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Novel circular DNA viruses identified in *Procordulia grayi* and *Xanthocnemis zealandica* larvae using metagenomic approaches

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ABSTRACT

Recent advances in sequencing and metagenomics have enabled the discovery of many novel single stranded DNA (ssDNA) viruses from various environments. We have previously demonstrated that adult dragonflies, as predatory insects, are useful indicators of ssDNA viruses in terrestrial ecosystems. Here we recover and characterise 13 viral genomes which represent 10 novel and diverse circular replication associated protein (Rep)-encoding single stranded (CRESS) DNA viruses (1628–2668 nt) from *Procordulia grayi* and *Xanthocnemis zealandica* dragonfly larvae collected from four high-country lakes in the South Island of New Zealand. The dragonfly larvae associated CRESS DNA viruses have different genome architectures, however, they all encode two major open reading frames (ORFs) which either have bidirectional or unidirectional arrangement. The 13 viral genomes have a conserved NAGTATTAC nonanucleotide motif and in their predicted Rep proteins we identified the rolling circle replication (RCR) motif 1, 2 and 3, as well as superfamily 3 (SF3) helicase motifs. Maximum likelihood phylogenetic and pairwise identity analysis of the Rep amino acid sequences reveal that the dragonfly larvae novel CRESS DNA viruses share <63% pairwise amino acid identity to the Reps of other CRESS DNA viruses whose complete genomes have been determined and available in public databases and that these viruses are novel. CRESS DNA viruses are circulating in larval dragonfly populations; however, we are unable to ascertain whether these viruses are infecting the larvae directly or are transient within dragonflies via their diet.

1. Introduction

Metagenomic approaches coupled with new sequencing technologies have allowed the exploration of novel and known viral communities in various ecosystems. Novel small circular DNA viruses with different genome architectures have been recovered from a variety of sources including environmental samples (soil, water, river sediments), invertebrates and birds, as well as faecal sources. The majority of these viruses are yet to be properly classified within the viral taxonomy structure, given that in most cases the pathway of transmission and hosts species have not been identified. Nonetheless, environmental sampling has enabled the exploration of the viral sequence space, enabling identification and the occurrence of viruses in natural ecosystems, beyond what has been discovered to be pathogenic to humans, plants and animals.

Over the last couple of years our research group has recovered a variety of ssDNA viruses from adult dragonfly species (*Anax junius*, *Diplacodes bipunctata*, *Erythemis fusca*, *Erythemis simplicicollis*, *Erythemis vesiculosa*, *Erythrodiplax umbrata*, *Myathiria Marcella*, *Orthetrum sabina*, *Pantala flavescens*, *Rhionaeschna multicolour*, *Somatochlora meridionalis*, *Tholymis tillarga* and *Xanthocnemis zealandica*) from Kingdom of Tonga, Australia, USA, Bulgaria, Puerto Rico, and New Zealand (Dayaram et al., 2013; Rosario et al., 2011, 2012a, 2013). Interestingly, most of the viruses recovered from these dragonfly species have mainly been cycloviruses. However, a few gemycircularviruses, mastreviruses, diverse circular replication associated protein (Rep)-encoding single stranded (CRESS) DNA viruses and a microphage were also recovered from adult dragonflies. Recently, Padilla-Rodriguez et al. (2013) recovered the a novel cyclovirus from *Eurycotis floridana* (Florida woods cock-

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roach) while Pham et al. (2013a,b) have recently recovered the genomes of circular ssDNA volvoviruses from *Acheta domesticus* and *Gryllus assimilis*.

The isolation and characterisation of these novel viruses provides intriguing suggestions that these circular ssDNA viruses may be commonplace in terrestrial insect and invertebrate species especially top-end predators. We have previously suggested that predatory insects may be acting as bio-accumulators in their given environment. Recently, our research group identified plant-infecting geminiviruses and an associated DNA satellite (associated with begomoviruses) in dragonflies sampled at an agricultural field in Puerto Rico (Rosario et al., 2013). This demonstrates that top-end insect predators may act as viral reservoirs for ssDNA viruses and could be used as ssDNA viral sampling tool in ecosystems for virus surveillance (Rosario et al., 2012a).

Dragonfly larvae are well documented predators (prey include aquatic invertebrates, tadpoles, small fish, and other dragonfly larvae) in their aquatic environments (Corbet and Brooks, 2008). Hence, just like adult dragonflies in the terrestrial environment, their larvae could be ideal indicators of viruses in aquatic environments. Over the last 5 years numerous aquatic environments (freshwater lakes, hypersaline lakes, reclaimed water, and oceans) have been analysed using metagenomic approaches and in all cases a high diversity of ssDNA viruses have been identified (Culley et al., 2006; Emerson et al., 2012; Hewson et al., 2012; Labonte and Suttle, 2013; Lopez-Bueno et al., 2009; Rosario et al., 2009a,b; Roux et al., 2012).

In order to determine whether we could detect ssDNA viruses in dragonfly larvae, we undertook a pilot study in the South Island of New Zealand where commonly found species of Odonata larvae include *Procordulia grayi* (Selys) and *X. zealandica* (McLachlan).

