Characterization of a novel circo-like virus in Aedes vexans mosquitoes from Germany: evidence for a new genus within the family Circoviridae

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Over recent decades, metagenomic studies have expanded the number of newly described, often unclassified, viruses within the family Circoviridae. Using broad-spectrum circovirus and cyclovirus PCRs, we characterized a novel circo-like virus in Aedes vexans mosquitoes from Germany whose main putative ORFs shared very low amino acid identity with those of previously characterized circoviruses and cycloviruses. Phylogenetic and genetic distance analysis revealed that this new virus species defined, together with previously described mosquito- and bat faeces-derived circo-like viruses, a different genus, tentatively called Krikovirus, within the family Circoviridae. We further demonstrated that viruses of the putative genus Krikovirus all shared a genomic organization that was unique among the family Circoviridae. Further investigations are needed to determine the host range, tissue tropism and transmission route(s). This report increases the current knowledge of the genetic diversity and evolution of the members of the family Circoviridae.

Members of the family Circoviridae are small non-enveloped ssDNA viruses with a circular genome of ~2 kb (Biagini et al., 2011). Two genera, Circovirus and Gyrovirus, are currently recognized by the International Committee on Taxonomy of Viruses (Biagini et al., 2011). A proposal has recently been made to include a new genus, Cyclovirus, in the family Circoviridae (International Committee on Taxonomy of Viruses, 2014). The genomes of circoviruses and cycloviruses exhibit ambisense organization with two major ORFs encoding a replication-associated protein (Rep) and a capsid protein (Cap) (Li et al., 2010a). A putative origin of replication with a conserved nonamer motif (5’S’-NANTA-TTAC-3’S’) is found between the 5’ ends of these two ORFs, but on the Rep-encoding strand for circoviruses and the Cap-encoding strand for cycloviruses (Rosario et al., 2012). Circoviruses have been associated with various diseases in pigs and avian species (Mankertz et al., 2004; Stewart et al., 2006). Cyclovirus infection has so far not been unequivocally shown to cause disease, but some species were detected in the cerebrospinal fluid of patients with neurological conditions (Smits et al., 2013; Tan et al., 2013) and in respiratory secretions of infants with respiratory disease (Phan et al., 2014). Numerous metagenomic studies have led to the description of increasing numbers of ‘unclassified circoviruses’ from various vertebrate and invertebrate hosts or from wastewater (Blinkova et al., 2009; Li et al., 2010a, b; Ge et al., 2011; Ng et al., 2011; Cheung et al., 2014; Smits et al., 2014; Zhang et al., 2014). In this study, we describe a novel mosquito-derived circo-like virus which defines, together with previously described mosquito and bat faeces circo-like viruses, a new phylogenetic lineage amongst the family Circoviridae. These viruses share a unique genomic organization distinct from that of circoviruses and cycloviruses. We propose their inclusion into a new genus tentatively named Krikovirus within the family Circoviridae with the earliest published genome TM-6c (GenBank accession number HM228875) as the prototype.

Using degenerate primers targeting conserved regions of the Rep ORF from circoviruses and cycloviruses described
by Li et al. (2010a), we identified new circo-like virus sequences in two pools of 250 Aedes vexans mosquitoes collected in 2013 close to the Lake Constance (Bodensee) region in Germany (GMB19, Böhringen 47° 45′ 10″ N 8° 55′ 58″ E; GMB51, Böhringen 47° 43′ 43″ N 8° 59′ 37″ E). Mosquitoes were originally collected for the German Arbovirus Surveillance Program (Becker et al., 2014). The full circular krikovirus genomes, named GMB19 and GMB51, were then amplified using inverse PCR with specific primers and Sanger sequencing using primer walking (see Table S1, available in the online Supplementary Material). PCR was performed in 25 μl PCRs consisting of 10 μl HotStarTaq Plus Master Mix (Qiagen), 1 μl each primer (20 pmol), 3 μl DNA template and double-distilled H2O up to 25 μl. The reaction was conducted with denaturation for 5 min at 95°C followed by 55 cycles of 95°C for 20 s, 55°C for 45 s and 72°C for 2 min 30 s, and a final elongation for 5 min at 72°C. The full viral sequences were deposited in GenBank under accession numbers KM972725 (GMB19 strain) and KM972726 (GMB51 strain). Putative ORFs in the krikovirus genome were predicted using Geneious version 7.1.7 (Biomatters) and ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder). Rep of GMB19 and GMB51 strains had the greatest identity to mosquito-derived SDWAP 1 strain (GenBank accession number HQ335042) (62%) and bat faeces-derived TM-6c (GenBank accession number HM228875), 00813 (GenBank accession number JN377580) and batCV-SC703 (GenBank accession number JN857329) strains (39–45%) – all recent mosquito- and bat faeces-derived krikovirus species (Fig. 1a). The previously described mosquito (SDWAP 1; GenBank accession number HQ335042) and bat (TM-6c, GenBank accession number HM228875; 00813, GenBank accession number JN377580; batCV-SC703, GenBank accession number JN857329) faeces-derived viruses, which we propose to include in this new genus together with both German mosquito krikovirus (GMB19 and GMB51), all encode the nanomer ORF also spans the origin of replication for some (00813 and batCV-SC703 bat faeces-derived genomes) but not all krikoviruses. Highly conserved motifs in the Rep protein of circoviruses and cycloviruses (WWDGY, DDFYGW and DRYP) were also conserved in krikoviruses, but in the latter the second motif was modified. Whilst the first three residues (DDF) were conserved, the next three were replaced by R/T, A/S/G and S/G, respectively, depending on the strain. Motifs associated with rolling circle amplification and dNTP binding were identified as overall conserved, with some inconsistent modifications (F/WTLNN, TPHLQG, CKK and G/E-GKS; modified residues underlined) (Li et al., 2010a). The highly basic and arginine-rich region at the N terminus, which is characteristic of circovirus Cap proteins (Stewart et al., 2006), was also found in all members of the proposed genus Krikovirus (Fig. S2).

