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Endogenous Brevidensovirus-like elements in *Cherax quadricarinatus*: Friend or foe?

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ABSTRACT

Nine endogenous Brevidensovirus-like elements (EBreVE) were identified in redclaw freshwater crayfish *Cherax quadricarinatus* from different sources suggesting that these elements are widespread in redclaw in northern Queensland, Australia. These endogenous virus-like elements shared nucleotide identities (70–100%) and amino acid similarities (34–100%) with infectious hypodermal and haematopoietic necrosis virus (IHHNV) scientifically classified as *Penaeus stylirostris* densovirus (PstDNV). They may not have originated from IHHNV genomes, but could be derived from another uncharacterised member of the genus Brevidensovirus that share nucleotide similarities with IHHNV. The most striking feature of EBreVEs was that in each case, the segment (portion) of viral sequences inserted into the host genomes was from the same region of the viral genome and most likely derived from non-structural protein regions of ancestral virus, but they cannot be assembled into one consensus sequence. The EBreVEs may be inserted into the redclaw genomes following chronic or persistent infection by a corresponding virus that may have occurred as multiple independent integration events years ago leading to the accumulation of several integrated elements in their genomes. Histological examination and polymerase chain reaction (PCR) suggested that these insertions may have a protective function to their host.

1. Introduction

Viral fragments integrated into host genomes are called endogenous viral elements, EVEs (Katzourakis and Gifford, 2010), and have been recognised for many years (Benveniste and Todaro, 1974; Jaenisch, 1976; Zhdanov, 1975). Endogenisation of viral elements occurs when a double stranded DNA copy of the viral genome is inserted into the germ line of the host. They can potentially be transmitted vertically to the offspring and become fixed in the population of the host (Feschotte and Gilbert, 2012; Holmes, 2011; Kapoor et al., 2010). Integration of viral genomes into the host cells involves a broad range of virus families both RNA and DNA viruses from various organisms (Belyi et al., 2010a,b; Feschotte and Gilbert, 2012; Holmes, 2011; Horie et al., 2010; Kapoor et al., 2010; Katzourakis and Gifford, 2010; Liu et al., 2011). This phenomenon has been detected in bacteria (Salanoubat et al., 2002; Simpson et al., 2000), fungi (Frank and Wolfe, 2009; Liu et al., 2010; Taylor and Bruenn, 2009), algae (Cock et al., 2010; Delaroque et al., 1999), plants (Bejarano et al., 1996; Chiba et al., 2011; Harper et al., 2002; Liu et al., 2010; Tanne and Sela, 2005) and protozoa (Liu et al., 2010).

In animals, viral inserts are commonly found in vertebrates such as mammals, birds and fish (Belyi et al., 2010a,b; Benveniste and Todaro, 1974; Fort et al., 2012; Horie et al., 2010; Kapoor et al., 2010; Katzourakis and Gifford, 2010; Liu et al., 2011; Zhdanov, 1975). In invertebrates, virus-related sequences have been reported in trematodes (Liu et al., 2011), nematodes (Fort et al., 2012; Liu et al., 2010; Malik et al., 2000), gastropods (Liu et al., 2010), tunicates (Liu et al., 2011), but primarily in arthropods such as insects, arachnids and copepods (Crochu et al., 2004; Cui and Holmes, 2012; Fort et al., 2012; Kapoor et al., 2010; Katzourakis and Gifford, 2010; Lin et al., 1999; Liu et al., 2010, 2011; Malik et al., 2000; Maori et al., 2007; Roiz et al., 2009). Endogenous viral sequences that have been identified in decapod crustacea include endogenous *Penaeus stylirostris* densovirus-like elements (EPstDNVE) in *Penaeus monodon* (Saksmerprome et al., 2011; Tang and Lightner, 2006) and endogenous white spot syndrome virus-like elements (EWSSVE) in *P. monodon* (Huang et al., 2011) and *Penaeus japonicus* (Dang et al., 2010; Koyama et al., 2010).

The insertion of endogenous viral elements in the host cells may provide an antiviral protection against infection by closely-related,

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present day exogenous viruses (Belyi et al., 2010b; Flegel, 2009; Griffiths, 2001). This may be achieved by synthesising dominant negative fragments or antisense RNAs that inhibit viral replication cycles (Flegel, 2009; Fort et al., 2012), synthesising a new phenotype of immune response that could recognise and prevent similar infection (Liu et al., 2010), activating the innate immunity of the host or via the protein expression that act as immunogens (Holmes, 2011). This may be true if some components of the elements are still active and are able to produce proteins that have protective immunity to exogenous viral infection (Feschotte and Gilbert, 2012). In this study, the presence of endogenous Brevidensovirus-like elements (EBreVE) in *Cherax quadricarinatus* is reported for the first time as a consequence of an investigation into the viral aetiology of "signet ring" hyperthrophied nuclei with marginated chromatin but without Cowdry type A (CA) intranuclear inclusion bodies within the gills of redclaw freshwater crayfish.

