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Data mining cDNAs reveals three new single stranded RNA viruses in *Nasonia* (Hymenoptera: Pteromalidae)

D. C. S. G. Oliveira*, W. B. Hunter†, J. Ng*, C. A. Desjardins*, P. M. Dang‡ and J. H. Werren*

**Department of Biology, University of Rochester, Rochester, NY, USA;* †*United States Department of Agriculture, Agricultural Research Service, US Horticultural Research Laboratory, Fort Pierce, FL, USA; and* ‡*United States Department of Agriculture, Agricultural Research Service, NPRU, Dawson, GA, USA*

Abstract

We report three novel small RNA viruses uncovered from cDNA libraries from parasitoid wasps in the genus *Nasonia*. The genome of this kind of virus is a positive-sense single-stranded RNA with a 3′ poly(A), which facilitates cloning from cDNAs. Two of the viruses, NvitV-1 and NvitV-2, possess a RNA-dependent RNA polymerase that associates them with the family *Iflaviridae* of the order *Picornavirales*. A third virus, NvitV-3, is most similar to the Nora virus from *Drosophila*. A reverse transcription-PCR method developed for NvitV-1 indicates that it is a persistent commensal infection of *Nasonia*.

Keywords: picorna-like virus, *Picornavirales*, insect RNA virus, RNA-dependent RNA polymerase.

Introduction

Current genomics efforts have expanded our understanding of animal, plant and microbial biology in many ways, quite frequently by providing new discoveries of associated, previously unknown microorganisms, such as viruses (Valles *et al*., 2004, 2008; Hunnicutt *et al*., 2006; Hunter *et al*., 2006; Katsar *et al*., 2007).

Correspondence: John W. Werren, 306 Hutchinson Hall, Department of Biology, University of Rochester, Rochester, NY 14627, USA. Tel.: +1 585 275 3889; fax: +1 585 275 2070; e-mail: werr@mail.rochester.edu

Present address: Deodoro C. S. G. Oliveira, Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, BCN 08193, Spain.

Small viruses with a positive-sense single-stranded RNA (ssRNA) genome, and no DNA stage, are known as picornaviruses (infecting vertebrates) or picorna-like viruses (infecting non-vertebrates). Recently, the order *Picornavirales* was formally characterized to include most, but not all, ssRNA viruses (Le Gall *et al*., 2008). Among other typical characteristics – e.g. a small icosahedral capsid with a pseudo-T = 3 symmetry and a 7–12 kb genome made of one or two RNA segments – the *Picornavirales* genome encodes a polyprotein with a replication module that includes a helicase, a protease, and an RNA-dependent RNA polymerase (RdRp), in this order (see Le Gall *et al*., 2008 for details). Pathogenicity of the infections can vary broadly from devastating epidemics to apparently persistent commensal infections. Several human diseases, from hepatitis A to the common cold (e.g. rhinovirus, Hughes *et al*., 1988), are caused by members of *Picornavirales*.

Besides vertebrates, ssRNA viruses also infect a broad range of hosts, including arthropods, e.g. flies (Johnson & Christian, 1998), moths (Wu *et al*., 2002), aphids (Moon *et al*., 1998), leafhoppers (Hunnicutt *et al*., 2006; Hunter *et al*., 2006) and others. Within the Hymenoptera, ants (Valles *et al*., 2004, 2008), bees (Ellis & Munn, 2005), and wasps (Reineke & Asgari, 2005) have been shown to be infected with ssRNA viruses. The honey bee is known to be infected by at least 18 different viruses (Allen & Ball, 1996), most of which are ssRNA viruses, and up to 4 viruses simultaneously (Chen *et al*., 2004, 2005). Parasitic wasps are frequently associated with viruses or virus-like entities that enable them to evade or directly suppress their hosts' immune system. The wasp *Venturia canescens*, family Ichneumonidae, has a picorna-like virus (VcSRV) which was proposed to contribute to the wasp endoparasitism of its host larvae (Reineke & Asgari, 2005).

Here, we describe the existence of three ssRNA viruses identified by mining expressed sequences tags (ESTs) from the wasp *Nasonia vitripennis* (Pteromalidae), provisionally named herein as NvitV-1, NvitV-2, and NvitV-3. Sequence analyses of RdRp of these viruses indicate that they are novel insect infecting viruses (Baker &

Table 1. Number of expressed sequence tags of viral origin in cDNAs prepared from two life-stages of *Nasonia vitripennis*

Virus*	Contig	cDNA from adult and pupae	cDNA from larvae	Total
NvitV-1	NVCL18Contig1	43	36	79
NvitV-2	NVCL278Contig1	0	10	10
NvitV-3†	NVCL3Contig1	374	1	375
NvitV-3	NVCL1797Contig1	2	0	2
NvitV-3	NVPDU05TR	1	0	1
Total viral reads		420	47	467
Total NV EST library‡		8306	10 381	18 687

*GenBank accession numbers for NvitV-1 (FJ790486); NvitV-2 (FJ790487); NvitV-3 (FJ790488).
†Three reads were also detected in expressed sequence tags (ESTs) of *Nasonia girault*i prepared from cDNA of pupae and adults.
‡Total good quality reads excluding mitochondrial sequences.