The genome consisted of two major ORFs encoding Rep and Cap. Secondary DNA structure predictions were performed with the web-based version of mfold (http://mfold.rit.albany.edu/?q=mfold/DNA-Folding-Form). The typical stem–loop and 5′-NANTATTAC-3′ nonamer sequence conserved in circoviruses and cycloviruses were present (Fig. 1b). Strikingly, the stem–loop structure of krikoviruses was located differently compared with that of circoviruses and cycloviruses. In addition, the 3′ end (and not the 5′ end) of both ORFs (Fig. 1b). This stem–loop structure was on the Cap-encoding strand, as in cycloviruses. In addition, the 3′ end of the Rep ORF spanned the putative origin of replication for both GMB19 and GMB51 strains and some bat faeces-derived krikovirus strains (Fig. 1b). Sequence alignment was performed using CLUSTAL_W implemented in Geneious version 7.1.7. Maximum-likelihood and Bayesian phylogenetic analysis of the Rep nucleotide and amino acid sequences were performed using MEGA6 (Tamura et al., 2013) and BEAST (Drummond et al., 2012) based on representative members of the genera Circovirus, Cyclovirus and Gyrovirus, and unclassified circo-like strains of the family Circoviridae. Bootstrap values (≥70%, based on 1000 replicates) and Bayesian posterior probability (≥90%) for each node are given (Figs 1a and S1). The phylogenetic analysis revealed that both mosquito-derived circo-like viruses detected in Germany formed, together with another previously described mosquito circovirus from the USA and several ‘unclassified bat faeces-derived circoviruses’ from China and the USA, a highly supported clade distinct from circoviruses, cycloviruses and gyroviruses, within the family Circoviridae (Figs 1a and S1).

We propose to call this new genus Krikovirus (krikos means ‘ring’ in Greek, as a reference to the circular nature of the genome). A Rep amino acid distance matrix analysis produced with Sequence Demarcation Tool version 1.2 (Muhire et al., 2014) confirmed the clear demarcation between krikoviruses and members of other genera within the family Circoviridae (Fig. 2). Genetic distance analysis also indicated that the krikoviruses could be further subgraded into two highly supported clades: mosquito- (MoKrV) and bat faeces-derived (BtKrV) krikovirus species (Fig. 1a). We previously described mosquito (SDWAP 1; GenBank accession number HQ335042) and bat (TM-6c, GenBank accession number HM228875; 00813, GenBank accession number JN377580; batCV-SC703, GenBank accession number JN857329) faeces-derived viruses, which we propose to include in this new genus together with both German mosquito krikovirus (GMB19 and GMB51), all encode the nanomer sequence on their Cap-encoding DNA strand. The Rep ORF also spans the origin of replication for some (00813 and batCV-SC703 bat faeces-derived genomes) but not all krikoviruses. Highly conserved motifs in the Rep protein of circoviruses and cycloviruses (WWDGY, DDFYGW and DRYP) were also conserved in krikoviruses, but in the latter the second motif was modified. Whilst the first three residues (DDF) were conserved, the next three were replaced by R/T, A/S/G and S/G, respectively, depending on the strain. Motifs associated with rolling circle amplification and dNTP binding were identified as overall conserved, with some inconsistent modifications (F/WTLNN, TPHLQG, CKK and G/E-GKS; modified residues underlined) (Li et al., 2010a). The highly basic and arginine-rich region at the N terminus, which is characteristic of circovirus Cap proteins (Stewart et al., 2006), was also found in all members of the proposed genus Krikovirus (Fig. S2).

