Short Communication

First isolation of an *Entomobirnavirus* from free-living insects

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Drosophila X virus (DXV), the prototype *Entomobirnavirus*, is a well-studied RNA virus model. Its origin is unknown, and so is that of the only other entomobirnavirus, Espirito Santo virus (ESV). We isolated an entomobirnavirus tentatively named Culex Y virus (CYV) from hibernating *Culex pipiens* complex mosquitoes in Germany. CYV was detected in three pools consisting of 11 mosquitoes each. Full-genome sequencing and phylogenetic analyses suggested that CYV and ESV define one sister species to DXV within the genus *Entomobirnavirus*. In contrast to the laboratory-derived ESV, the ORF5 initiation codon AUG was mutated to $_{1927}$GUG in all three wild-type CYV isolates. Also in contrast to ESV, replication of CYV was not dependent on other viruses in insect cell culture. CYV could provide a wild-type counterpart in research fields relying on DXV and other cell culture-adapted strains.

Drosophila X virus (DXV) is the prototype species of the monospecific genus *Entomobirnavirus* within the family *Birnaviridae* (Delmas *et al.*, 2011). DXV was isolated as a contaminant during infection studies with Sigma virus (family *Rhabdoviridae*) in *Drosophila* cell lines (Dobos *et al.*, 1979; Teninges, 1979; Teninges *et al.*, 1979). Infection with DXV causes sensitivity to anoxia and death in *Drosophila melanogaster* (Teninges *et al.*, 1979). Nevertheless, DXV has never been found as a natural infection in *Drosophila* flies and no wild-type strains are known. The DXV origin is unknown and it was hypothesized that it might have originated as a contaminant from FCS used in the original infection studies (Plus, 1979; Teninges *et al.*, 1979). However, trials to culture DXV in vertebrate cell lines and suckling mouse brain were unsuccessful. To date, entomobirnaviruses have not been found in humans or any other vertebrates and are believed to infect insects only (Delmas *et al.*, 2011).

Most of our current knowledge of the insect-specific innate immune response and RNA interference (RNAi) has been gained through infection studies with DXV in *Drosophila* flies and cultured *Drosophila* (S2) cells (Brennan & Anderson, 2004; van Rij & Berezikov, 2009). For example, the Toll pathway was shown to inhibit the replication of DXV in *Drosophila* (Zambon *et al.*, 2005). RNAi has been shown to constitute a major antiviral effector mechanism using the example of DXV in *Drosophila* (Zambon *et al.*, 2006). These studies on *Drosophila* have strongly influenced research on mosquito innate immune response toward infections with arthropod-borne viruses, such as dengue virus (DENV), one of the most important emerging human pathogens. DENV is modulated by RNAi in *Drosophila* cells (Mukherjee & Hanley, 2010). Interaction studies between DENV and RNAi are important as small interfering (si)RNAs may be used to treat flavivirus infections (Kumar *et al.*, 2006) and genetically modified mosquitoes expressing siRNAs may reduce DENV replication in mosquitoes (Franz *et al.*, 2006; Haasnoot *et al.*, 2003).

Recently, a novel entomobirnavirus with distant relationship to DXV, named Espirito Santo virus (ESV), was detected in an *Aedes albopictus* (C6/36) cell culture inoculated with serum from a patient infected with DENV-2 (Vancini *et al.*, 2012). The origin of this virus was not investigated but it was reported that ESV replication depended on the replication of virulent DENV-2 in the same cells. Here, we demonstrate direct isolation of an entomobirnavirus from mosquitoes in close (probably conspecific) relation to ESV that does not depend on any co-infecting virus.

In March 2010, hibernating mosquitoes were collected for viral surveillance from a cave in Bad Segeberg, Germany (53°56′7.75″N/10°19′0.00″E). Mosquitoes pertaining upon morphological determination to the *Culex pipiens* complex were homogenized individually, and pools consisting of 11 mosquitoes each were generated. Four pools were inoculated in C6/36 and Vero E6/7 (African green monkey kidney) cells (Junglen *et al.*, 2009). Three pools (P1, P3 and

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are JQ659254–JQ659255.