Schroeder, 2008; Le Gall *et al*., 2008). Two of them, NvitV-1 and NvitV-2, belong to the order *Picornavirales*, and probably to the family *Iflaviridae*. All current members of the *Iflaviridae* are placed in a single genus *Iflavirus* (Fauquet *et al*., 2005), however, similarities of NvitV-1 and VcSRV from the ichneumonid wasp suggest that they may form a new genus. NvitV-3, the only virus also found in *Nasonia girault*i ESTs, is most closely related to the Nora virus from a dipteran host, *Drosophila melanogaster* (Habayeb *et al*., 2006), and do not fall in the order *Picornavirales* (Le Gall *et al*., 2008). NvitV-3 was detected only in ESTs prepared from cDNA of pupae and adult wasps, while NvitV-2 was only detected in ESTs prepared from cDNA of larvae. NvitV-1 was further characterized by a reverse transcription-PCR (RT-PCR) assay, with results indicating that NvitV-1 is a persistent infection found in all tissues and life-stages tested. No detrimental symptoms were noted on wasp colonies infected with these viruses.

Results

Bioinformatic detection and annotation of three viruses in the Nasonia *expressed sequence tags*

Three novel ssRNA viruses were identified through mining ESTs of *N. vitripennis* (Table 1). Bioinformatic analysis of a set of sequences not matching the assembled *N. vitripennis* genome (NV genome version 1.0; Werren *et al*., 2010) revealed high similarity to picorna-like viruses (see below).

A sequence of 2789 bp, not including the poly(A), was assembled for one of the viruses, NvitV-1 (GenBank accession number FJ790486). NvitV-1 has an open reading frame (ORF) of 2366 bp incomplete at the 5′. The predicted polyprotein includes the partial sequence of a protease and the complete RdRp, with an additional 423 bp at the 3′ untranslated region (3′ UTR; Fig. 1). NvitV-1 was found in roughly equal frequencies in ESTs from larval and pupal/adult stages (Table 1).

For NvitV-2, a sequence of 1523 bp (not including the polyA) was assembled (GenBank accession number FJ790487) which consists of an 1161 bp ORF and 362 bp at the 3′ UTR (Fig. 1). The translated ORF has only a partial sequence of the RdRp. There is a 48 bp tandem repeat (three times) at the 3′UTR with unknown function. All 10 NvitV-2 reads were present in ESTs generated from larvae (Table 1).

Sequences of the third virus, NvitV-3, were assembled in two contigs and one singleton (GenBank accession FJ790488; Table 1), all of which present higher similarity to the *Drosophila* Nora virus (Habayeb *et al*., 2006), and

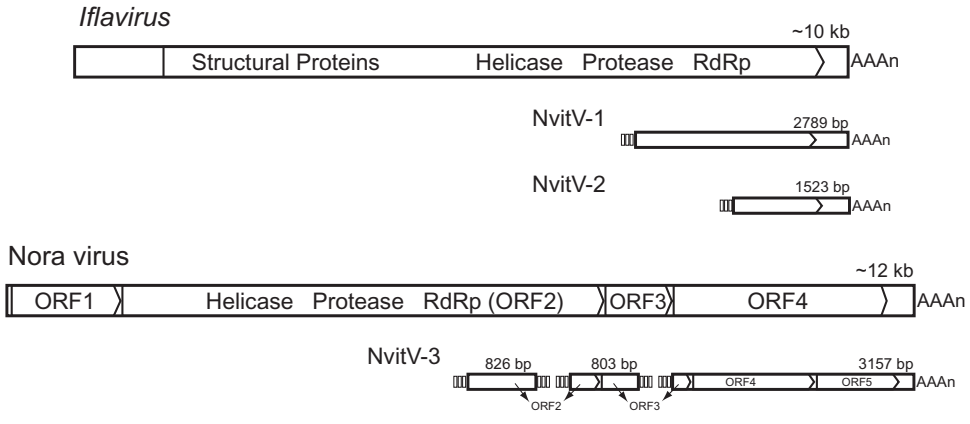

Figure 1. Schematic diagram showing genome organization of two kinds of ssRNA virus, an *Iflavirus* and the Nora virus. *Nasonia* single stranded RNA viral sequences are aligned to the homologous regions. RdRp is RNA-dependent RNA polymerase.

we assume they are all from a single virus. The larger 3′contig has one partial (tentatively ORF 3) and two complete ORFs (tentatively ORFs 4 and 5); ORFs 4 and 5 are homologous to ORF 4 of the Nora virus and they encode uncharacterized products (Fig. 1). The other contig and the singleton have partial sequences of the RdRp. This is the only virus also found in the *N. giraulti* ESTs, however, with a significantly smaller number of reads, only three. The *N. giraulti* sequences are identical to those that originated from *N. vitripennis*. Interestingly, all of the reads, but one, came from the pupal/adult cDNA libraries (Table 1).

The phylogenetic position of the Nasonia *picorna-like viruses*

The conserved RdRp sequences have been used to assist virus classification (Zanotto *et al*., 1996; Baker & Schroeder, 2008). Phylogenetic inferences were performed for the three *Nasonia* viruses along with another 22 diverse picornaviruses and picorna-like viruses (Table 2). Figure 2 shows a Maximum Parsimony (MP) reconstruction using the amino acid sequences of the most conserved regions of RdRp.

The phylogenetic analysis showed that NvitV-1 forms a well-supported clade with VcSRV, suggesting that this cluster might represent a new genus within the family *Iflaviridae* (Fauquet *et al*., 2005). However, NvitV-1 and VcSRV have not been fully sequenced and await a full diagnosis for taxonomic placement. NvitV-2 is also likely to be a member of the *Iflaviridae*. As suggested by genomic structure and despite the fact that NvitV-3 had a significant amount of missing data (Table 3), the clustering with Nora is well supported. The genomic structure of NvitV-3 and Nora indicates they are not *Picornavirales* (as defined by Le Gall *et al*., 2008).