Their unique genomic organization and the phylogenetic clustering of their Rep and of their full-length genome (data not shown) indicate the presence of a new genus consisting so far of two mosquito-derived circo-like viruses described in this study (GMB19 and GMB51) and mosquito (SDWAP 1) and bat faeces-derived viruses (00813, batCV-SC703 and TM-6c) described previously (Li et al., 2010b; Ng et al., 2011) that we tentatively called Krikovirus in the family Circoviridae. Krikoviruses have so far only been identified in mosquitoes and bat faeces.
Fig. 1. Phylogenetic analysis and genomic features of the members of the family Circoviridae. (a) The maximum-likelihood tree was conducted based on Rep protein sequences depicting relationships amongst representative members of the genera Circovirus, Cyclovirus and Gyrovirus and the proposed genus Krikovirus within the family Circoviridae. Maximum-likelihood bootstrap replicate scores (>70%) and posterior probabilities of the Bayesian analysis (>90%) are shown next to each node. Taxon information includes GenBank accession number, species and strain designation (only for krikoviruses): MiCV, Mink circovirus; BtCV, Bat circovirus; DuCV, Duck circovirus; GooCV, goose circovirus; CiCV, catfish circovirus; PCV1, Porcine circovirus-1; PCV2, Porcine circovirus-2; BaCV, barbel circovirus; BFDV, Beak and feather disease virus; CoCV, columbid circovirus; ChCV, chicken circovirus; GuCV, Gull circovirus; FiCV, Finch circovirus; CaCV, Canary circovirus; ChiCV, chimpanzee circovirus; HuCyCV, human cyclovirus; GoCyCV, goat cyclovirus; FeCyCV, feline cyclovirus; FwcaCyCV, Florida woods cockroach-associated cyclovirus; DiCyCV, dragonfly cyclovirus; ChiCyCV, chimpanzee cyclovirus; ChCyCV, chicken cyclovirus; BtCyCV, bat cyclovirus; BtKrV, bat krikovirus; MoKrV, mosquito krikovirus; HuGyV, human gyrovirus; CAV, Chicken anemia virus; AGyV, avian gyrovirus. Mosquito krikoviruses generated during this study are highlighted in grey. The main clades (genera) are indicated to the right of the tree. Bar, amino acid substitutions per site. (b) Genome organization and stem–loop structure of the krikoviruses and representative members of the genera Circovirus, Cyclovirus and Gyrovirus. The locations and directions of the putative Rep and Cap genes are indicated by arrows.
Figure 1. cont.

Circovirus

Origin of replication

Rep

BIV1/VTM-6c (M228370) Krikovirus 1668 bp

BIV/VTM-6c (M228370) Krikovirus 1668 bp

Origin of replication

Rep

Cyclicovirus

Origin of replication

Rep

POV1 (AY660574) Circovirus 1759 bp

POV1 (AY660574) Circovirus 1759 bp

Origin of replication

Rep

Gyrovirus

Origin of replication

Rep

CAV (AF285882) Gyrovirus 2298 bp

CAV (AF285882) Gyrovirus 2298 bp

Origin of replication

Rep

Krikovirus

Origin of replication

Rep

BtKrV/TM-6c (HM228875) Krikovirus 1696 bp

BtKrV/batCV-SC703 (JN857329) Krikovirus 1578 bp

BtKrV/00813 (JN377580) Krikovirus 1562 bp

BtKrV/00813 (JN377580) Krikovirus 1562 bp

Origin of replication

Rep

MoKrV/GMB19 (KM972725) Krikovirus 1759 bp

MoKrV/SDWAP I (HQ335042) Krikovirus 2000 bp

MoKrV/SDWAP I (HQ335042) Krikovirus 2000 bp

Origin of replication

Rep

Fig. 1. cont.
However, they formed two distinct sister clades within the krikovirus lineage and seem to represent two distinct species within the genus. Our analysis indicated that these sequences are distantly related to known circovirus/cyclovirus/gyrovirus genomes and represent novel viral species that belong to the proposed genus *Krikovirus* within the family *Circoviridae*. Further investigations are needed to determine the host range, tissue tropism and the transmission route(s).

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**References**