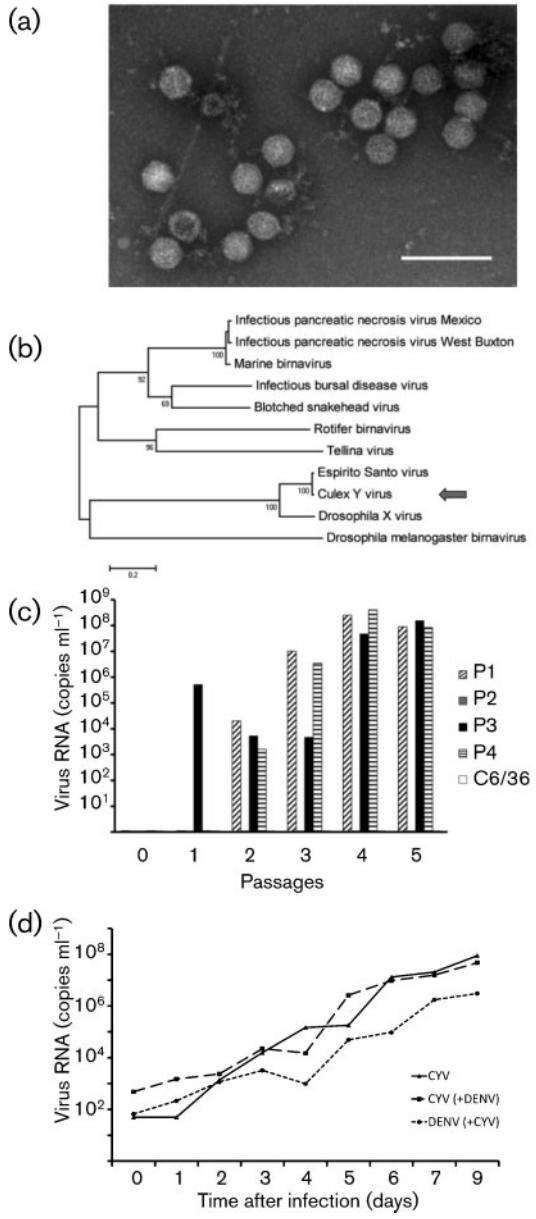

Fig. 1. CYV morphology, phylogenetic relationship and growth. (a) Negative-staining electron micrograph of virions purified by ultracentrifugation. Bar, 100 nm. (b) Maximum-likelihood phylogenetic tree of the VP1 gene of CYV and other representative birnaviruses generated with the WAG substitution model, uniform rates and complete deletion option in MEGA version 5 (Tamura *et al.*, 2011). Confidence levels according to 1000 bootstrap replicates are indicated at the nodes. (c) Virus growth in C6/36 cells over the first five passages observed by real-time RT-PCR. (d) Numbers of genome copies ml^{-1} of cell culture supernatant of C6/36 cells infected with an m.o.i. of 0.001 with either CYV or CYV and DENV measured by real-time RT-PCR for 9 days.

P4) induced strong cytopathic effects (CPE) involving clumping and stretching 6 days post-infection in C6/36 cells. No signs of CPE were observed in Vero cells. Infectious cell culture supernatants of C6/36 cells were passaged five times in fresh cells. In cell cultures from P1, P3 and P4 icosahedral, unenveloped virus-like particles 70 nm in diameter with birnavirus-like morphology (Böttcher *et al.*, 1997; Pous *et al.*, 2005) were observed by electron microscopy in ultracentrifuged supernatant (Fig. 1a). No such particles were detected in P2. The entire genome was determined by adaptor-based random amplification and rapid amplification of cDNA-ends using purified particles from P1 (Zirkel *et al.*, 2011). Like other birnaviruses, the tentatively named Culex Y virus (CYV) was found to contain a bisegmented dsRNA genome (GenBank accession nos JQ659254–JQ659255). Segment A (3429 bp) encodes a putative polyprotein precursor of 1058 aa containing the predicted proteins pVP2–VP4–VP3 (Chung *et al.*, 1996; Nagy & Dobos, 1984a, b). A putative VP5 is encoded by an additional small overlapping ORF (Chung *et al.*, 1996; Da Costa *et al.*, 2003; Magyar & Dobos, 1994; Mundt *et al.*, 1995; Vancini *et al.*, 2012). In contrast to other birnaviruses the ORF5 initiation codon AUG was mutated to $_{1927}$GUG in all three CYV isolates and cell culture passage supernatants. Mutation of the ORF5 initiation codon was also observed in ESV (Vancini *et al.*, 2012), while the full-genome sequence showed a canonical AUG codon in this position (GenBank accession no. NC_016518). Segment B of CYV (3254 bp) codes for VP1 (999 aa), the putative RNA-dependent RNA polymerase (Shwed *et al.*, 2002; von Einem *et al.*, 2004). Full-genome comparison yielded distant relationships at the nucleotide level to DXV (71–72 % identity) and a very close relationship to ESV (99 % identity). Location of nucleotide and amino acid differences found between CYV and ESV are presented in Table 1. Together with the phylogenetic analysis shown in Fig. 1(b), this suggested that CYV and ESV define one sister species to DXV within the genus *Entomobirnavirus*.

Findings of the same virus species in two continents suggest ubiquitous distribution. Alternatively, *A. albopictus* cells may have become contaminated at some point with either ESV or CYV. In order to investigate the origin of CYV, cell culture supernatants of the first five passages of P1–P4 and of mock-infected cells were tested for CYV replication by real-time RT-PCR, indicating active CYV replication in all virus-positive cultures starting from the first or second passages (P1, P3 and P4), but neither in P2 nor in mock-inoculated C6/36 cells (Fig. 1c). Virus RNA was also detected in those three original mosquito pools yielding virus isolates, indicating that the mosquitoes but not the C6/36 cells had been infected with CYV originally.