The paraphyly of the *Iflaviridae* is somewhat surprising because of the position of infectious flacherie virus (IFV). It is worth noting that IFV is the prototype of the family *Iflaviridae* (Isawa *et al*., 1998; Fauquet *et al*., 2005). Reconstruction of basal nodes was problematic; basal branches are poorly supported and reconstruction was likely to be confounded by a high number of homoplasies. Major clades were well-supported and congruent across all phylogenetic analyses that were conducted, either using DNA or protein sequences (data not shown). In general, most well supported groupings are in agreement with previous similar phylogenies (Habayeb *et al*., 2006; Le Gall *et al*., 2008).

The eight conserved domains in the RNA-dependent RNA polymerase protein

The predicted RdRp amino acid sequence of the three *Nasonia* viruses were compared with seven other ssRNA viruses (Table 3) and shown to contain the eight conserved domains identified as common to the RdRp of positive-strand RNA viruses (Baker & Schroeder, 2008).

The RdRp region across all eight characteristic protein domains indicated that NvitV-1 and NvitV-2 are more closely related to viruses in the family *Iflaviridae* than to

Table 2. Picorna-like virus included in this study

Virus name	Abbreviation	Family	GenBank Accession Number*
Drosophila Nora virus	Nora	Unclassified	ABC55268
Silkworm infectious flacherie virus	IFV	*Iflaviridae*	BAA25371
Wasp *Venturia canescens* virus	VcSRV	*Iflaviridae*	AAS37668
Moth *Perina nuda* virus	PnPV	*Iflaviridae*	AAL06289
Honey bee Sacbrood virus	SBV	*Iflaviridae*	AAD20260
Mite *Varroa destructor* virus 1	VDV-1	*Iflaviridae*	AAP51418
Honey bee deformed wing virus	DWV	*Iflaviridae*	CAD34006
Honey bee Kakugo virus	KaV	*Iflaviridae*	BAD06930
Moth *Ectropis obliqua* virus	EoPV	*Iflaviridae*	AAQ64627
Aphid *Rhopalosiphum padi* virus	RhPV	*Dicistroviridae*	AAC95509
Honey bee black queen-cell virus	BQCV	*Dicistroviridae*	AAF72337
Drosophila C virus	DCV	*Dicistroviridae*	AAC58807
Cricket paralysis virus	CrPV	*Dicistroviridae*	AAF80998
Kashmir bee virus	KBV	*Dicistroviridae*	AAP32283
Honey bee Israel acute paralysis virus	IAPV	*Dicistroviridae*	ACD01403
Acute bee paralysis virus	ABPV	*Dicistroviridae*	AAN63804
Ant *Solenopsis invicta* virus	SINV-1	*Dicistroviridae*	AAU85375
Human hepatitis A virus	HAV	*Picornaviridae*	P08617
Human rhinovirus 1B	HRV-1B	*Picornaviridae*	BAA00168
Cowpea mosaic virus	CPMV	*Comoviridae*	CAA25029
Maize chlorotic dwarf virus	MCDV	*Sequiviridae*	AAB58882
Rice tungro spherical virus	RTSV	*Sequiviridae*	AAA66056

*GenBank numbers refer to the polyprotein.

