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Rapidly expanding genetic diversity and host range of the *Circoviridae* viral family and other Rep encoding small circular ssDNA genomes

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Abstract

The genomes of numerous circoviruses and distantly related circular DNA viruses encoding a rolling circle replication initiator protein (Rep) have been characterized from the tissues of mammals, fish, insects, and plants (geminivirus and nanovirus), human and animal feces, in an algae cell, and in diverse environmental samples. We review the genome organization, phylogenetic relationships and initial prevalence studies of cycloviruses, a proposed new genus in the *Circoviridae* family. Viral fossil *rep* sequences were also identified integrated on the chromosomes of mammals, frogs, lancelets, crustaceans, mites, gastropods, roundworms, placozoans, hydrozoans, protozoans, land plants, fungi, algae, and phytoplasma bacterias and their plasmids, reflecting their past host range. An ancient origin for viruses with *rep*-encoding single stranded small circular genomes, predating the diversification of eukaryotes, is discussed. The cellular hosts and pathogenicity of many recently described *rep*-containing circular genomes remain to be determined. Future studies of the virome of single cell and multi-cellular eukaryotes are likely to further extend the known diversity and host-range of small *rep*-containing circular viral genomes.

Keywords

circovirus; cyclovirus; *Circoviridae*; Rep protein; deep sequencing; circular ssDNA genome

Introduction

Members of the genus *Circovirus* in the family *Circoviridae*, are non-enveloped, icosahedral viruses with a single-stranded circular DNA (ssDNA) genome of approximately 2 kb, the smallest known autonomously replicating viral genomes (Todd et al 2005). Circoviruses infect numerous bird species including parrots, pigeons, gulls, ducks, geese, swans, ravens, canaries, finches, starlings, and chickens (Niagro et al, 1998; Mankertz et al, 2000; Todd et al, 2001; Todd et al, 2007; Hattermann et al, 2003; Johne et al, 2006; Stewart et al, 2006; Halami et al, 2008; Li et al, 2011). To date only two circoviruses have been extensively documented to replicate in a mammal, Porcine circovirus 1 and 2 (PCV1 and PCV2) (Allan

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and Ellis, 2000; Mankertz et al, 2004). PCV1 is generally considered non-pathogenic while PCV2 infection can be either asymptomatic or cause a variety of clinical symptoms with significant economic impact (Finsterbusch and Mankertz, 2009; Todd et al, 2001; Segales et al, 2005; Chae, 2005; Opriessnig et al, 2007; Grau-Roma et al, 2011).

Circoviruses have an ambisense genome organization containing two major inversely arranged open reading frames, encoding the rolling circle replication initiator protein gene (*rep*) and capsid protein gene (*cap*) (Tood et al, 2005). A stem–loop structure with a conserved 9 bases motif in the loop, located between the 5'-ends of the two main ORFs, is required to initiate the replication of the viral genome. The replication complex consists of Rep and a shorter Rep' protein with a different carboxy termini derived from a spliced transcript. Following cell infection, a double stranded template genome is first generated by cellular DNA polymerase 1 extending a small RNA primer. Rep and Rep' bind the stem loop, cutting a nick in the plus strand and a host-encoded DNA polymerase then extends the 3' hydroxyl to copy the complementary circle using a rolling circle replication mechanism (Steinfeldt et al, 2001; Steinfeldt et al, 2007; Faurez et al, 2009). The rolling circle replication strategy of PCV is similar to that of plant Geminivirus and Nanovirus and of bacterial plasmids in the pT181 family (Timchenko et al, 1999; del Solar et al, 1998; Gutierrez, 1999).

There has been a recent surge of small circular DNA genomes containing a *rep* gene discovered from different sources using different methods. *In vitro* rolling circle amplification (Haible et al, 2006; Rector et al, 2004), high-throughput sequencing (Blinkova et al, 2009; Rosario et al, 2009; Ng et al, 2011; Li et al, 2010) and/or degenerate/consensus PCR have all been extensively used to identify novel *rep* containing circular DNA genomes in tissues (Li et al, 2011; Li et al, 2010) and feces of mammals (Ge et al, 2011; Li et al, 2010), fish (Lorincz et al, 2011), insects (Ng et al, 2011; Rosario et al, 2011) and in environmental samples (Rosario et al, 2009; Rosario et al, 2009; Blinkova et al, 2009; López-Bueno et al, 2009; Kim et al, 2008). Our understanding of the extensive genetic diversity of the *Circoviridae* and of distantly related viral families of *rep* bearing small circular ssDNA genome has therefore rapidly increased.

Ancient origin of small single stranded circular DNA genomes encoding Rep

Multiple lines of evidence point to an ancient origin for circoviruses and related genomes. The recent detection of genetically decayed fossil circovirus-like sequences integrated into the chromosomes of various mammals (as well as a frog) yielded estimates that these genomes replicated in mammals at least and possibly more than 100 million years ago (Katzourakis and Gifford, 2010; Belyi et al, 2010). The detection of Rep-encoding ORFs integrated in the chromosomes of the common parasitic protozoa *Giardia duodenalis* and *Entamoeba histolytica* may reflect past replication of related genomes and integration in those hosts possibly due to the DNA binding, cutting and ligating Rep activity normally used for rolling circle replication (Gibbs et al, 2006). A geminivirus-like genome was similarly observed in the genome of tobacco plants (Bejarano et al, 1996). A recent comprehensive search of NCBI databases has also greatly increased the number of eukaryotic genomes known to contain viral *rep*-like genes (Liu et al, 2011).