2. Materials and methods

2.1. Experimental Animals

Redclaw crayfish, *C. quadricarinatus*, were sourced from two stocks at the School of Veterinary and Biomedical Sciences (SVBMS), James Cook University (JCU). One population of these crayfish developed hyperthrophied nuclei with rarefied chromatin but without Cowdry type A (CA) intranuclear inclusion bodies in the gills (hypertrophied nuclei population), while another population did not have these nuclear changes (non-hypertrophied nuclei population). Redclaw crayfish from the Marine and Aquaculture Research Facilities Unit (MARFU) at School of Marine and Tropical Biology, JCU that had experienced chronic mortality were sampled. Redclaw crayfish were also taken from two geographically separated crayfish farms (Farm A and Farm B) in northern Queensland. The crayfish were transported to the Aquatic Pathology Laboratory of SVBMS, JCU for extraction of material from the gills (20 animals from each population). In addition, extracted DNA from the gills of crayfish that had developed characteristics of hypertrophied nuclei with Cowdry type A intranuclear inclusion bodies (*C. quadricarinatus* parvo-like virus, CqPV) in the gills (Bowater et al., 2002) from Tropical and Aquatic Animal Health Laboratory, Biosecurity Queensland (North), Department of Agriculture, Fisheries and Forestry (DAFF) was examined. Crayfish were anaesthetised by submerging in iced water. The cephalothorax was cut midsagitally, one half was preserved in 95% ethanol for nucleic acid isolation and the remaining half was fixed in Davidson's fixative (below) for histological examination.

2.2. Histology

Gills were fixed in Davidson's fixative (formaldehyde 220 ml, acetic acid 115 ml, absolute ethanol 313 ml and tap water 352 ml) for 48 h, transferred to 70% ethanol and then processed for routine histological examination using standard wax embedding procedure (Bell and Lightner, 1988). Sections were cut at 5 μM using a rotary microtome and mounted on glass slides. Tissue sections were stained with haemotoxylin and eosin (H & E), unless otherwise stated.

2.3. Nucleic acids extraction

Deoxyribonucleic acid (DNA) was extracted from the gills of crayfish using a High Pure PCR Template Preparation Kit (Roche Diagnostics) or Wizard® SV Genomic DNA Purification System (Promega) following the manufacturer's protocols. To obtain a positive control for the parvovirus primers, DNA was extracted from the pleopods of banana prawn, *Penaeus merguiensis* known to be positive for Australian *P. merguiensis* densovirus (PmergDNV). Total RNA was also extracted from the gills of hypertrophied and non-hypertrophied nuclei crayfish (20 crayfish from each population) using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions and kept at −80 °C until used.

2.4. Polymerase chain reaction (PCR) amplification with parvovirus primers and reverse transcription-PCR (RT-PCR) with bunyavirus primers

Several parvovirus primers (Supplementary Table 1) were designed from various penaeid densoviruses, insect densoviruses and a Brevidensovirus (IHHNV) of penaeids to amplify any relevant viral sequences from the nuclear changes in the hypertrophied nuclei population. To investigate the possibility of the aetiological agent of the nuclear changes caused by virus with no poly(A) tail, several bunyavirus primers (Supplementary Table 1) were designed from members of the related-genus Phlebovirus within the Family Bunyaviridae (van Regenmortel et al., 2000), including Mourilyan virus (AY927991), Uukuniemi virus (M17417) and Toscana virus (EU003175). Sequences were aligned and primers were designed in areas of genetic similarity using Vector NTI software or Oligo 7 software. Primers were synthesised by Sigma-Aldrich Pty Ltd, Australia or Macrogen, Korea. Some published primers were also used in this study (Supplementary Table 1).

Complementary DNA (cDNA) was synthesised from total RNA using random primers of ImProm-II™ Reverse Transcription System (Promega) according to manufacturer's instruction. Reverse transcriptase (RT)-nested PCR was performed using primers MoV24F/MoV25R in the first PCR. Templates with amplicons that appeared in the gel electrophoresis produced from the primary PCR were subjected to secondary amplification (nested PCR) using primers MoV148F/MoV149R.

Polymerase chain reaction mixture contained 12.5 μl of GoTaq®Green Master Mix (Promega), 1 μl of DNA templates (PCR amplification) or 1–2 μl of cDNA templates (RT-PCR amplification), and 0.75 μl (10 μM) of each primer. The PCR reaction volume was adjusted with nuclease free water (Promega) to a final volume of 25 μl. The PCR amplification (Supplementary Table 1) was performed in a Mastercycler gradient 5333 (Eppendorf, Germany). Amplified products (10 μl) were visualised in 1.2% agarose-TAE gels containing GelRed (10,000× in water) at a concentration of 0.5:10,000. Gels were visualised and photographed using an InGenius LHR, gel documentation and analysis system (Syngene, UK).

2.5. Polymerase chain reaction (PCR) amplification of endogenous virus-like elements in C. quadricarinatus

Primers QPF1 and QPR1 (sequences kindly provided by Dr. Jeff A. Cowley) were used to amplify the putative endogenous virus-like elements in *C. quadricarinatus*. The QPF1 and QPR1 are at nucleotide (nt) 1519–1538 and 1601–1622 of Hawaiian isolate of IHHNV (AF218266), respectively. Thus these primers are expected to yield a 104-bp amplicon in the non-structural (NS) protein region of IHHNV. Two sets of primers were also designed. The 101F21 and 314R23 primers were intended to yield a 236-bp nucleotide portion of Fragment 1 of endogenous virus-like element. Primers 832F21 and 2585R21 were expected to amplify up and down stream of Fragment 7 (Table 1).

The PCR mixture contained 12.5 μl of GoTaq®Green Master Mix, 1 μl of DNA template, 0.75 μl (10 μM) of primers QPF1/QPR1 and 101F21/314R23 or 1.0 μl (10 μM) of primers 832F21/2585R21, 832F21/QPR1 and QPF1/2585R21. This mixture was adjusted with nuclease free water to a final volume of 25 μl. The PCR amplification profile of primers QPF1/QPR1 and 101F21/314R23 consisted of initial denaturation at 94 °C for 1 min, 40 cycles denaturation at 94 °C for 25 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and followed by final extension at 72 °C for 7 min. The amplification profile was similar for primers 832F21/2585R21, 832F21/QPR1 and QPF1/