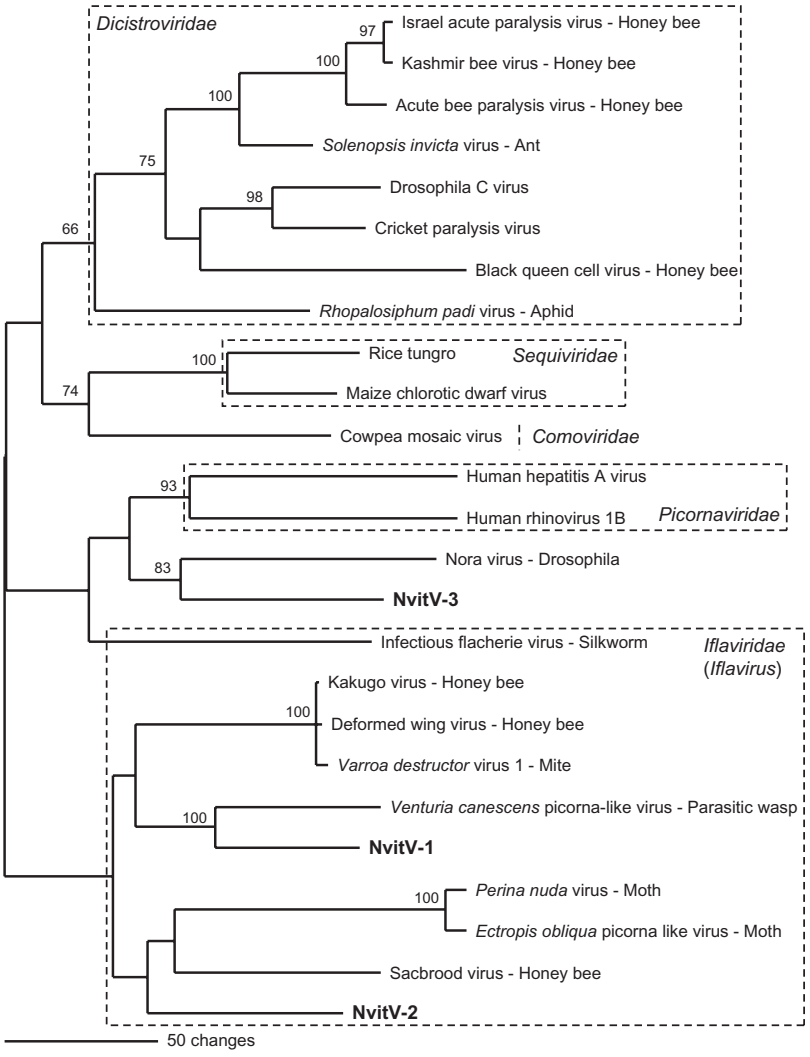

Figure 2. Phylogenetic inference of picorna-like viruses. The tree is the consensus of the two most parsimonious trees (1621 steps) obtained using conserved RNA-dependent RNA polymerase amino acid sequences – 202 characters, 179 parsimony-informative; CI = 0.665, RI = 0.618. The tree shown is midpoint rooted. Bootstrap support >50% is shown above the branches. GenBank accession numbers are presented in Table 2.

viruses of other families (Table 3). NvitV-1 was found to have the greatest overall similarity to RdRp of VcSRV (found in an ichneumonid wasp), and then to VDV-1, and deformed wing virus (DWV) from honey bees with 64%, 47% and 46% identities, respectively (for the conserved regions used for phylogenetics). Only partial sequence of the RdRp is available for the other two new *Nasonia* viruses. NvitV-2 is most similar to SBV and clearly also belongs to the family *Iflaviridae.* NvitV-3 is fairly distinct at the amino acid sequences level, but shows higher similarity to the Nora virus. These two viruses, NvitV-3 and Nora, are not members of the order *Picornavirales*, in agreement with their genomic structure.

Reverse transcription-PCR based detection of NvitV-1

VcSRV, the closest virus to NvitV-1, was found to be transmitted from the infected wasp *V. canescens* to the parasitized caterpillar (Reineke & Asgari, 2005). A RT-PCR assay was developed to diagnose the presence of NvitV-1 and to test the hypothesis that it is transmitted to the host fly pupae during parasitization by *Nasonia*. Results show that NvitV-1 is present in all life stages in both males and females (Fig. 3). The flesh fly host *Sarcophaga bullata* was negative for NvitV-1 infection after being stung by infected *Nasonia* (data not shown). Therefore, NvitV-1 neither appears to be passed through the venom nor passed during oviposition, although it is clearly present in the *Nasonia* female reproductive tract (Fig. 3).

Viral infection of NvitV-1 was not detected in two sibling species of *Nasonia*, *N. giraulti* and *Nasonia longicornis*. A closely related wasp *Trichomalopsis sarcophagae* also tested negative for NvitV-1. Strains of the species tested have been reared in the laboratory in close proximity with the infected *N. vitripennis* strain for many years, suggesting that either the NvitV-1 is species specific or requires a more direct contact mode of transmission.

Table 3. Amino acid sequence alignments of the eight conserved domains of RNA-dependent RNA polymerase of the three *Nasonia* viruses (NvitV-1, -2, -3) to members in the order *Picornavirales*: families: *Sequiviridae*, *Dicistroviridae,* and *Iflaviridae*

Virus	Family	Domain I	Domain II	Domain III	Domain IV
MCDV	Sequiviridae	ECPKDERRKLSK	TFTILPPEINILFRQYFGDFAAMIM	VGINPENMEWSD	GDYSKFDGIGD
ABPV	Dicistroviridae	DTLKDERRPIEK	VFSNGPMDFSITFRMYYLGFIAHLM	IGTNVYSQDWNK	GDFSTFDGSLN
DCV	Dicistroviridae	DTLKDERRDIAK	VFSAGPQHFVVAFRQYFLPFAAWLM	VGTNVYSSDWER	GDFGNFDGSLV
KaV	Iflaviridae	DCLKDTCLPVEK	IFSISPVQFTIPFRQYYLDFMASYR	IGIDVNSLEWTN	GDYKNFGPGLD
VcSRV	Iflaviridae	ACLKDARIPNRK	VFEMSPVDLTIAQRQFFMDFTVAYR	IGINPDGKEWTQ	ADYSGYGPRLS
NvitV-1	Iflaviridae	SCLKDARIPIYK	VFEMSPVDFTISQRQYFLDFYVAYQ	IGINPDGPEWTE	ADYSAYGPRLL
SBV	Iflaviridae	DTLKDERKLPEK	VFCNPPIDYIVSMRQYYMHFVAAFM	VGINVQSTEWTL	IDYSNFGPGFN
NvitV-2	Iflaviridae	??????????EK	VFSMSPVTASIVLRQYTLDLTSYLR	IGINPDGPEWGK	IDFSNFGPGLN
Nora	Unclassified	SKLKDQPIKIAQ	VFHCIPVDLILFSGALYGPYKEAYT	VGIDPKSVGWQQ	ADYKNYDKYLH
NvitV-3	Unclassified	SKLKDELVKPSK	IFQSSPVEYVIYAKGLFNNFIRFFR	MAIDPISYDWQE	IDYKNFDKRTS
	consensus	xxxKDxxxxxx:	xFxxxPxxx:xxxxxxxxxxxxxxx	:.x:xx.xxWxx	xD:xx:.xxxx