2. Materials and methods

2.1. Sample collection

We collected larvae from four high-country lakes with minimal human impact: Lake Donne (43°36′30.34″S, 171°6′56.73″E; 663 m alt.), Lake Grasmere (43°3′48.28″S, 171°46′30.33″E; 584 m alt.), Lake Sarah (43°2′57.11″S, 171°46′35.04″E; 577 m alt.) and Lake Hawdon (43°6′12.83″S, 171°50′57.58″E; 576 m alt.). Late instar larvae were collected in the austral summer (February and December 2012) using 1-meter D-net sweeps (1 mm mesh) along the shallow lake bottoms and emergent aquatic vegetation. Repeated sweeps in these habitats were undertaken until 10–20 larvae of each species were collected. For virus analyses, live larvae were sorted in the field and kept in their lake water for transport back to the laboratory. Other larvae were collected and preserved in 80% ethanol for gut analyses. Species identifications were confirmed in the laboratory using identification keys outlined for New Zealand Odonata species (Rowe, 1987).

2.2. Viral purification and DNA extraction

Larvae of each species within each lake was homogenized in SM buffer [0.1 M NaCl, 50 mM Tris/HCl (pH 7.4), 10 mM $MgSO_4$] at a ratio of 5 ml SM buffer to 2.5 g of larvae tissue. The homogenate was processed as previously described (Dayaram et al., 2013; Rosario et al., 2012a). In brief, the homogenate was pelleted and the supernatant was then filtered sequentially through a 0.45 μm pore size syringe filter (Sartorius Stedim Biotech, Germany) followed by a 0.2 μm filter and finally viral DNA was then extracted from the filtrate using the High Pure Viral Nucleic Acid kit (Roche, USA).

2.3. Enrichment of circular DNA, viral identification and cloning of viral genomes

The viral nucleic acid was enriched for circular DNA using rolling circle amplification (RCA) using Illustra TempliPhi Amplification kit (GE Healthcare, USA). The RCA concatenated DNA from each sample group was digested with either *Bam*H1, *Sma*1, *Eco*R1 and *Xmn*1 in separate reactions resulting in DNA fragments between ~1.7 and 2.6 KB. The resulting fragments were gel purified and then cloned into *Eco*R1 pGEM3ZF (+) (Promega, USA) restricted plasmid or into pUC-19 plasmid vector restricted with *Bam*H1 or *Sma*1 (Fermentas, USA). The clones were sequenced by primer walking at Macrogen Inc. (South Korea). The putative genomes were verified either by restriction mapping or by designing back-to-back primer followed by PCR amplification of the genomes using Kapa HiFi HotStart DNA polymerase (Kapa Biosystems, USA), then cloning the amplicon into pJET1.2 (Thermo Fisher, USA) and sequencing the recombinant plasmid.

We also implemented a next-generation sequencing informed approach to explore the viral diversity amongst the larvae. All enriched RCA products were also grouped together and sequenced on an Illumina HiSeq 2000 (Illumina) platform at the Beijing Genomics Institute (Hong Kong). The resulting paired end reads were then assembled using ABySS V1.3.5 (Simpson et al., 2009) with kmer = 64. A full BLASTx (Altschul et al., 1990) analysis was performed of the assembled contigs using KoriBlast v4.1 (Korilog SARL, France). Contigs >750 nt with credible hits to potential ssDNA viruses were flagged and for these we designed back-to-back primers to recover full genomes (Table 1) and used these to amplify viral genomes using Kapa HiFi HotStart polymerase (Kapa Biosystems USA). The amplicons were gel purified, cloned into pJET1.2 (Thermo Fisher, USA) and the clones were sequenced at Macrogen Inc. (South Korea) by primer walking.

Table 1
Detail of primer sequences and restriction enzymes to recover complete dragonfly larvae associated CRESS DNA viral genomes.

	Initial restriction enzyme	Second restriction enzyme	Forward primer	Reverse primer
DflaCV-1 [NZ-PG11-LD]	*Sma*1	*Hind*III	–	–
DflaCV-2 [NZ-PG8-LS]			GACTGGCACTGGGAAATCTAGAACT	GAACCCCAGAACACATAGCAGG
DflaCV-3 [NZ-PG1-LG]			GACGAGCCATATGGTCCTTGG	CAGATGGTTTGGATGGTGTTTGTGG
DflaCV-3 [NZ-PG4-LH]			GACGAGCCATATGGTCCTTGG	CAGATGGTTTGGATGGTGTTTGTGG
DflaCV-4 [NZPG3-LG]			AAGAGGCGAATGGTGGGAT	GGCTTGGGATAGGGATTCT
DflaCV-5 [NZ-PG2-LG]	*Eco*RI		CTAACCCACAAATGAATAATGTTGCC	GGTAATGGATGATGTGATAGATGG
DflaCV-5 [NZ-PG7-LS]	*Xmn*I		GTGGTGGGTCATGAGATCTG	GATGTACTTGCAGGCGATGTC
DflaCV-6 [NZ-PG9-LD]			AATGGTGCACGGATAGGATTG	TTTGTCTTACGGATCCAACG
DflaCV-7 [NZ-PG5-LH]			CAGGGGTATCTGGAGCTGAA	CCAATGCTCCCTCCCCG
DflaCV-8 [NZ-PG5-LS]			GAGAGCAGAGGGACATAGTACGG	CGGGTAGGGATAGTACGGGGTA
DflaCV-9 [NZ-PG10-LD]	*Xmn*I	*Eco*RV	–	–
DflaCV-10 [NZ-XZ2-LS]	*Xmn*I		GGGGTATCTAGTGATTGTCTTTC	GGTGGTAAACTGCTGATCTCC
DflaCV-10 [NZ-XZ1-LH]	*Pst*I		GCTAGTGATGTCAACAATCTTCCCG	ATTCATTGAGAGACAGTCGGTATAGATG