To investigate virus growth, virus titres were determined by $TCID_{50}$ end-point dilution (Reed & Muench, 1938) and C6/36 cells were inoculated at an m.o.i. of 0.001. RNA concentrations peaked at 1×10^8 genome copies at day 6 (Fig. 1d).

Vancini *et al.* (2012) suggested that ESV depended on co-infection with DENV for its replication in C6/36 cells. The

Table 1. Nucleotide (nt) and amino acid (aa) differences between CYV and ESV

Segment/ putative gene	Location*	nt (CYV–ESV)	aa (CYV–ESV)
A/preVP2	127	A–G	
	152	A–G	T–A
	206	U–G	S–A
	469	U–C	
	503	G–A	D–N
	935	U–A	S–T
	927	A–G	
	1055	A–U	T–S
	1383	A–G	D–G
	1513	G–A	A–T
A/VP4	1654	U–C	
	1927	G–A	
	2177–2179	AAG–Δ†AAG	K–Δ†K
	2217	G–A	G–D
	2281	C–G	
A/VP3	2612	U–G	L–V
	2638	G–U	E–D
	2675	G–A	V–I
	2681	A–C	N–H
	2769	G–U	S–I
	3084	A–U	D–V
	3183	C–A	A–E
B/VP1	153	U–A	S–T
	162	C–A	L–I
	395	A–G	
	447	G–A	G–S
	513	U–A	L–I
	617	A–G	
	878	A–C	
	915	A–G	I–V
	980	A–U	
	1226	U–C	
	1227–1229	AUC–GUU	I–V
	1961	C–U	
	1995	A–G	I–V
	2014	G–A	R–K
	2025	A–G	N–D
	2075	G–A	
	2205	U–C	
	2388	G–A	G–S
	2601	U–A	Y–N
	2621	U–C	
	2687	U–C	
	2867	G–U	E–D
	3017	C–A	

*Location from the 5′ terminus of CYV.
†Deleted.

level of replication seemed to correlate with the virulence of the co-infecting DENV strain (Vancini *et al.*, 2012). To test whether also CYV grew to higher titres when co-cultivated with DENV we performed co-infection experiments with DENV-2 strain 16681 (Kinney *et al.*, 1997) at an m.o.i. of 0.001 for both viruses. No difference in CYV replication was observed between single- and co-infected C6/36 cells (Fig. 1d).

Based on its autonomous replication and confirmed insect origin it is suggested that CYV exemplifies wild-type entomobirnavirus properties. Comparing insect antiviral responses between wild-type and laboratory-adapted strains can reveal critical elements of host signalling pathways, as well as viral factors such as RNA silencing suppressors (Li *et al.*, 2004; Nayak *et al.*, 2010; Qi *et al.*, 2012; Singh *et al.*, 2009; van Cleef *et al.*, 2011). For flock house virus (family *Nodaviridae*), another common model to study virus–insect interactions, significant contrasts have already been identified with its wild-type counterpart Wuhan nodavirus (Cai *et al.*, 2010; Liu *et al.*, 2006a, b; Qi *et al.*, 2011; Qiu *et al.*, 2011). Comparisons with DXV may also promote studies on antiviral RNAi based on CYV (Khoo *et al.*, 2010; Myles *et al.*, 2008; Pacca *et al.*, 2009; Sánchez-Vargas *et al.*, 2009; Scott *et al.*, 2010; Zambon *et al.*, 2005, 2006). One interesting target is the non-AUG start codon in ORF5. Initiation of translation at GUG has been reported in bacteria (Gold, 1988; Kozak, 1989), yeast (Chang *et al.*, 2010) and plants (Bock *et al.*, 1994), as well as *Drosophila* (Sugihara *et al.*, 1990). Initiation activity at non-AUG codons is much lower and may be used to regulate translation, indicating for ORF5 a function that might be important in entomobirnavirus host interactions (Chang *et al.*, 2010; Sugihara *et al.*, 1990). Expression of ORF5 may also be mediated by ribosomal frameshift into the −1 reading frame (Firth & Brierley, 2012). Programmed ribosomal frameshift has been reported to regulate protein expression ratios. The only X_XXY_YYZ-like sequence identified upstream of ORF5 was $_{1897}$UUUUUUA. This heptanucleotide was also found in ESV and DXV and shown to promote efficient −1 ribosomal frameshifting in human immunodeficiency virus type 1 (Jacks *et al.*, 1988; Wilson *et al.*, 1988). However, further experimental *in vitro* studies are needed to investigate if and by which translational strategies ORF5 may be expressed.

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