Virus	Family	Domain V	Domain VI	Domain VII	Domain VIII
MCDV	Sequiviridae	CQGMPSGFAMTVIFNSFVNYYYLAMAW	VVVYGDDNIV	TSVSFLKRR	PLDKTSIEER
ABPV	Dicistroviridae	THSQPSGNPATTPLNCFINSMGLRMVF	IVSYGDDNVI	EDVQYLKRK	PLSMDTILEM
DCV	Dicistroviridae	THSQPSGNPFTVIINCLYNSIIMRLSW	LITYGDDNVL	EDIFFLKRK	PLKIEVIYEM
KaV	Iflaviridae	PCGIPSGSPITDILNTISNCLLIRLAW	LVCYGDDLIM	QTATFLKHG	NLDKVSVEGT
VcSRV	Iflaviridae	TCGLPSGNCETVERNSQTNSLYIRIAF	LVTNGDDLIA	EEASYLKRG	PLEEASITDT
NvitV-1	Iflaviridae	NTGMPSGNAGTVITNSECNSIYIRCAY	MFSNGDDLIM	EEATYLKRG	PLERASITDT
SBV	Iflaviridae	KCGSPSGAPITVVINTLVNILYIFVAW	LFCYGDDLIM	LNSTFLKHG	ALAWSSINDT
NvitV-2	Unclassified	KSGSPSGAAITVEINSFVHLMYINICW	GVVYGDDGIF	SEMTFLKRS	PIDPDSIVEC
Nora	Unclassified	NRGNKSGSYTTTIDNCLANDIYGLYAW	SVAFGDDIIK	ENLQFLKRG	PLLQRSIEGP
NvitV-3	Unclassified	??????????????????????????????	??????????	ENVTFLKRY	PLEKASIEAP
	consensus	xx:xxSGxxxTxxxNxxx:xxxxxxx:	x.xxGDDx:x	xxxx:LK:x	x:xxxx:xxx

We failed to detect via RT-PCR the other two viruses (data not shown). Some possible reasons are that they could be transient infections or they could actually be infections of the fly host. An alternative explanation is that, in some cases, the viral titres are too low to be detected by the method used. This later explanation seems not to apply for NvitV-3 because of the very large number of adult ESTs from this virus (Table 1).

Discussion

Insects and other arthropods are vectors of many human (and economically important vertebrate) diseases. Therefore, the association of virus with arthropods has long been of interest (Koonin & Dolja, 1993, 2006; Forterre, 2006). In addition, many studies are now revealing that arthropods harbour their own assemblage of viruses, some of which can be vectored between hosts by parasitoids (López *et al*., 2002), while others are implicated in suppression of host immunity or other host modifications during parasitization (Bigot *et al*., 1997a; Schmidt *et al*., 2001; Lawrence, 2002). The advent of genomics has sped up novel virus discoveries in arthropods, which certainly provide new subjects for investigations of viral/host interactions (e.g. Isawa *et al*., 1998; Ghosh *et al*., 1999; Leat *et al*., 2000; van Munster *et al*., 2002). Here, we described three viruses in the ESTs of the parasitoid wasps *Nasonia*.

All three viruses are ssRNA viruses that were unintentionally cloned during an EST project designed to identify transcribed genes in *Nasonia*. The method used to generate ESTs clearly favours the purification and cloning of ssRNA viruses, which have a single-stranded RNA genome polyadenylated at the 3′ end similar to the

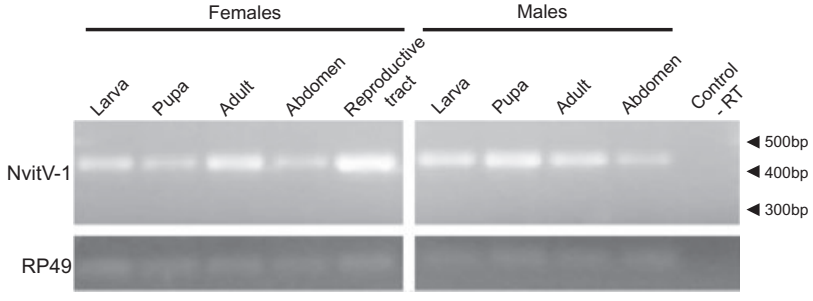

Figure 3. Reverse transcription-PCR (RT-PCR) assay for detection of NvitV-1. The virus NvitV-1 can be detected in *Nasonia vitripennis* males and females of different life stages, it is also detected in the abdomen and in the female reproductive tract. RT-PCR amplification of the ribosomal RP49 is shown for comparison.

targeted eukaryotic mRNA. The frequency of viral reads varies broadly depending on the type of virus, life stage, and species considered. However, it reaches 5% (NvitV-3 alone accounts for 4.5%) in the *N. vitripennis* pupal/adult cDNA library (Table 1). Far fewer were detected for the other two viruses and for the *N. giraulti* ESTs.

Modes of virus transmission vary widely, and host-to-parasitoid transmission of viruses has been reported for an iridovirus (López *et al*., 2002) and an ascovirus (Bigot *et al*., 1997a,b). In that regard, NvitV-1 clustered with VcSRV which has a proposed relationship with *V. canescens* favouring the development of the parasitoid larvae within parasitized host caterpillars (Reineke & Asgari, 2005). Such a relationship is well documented for polydnavirus (Shelby & Webb, 1999). Other types of parasitoid wasp associated viruses have been shown to be immune suppressors of the wasps' parasitized hosts. An ascovirus associated with the parasitoid wasp *Diadromus pulchellus* modulates the metabolism, development, and defence system of the wasp's lepidopteran host *Acrolepiopsis assectella* (Bigot *et al*., 1997a). Another example for a symbiotic virus–wasp relationship is an entomopoxvirus isolated from the braconid wasp *Diachasmimorpha longicaudata* that replicates in the wasp, but is pathogenic only to the wasp's dipteran host and may play a role in suppression of the hosts' immune system (Lawrence, 2002). The parasitoids above are all endoparasitic, laying their eggs within the host body, and therefore mechanisms to suppress host immunity are required. In contrast, *Nasonia* is an ectoparasite that lays its eggs on the surface of the host pupa, albeit under the puparial wall. Nevertheless, venoms are injected into the host that modifies cell physiology in complex ways (Rivers *et al*., 1993). However, RT-PCR of parasitized hosts did not detect the virus and, currently, there is no evidence that NvitV-1 is involved in these effects.

