen Virol* **66** (Pt 10), 2259–2263 (1985).
58. Tang, K. F. & Lightner, D. V. Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn Penaeus monodon from Africa and Australia. *Virus Res* **118(1–2)**, 185–191 (2006).

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Author Contributions

Data Acquisition S.F., D.F., D.B. and P.G.; Analysis and interpretation of data S.F., P.R., D.P. and M.O.; Manuscript preparation S.F., P.R., D.P. and M.O.; Study supervision S.F., P.R., R.F. and M.O.

Additional Information

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