Diseases which could be attributed to these viruses were never observed in any of the wasps nor in the dipteran host in laboratory cultures. Nevertheless they could still be pathogens of the parasitoid, *N. vitripennis*, with relatively mild, or latent effects. It seems, however, that the NvitV-1 virus described here is a persistent commensal infection.

The intracellular bacteria *Wolbachia* were recently shown to promote resistance to ssRNA viruses in *Drosophila* (Teixeira *et al*., 2008). *Nasonia vitripennis* is infected by two types of *Wolbachia* and *N. giraulti* by three different strains (Raychoudhury *et al*., 2009). The *N. vitripennis* and *N. giraulti* strains used for EST production were both infected with these *Wolbachia*. It is not known whether *Wolbachia* in *Nasonia* can modulate ssRNA virus levels. This will be an interesting area for future research and may explain the failure to detect NvitV-2 and NvitV-3 by RT-PCR.

Proper taxonomy relies on full viral genome sequencing and knowledge of virion structure. The partial sequence of two of the viruses, NvitV-1 and NvitV-2 contained sufficient information to let us confidently place them in the newly defined order *Picornavirales* (Baker & Schroeder, 2008; Le Gall *et al*., 2008). The systematic position of NvitV-3 is at the moment unclear, since there is only one other similar virus, the also unplaced Nora virus found in *Drosophila* (Habayeb *et al*., 2006). Further, phylogenetic analysis revealed that NvitV-1 was most closely related to VcSRV, which may provide evidence for the creation of a new genus (Table 3; Fig. 2). To our knowledge it is the only other picorna-like virus of a parasitic wasp for which sequence information is available. This kind of comparative genetic analysis of information provides evidence for evolutionary relationships among insect, mammalian and plant picorna-like viruses (Isawa *et al*., 1998) and have recently been used to re-evaluate members into the order *Picornavirales*. By comparison of the RdRp protein sequences, both NvitV-1 and VcSRV were more similar to each other than to other members of the genus *Iflavirus* (containing the insect-infecting RNA viruses; Table 3, Fig. 2) and would therefore represent another clade within the family *Iflaviridae*. Further support for this will depend on full viral genome comparisons plus the discovery of more viral members with strong homology to NvitV-1 and VcSRV (Mayo, 2002; Fauquet *et al*., 2005). Genomic comparisons will highlight contrasts to the genomes of the *Dicistroviridae* and other insect viruses from the *Picornavirales* where the capsid proteins are encoded in the 3′ part and the non-structural proteins including the RdRp are at the 5′ (Mayo, 2002; Fauquet *et al*., 2005).

The current genomic effort on the parasitoid *Nasonia* (Werren *et al*., 2010) continues to provide new findings from these small insects, which are emerging as a genetic model system. The newly discovered viruses reported here define new members and expand the taxonomy of ssRNA viruses, and provide evidence for consideration of creating a new virus genus within the *Iflaviridae.* The *Nasonia* viruses also may turn out to be a safe, easily manipulated system for the study of basic ssRNA viral features and more specific virus–hymenopteran interactions.

Experimental procedures

Nasonia *expressed sequence tags*

Details of ESTs generated from two species of *Nasonia*, *N. giraulti* and *N. vitripennis*, will be presented elsewhere (Werren *et al*., 2010). The strains used were RV2, *N. giraulti*, and AsymC(LbII), *N. vitripennis*, both *Wolbachia* infected. In brief, for each species two cDNA libraries were prepared, one from larvae and one pupae/adults. cDNA libraries were prepared using ZAP-cDNA library construction kit (Strategene, La Jolla, CA, USA), from

isolate poly (A+) RNA (MicroPoly(A) Purist Kit, Ambion, Austin, TX, USA) and directionally cloned into pBluescript II XR vector (Strategene). Clones were 5′ sequenced. After removal of sequences of mitochondrial origin (Oliveira *et al*., 2008), 18 687 good quality reads were assembled and annotated.

Wasps rearing, species and strains investigated

Pteromalidae wasps of the genus *Nasonia* and *Trichomalopsis* were cultured in standard condition using the flesh fly *Sarcophaga bullata* as host. Three *Nasonia* species, *N. vitripennis* (NV, strain AsymCX), *N. giraulti* (NG, strain RV2X), and *N. longicornis* (NL, strain IV7X), and one *Trichomalopsis*, *T. sarcophagae*, were investigated.

RNA purification

RNA was purified from the entire body or from dissected tissues pulled from 5 individual wasps. Three different life stages were investigated for both males and females: larvae, yellow pupae (10 days old), and adults. In addition, different body parts were assayed: male and female abdomens, and female reproductive tracts. Three extractions of each sample were conducted to produce independent biological replicates. In addition, RNA was purified from both a stung (with *Nasonia* eggs removed) and unstung single *S. bullata* pupa. Tissues were harvested and immediately placed in RNA*later* (Ambion) and stored at −20 °C. Total RNA was extracted either using Trizol (Invitrogen, Carlsbad, CA, USA) or with Invisorb Spin Tissue RNA purification Kit (Invitek, Berlin, Germany) and poly(A) RNA isolated using the mini volume protocol of the Dynabeads mRNA Direct Kit (Dynal Biotech, Oslo, Norway). RNA was then quantified using a Qubit fluorometer (Invitrogen) and a Quant-iT RNA Assay Kit (Invitrogen).

Primer design and RT-PCR screening of NvitV-1

To test for the presence of the NvitV-1 virus, RNAs were first converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen), then amplified by PCR using virus-specific primers Picorna-a (5′ATTTATATTAGGTGTGCGTATCTTG3′) and Picorna-b (5′CAGGACCGTGAGTATAAGCAAG3′). Thermal-cycling conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 68 °C for 60 s. This was followed by a 5 min final extension at 72 °C. Sequencing of the amplified product verified that the primers were correctly amplifying a fragment of the NvitV-1 virus sequence. Primers that amplify part of the ribosomal RP49 sequence were used as control for the RT-PCR assay (RP49F_1 5′CTTCCGCAAAGTCCTTGTTC3′ and RP49R_1 5′AACTCCATGGGCAATTTCTG3′).

Bioinformatic and phylogenetic analysis

The name, acronym, and sequence accession numbers of the 22 viruses used in this investigation are shown in Table 2. Protein (and corresponding nucleotide sequences) of insect and plant picorna-like viruses were obtained from GenBank. Blast searches of the National Center for Biotechnology Information (NCBI) databases were used to initially assign sequences homologies (Altschul *et al*., 1997). Computational sequence analysis was performed using either the GeneCodes software package (Sequencher™, Ann Arbor, MI, USA) or the BioEdit Sequence Alignment Editor (Hall, 1999).

Phylogenetic analyses were conducted on both amino acid and nucleotide sequences spanning the 8 conserved domains of the RdRp protein (Baker & Schroeder, 2008). ClustalW2 (Larkin *et al*., 2007) was used to generate initial alignments and sequences were manually adjusted (final alignment is available upon request). Phylogenetic relationships were reconstructed using a 1000 random addition (TBR swapping) in PAUP* v4.0b10 (Swofford, 2003). Branch support was accessed with 100 bootstrap replicates.

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Conflicts of interest

The authors have not declared any conflicts of interest.

References

Allen, M. and Ball, B.V. (1996) The incidence and world distribution of honey bee viruses. *Bee World* **77**: 141–162.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Baker, A.C. and Schroeder, D.C. (2008) The use of RNA-dependent RNA polymerase for the taxonomic assignment of Picorna-like viruses (order *Picornavirales*) infecting *Apis mellifera* L. populations. *Virol J* **5**: 10.

Bigot, Y., Rabouille, A., Doury, G., Sizaret, P.Y., Delbost, F., Hamelin, M.H. *et al.* (1997a) Biological and molecular features of the relationships between *Diadromus pulchellus* ascovirus, a parasitoid hymenopteran wasp (*Diadromus pulchellus*) and its lepidopteran host, *Acrolepiopsis assectella*. *J Gen Virol* **78**: 1149–1163.

Bigot, Y., Rabouille, A., Sizaret, P.Y., Hamelin, M.H. and Periquet, G. (1997b) Particle and genomic characteristics of a new member of the *Ascoviridae: Diadromus pulchellus* ascovirus. *J Gen Virol* **78**: 1139–1147.

Chen, Y., Zhao, Y., Hammond, J., Hsu, H.T., Evans, J. and Feldlaufer, M.J. (2004) Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *J Invertebr Pathol* **87**: 84–93.

Chen, Y., Pettis, J.S. and Feldlaufer, M.F. (2005) Detection of multiple viruses in queens of the honey bee *Apis mellifera* L. *J Invertebr Pathol* **90**: 118–121.

Ellis, J.D. and Munn, P.A. (2005) The worldwide health status of honey bees. *Bee World* **86**: 88–101.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. (2005) *Virus Taxonomy: VIIIth Report of the*

International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London.

Forterre, P. (2006) The origin of viruses and their possible roles in major evolutionary transitions. *Virus Res* **117**: 5–16.

Ghosh, R.C., Ball, B.V., Willcocks, M.M. and Carter, M.J. (1999) The nucleotide sequence of sacbrood virus of the honeybee, an insect picorna-like virus. *J Gen Virol* **80**: 1541–1549.

Habayeb, M.S., Ekengren, S.K. and Hultmark, D. (2006) Nora virus, a persistent virus in *Drosophila*, defines a new picorna-like virus family. *J Gen Virol* **87**: 3045–3051.

Hall, T.A. (1999) *BioEdit: a User-Friendly Biological Sequence Alignment Editor and Analysis*. Ibis Biosciences, Carlsbad, CA.

Hughes, P.J., North, C., Jellis, C.H., Minor, P.D. and Stanway, G. (1988) The nucleotide sequence of human rhinovirus 1B: molecular relationships within the rhinovirus genus. *J Gen Virol* **69**: 49–58.

Hunnicutt, L.E., Hunter, W.B., Cave, R.D., Powell, C.A. and Mozoruk, J.J. (2006) Complete genome sequence and molecular characterization of *Homalodisca coagulata virus-1*, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera: Cicadellidae). *Virology* **350**: 67–78.

Hunter, W.B., Katsar, C.S. and Chaparro, J.X. (2006) Nucleotide sequence of 3′ -end of *Homalodisca coagulata Virus-1*. A new leafhopper-infecting virus from the glassy-winged sharpshooter. *J Insect Sci* **6**: 28.

Isawa, H., Asano, S., Sahara, K., Iizuka, T. and Bando, H. (1998) Analysis of genetic information of an insect picorna-like virus, infectious flacherie virus of silkworm: evidence for evolutionary relationships among insect, mammalian and plant picorna(-like) viruses. *Arch Virol* **143**: 127–143.

Johnson, K.N. and Christian, P.D. (1998) The novel genome organization of the insect picorna-like virus Drosophila C virus suggests this virus belongs to a previously undescribed virus family. *J Gen Virol* **79**: 191–203.

Katsar, C.S., Hunter, W.B. and Sinisterra, X.H. (2007) Phytoreovirus-like sequences from glassy-winged sharpshooter salivary glands. *Fla Entomol* **90**: 196–203.

Koonin, E.V. and Dolja, V. (2006) Evolution of complexity in the viral world: the dawn of a new vision. *Virus Res* **117**: 1–4.

Koonin, E.V. and Dolja, V.V. (1993) Evolution and taxonomy of positive strand RNA viruses: implications of comparative analysis of amino acid sequences. *Crit Rev Biochem Mol Biol* **28**: 375–430.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H. *et al.* (2007) ClustalW and ClustalX version 2. *Bioinformatics* **23**: 2947–2948.

Lawrence, P.O. (2002) Purification and partial characterization of an entomopoxvirus (DlEPV) from a parasitic wasp of tephritid fruit flies. *J Insect Sci* **2**: 2.

Le Gall, O., Christian, P., Fauquet, C.M., King, A.M., Knowles, N.J., Nakashima, N. *et al.* (2008) *Picornavirale*s, a proposed order of positive-sense single-stranded RNA viruses with a pseudo-T = 3 virion architecture. *Arch Virol* **153**: 715–727.

Leat, N., Ball, B., Govan, B. and Davison, S. (2000) Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. *J Gen Virol* **81**: 2111–2119.

López, M., Rojas, J.C., Vandame, R. and Williams, T. (2002) Parasitoid-mediated transmission of an iridescent virus. *J Invertebr Pathol* **80**: 160–170.

Mayo, M.A. (2002) A summary of taxonomic changes recently approved by the ICTV. *Arch Virol* **147**: 1655–1656.

Moon, J.S., Domier, L.L., McCoppin, N.K., D'Arcy, C.J. and Jin, H. (1998) Nucleotide sequence analysis shows that *Rhopalosiphum padi* virus is a member of a novel group of insect-infecting RNA viruses. *Virology* **243**: 54–65.

van Munster, M., Dullemans, A.M., Verbeek, M., van den Heuvel, J.F.J.M., Clerivet, A. and van der Wilk, F. (2002) Sequence analysis and genomic organization of aphid lethal paralysis virus: a new member of the family Dicistroviridae. *J Gen Virol* **83**: 3131–3138.

Oliveira, D.C.S.G., Raychoudhury, R., Lavrov, D.V. and Werren, J.H. (2008) Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp *Nasonia* (Hymenoptera: Pteromalidae). *Mol Biol Evol* **25**: 2167–2180.

Raychoudhury, R., Baldo, L., Oliveira, D.C.S.G. and Werren, J.H. (2009) Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. *Evology* **63**: 165–183.

Reineke, A. and Asgari, S. (2005) Presence of a novel small RNA-containing virus in a laboratory culture of the endoparasitic wasp *Venturia canescens* (Hymenoptera: Ichneumonidae). *J Insect Physiol* **51**: 127–135.

Rivers, D.B., Hink, W.F. and Denlinger, D.L. (1993) Toxicity of the venom from *Nasonia vitripennis* (Hymenoptera: Pteromalidae) toward fly hosts, nontarget insects, different developmental stages, and cultured insect cells. *Toxicon* **31**: 755–765.

Schmidt, O., Theopold, U. and Strand, M. (2001) Innate immunity and its evasion and suppression by hymenopteran endoparasitoids. *Bioessays* **23**: 344–351.

Shelby, K.S. and Webb, B.A. (1999) Polydnavirus-mediated suppression of insect immunity. *J Insect Physiol* **45**: 507–514.

Swofford, D.L. (2003) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer Associates Inc., Sunderland, MA.

Teixeira, L., Ferreira, A. and Ashburner, M. (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol* **6**: 2753–2763.

Valles, S.M., Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H. *et al.* (2004) A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. *Virology* **328**: 151–157.

Valles, S.M., Strong, C.A., Hunter, W.B., Dang, P.M., Pereira, R.M., Oi, D.H. *et al.* (2008) Expressed sequence tags from the red imported fire ant, *Solenopsis invicta*: annotation and utilization for discovery of viruses. *J Invertebr Pathol* **99**: 74–81.

Webb, B.A. (1998) Polydnavirus biology, genome structure, and evolution. In *The Insect Viruses* (Miller, L.K. and Ball, L.A., eds), pp. 105–139. Plenum, New York, New York.

Werren, J.H., Richards, S., Desjardins, C.A., Niehuis, O., Gadau, J., Colbourne, J.K., *et al.* (2010) Functional and evolutionary insights from the genomes of three parasitoid *Nasonia* species. *Science*, doi:10.1126/science.1178028.

Wu, C.Y., Lo, C.F., Huang, C.J., Yu, H.T. and Wang, C.H. (2002) The complete genome sequence of *Perina nuda* picorna-like virus, an insect-infecting RNA virus with a genome organization similar to that of the mammalian picornaviruses. *Virology* **294**: 312–323.

Zanotto, P.M., Gibbs, M.J., Gould, E.A. and Homes, E.C. (1996) A reevaluation of the higher taxonomy of viruses based on RNA polymerases. *J Virol* **70**: 6083–6096.