The viral *rep* gene may have originated through recombination between unrelated viruses, with its N-terminal region being most related to that encoded by small single-stranded circular DNA viruses with segmented genomes of the *Nanoviridae* family (infecting plants) and a C-terminal region related to the RNA-binding 2C helicase protein of positive strand RNA picorna-like viruses (Gibbs and Weiller, 1999). The similar rolling circle replication

strategy employed by the *Geminiviridae* and *Nanoviridae* families infecting plants, the *Circoviridae* infecting mammals, birds and possibly fishes and insects, and of some bacterial plasmids, may also reflect a long and common evolutionary history for these genomes (Faurez et al, 2009). Sequence similarities between the Rep of some plasmids and those of geminiviruses and parvoviruses (which also replicate via a rolling circle DNA replication mechanism) led to the hypothesis that these eukaryotic viruses evolved from eubacterial replicons (Koonin and Ilyina, 1992; Ilyina and Koonin, 1992). The phylogenetic closeness of the *rep* of plant Geminiviruses to the *rep* of plasmids of wall-less, plant infecting, phytobacteria led to the proposal that a phytoplasma plasmid may have evolved into a Geminivirus virus (Krupovic et al, 2009). While no sequence similarity was detected between geminivirus capsids and other proteins, their predicted structure was most like that that of a capsid protein from a ssRNA plant virus (satellite tobacco necrosis virus), a helper virus-dependent genome encoding only a capsid gene (Krupovic et al., 2009). Geminiviruses may have therefore captured their capsid gene from a plant RNA virus (Krupovic et al., 2009). An alternative theory has been proposed that phytoplasmal plasmids acquired their *rep* by horizontal transfer from a geminivirus (Saccardo et al, 2011). The T=1 icosahedral structure of geminiviruses (Böttcher et al, 2004; Zhang et al, 2001) and circoviruses (Khayat et al, 2011) both consist of capsids with a canonical viral jelly roll structure (eight stranded beta-barrel fold) which may also reflect a common evolutionary path. Tandem repeats of either Geminivirus or porcine circovirus encoding intact *rep* genes and their stem loop origins of replication can generate replicative forms in bacterial hosts that are indistinguishable from those in their eukaryotic hosts (Rigden et al, 1996; Cheung, 2006; Gibbs and Weiller, 1999; Selth et al, 2002). Collectively these observations support the hypothesis that small circular ssDNA viral genomes originated from prokaryotic episomal replicons (Koonin and Ilyina, 1992; Ilyina and Koonin, 1992).

Cyclovirus, a new genus in the *Circoviridae* family

As part of a metagenomics based search for new viruses, the viral nucleic acids in the feces of children from developing countries were randomly amplified and sequenced (Victoria et al, 2009). The initial cyclovirus genome fragment identified encoded a partial Rep protein detected through BLASTx sequence similarity searches against all viral protein sequences (Victoria et al, 2009). Given that circoviruses have small circular DNA genomes, the rest of the viral genome was then amplified by inverse PCR (Li et al, 2010).

Like circoviruses, the cycloviruses have small circular ambisense DNA genomes of 1.7 to 1.9 Kb (Table 1) containing two major inversely arranged ORFs encoding the putative Rep and Cap proteins. The rep of one genome (CyCV-TN25) was interrupted by a small putative 171 bases intron (Li et al, 2010). Relative to circoviruses, the Rep and Cap proteins of cycloviruses are slightly shorter and the 3′-intergenic regions between the stop codons of the two major ORFs were either absent or consisted of only a few bases (Li et al, 2011; Li et al, 2010). The 5′-intergenic regions between the start codons of the *rep* and *cap* ORFs of cycloviruses were relatively larger than those of circoviruses and also contained a highly conserved stem-loop structure with a distinct loop nonamer sequence (Table 1). In the Rep N-terminus half several motifs associated with rolling circle replication (FTLNN, TPHLQG and YCSK) and dNTP-binding (GXGSK) were identified, with some alterations. Conserved amino acid motifs associated with 2C helicase function of some picorna-like viruses were also identified in the carboxy half of Rep (WWDGY, DDFYGW, and DRYP). The N-terminal region of the cyclovirus Cap proteins was highly basic and arginine-rich, as is typical for circoviruses capsid proteins. Cycloviruses are therefore distinguishable from circoviruses based on several unique genome characteristics and phylogenetically clustered into a related but separate clade (Figure 1).

Cycloviruses and circoviruses are common in human and chimpanzee feces

Using a consensus PCR approach targeting *rep* sequences conserved between circoviruses and cycloviruses (Ge et al, 2011; Li et al, 2011) cycloviruses were detected in 40 of 395 (10%) Pakistani, Nigerian, Tunisian human fecal samples tested, and in 6 of 44 (13%) wild African chimpanzee fecal samples. Cycloviruses were not detected in 247 human stool samples from the US but 12 (5%) of them contained PCV1 or PCV2 (Li et al, 2010). After measuring cyclovirus prevalence in the feces of healthy subjects no association was detected with non-poliovirus acute flaccid paralysis (AFP) in Pakistani and Tunisian children (Li et al, 2010). Disease association restricted to only a subset of the many different cyclovirus species found in human stool may not have been detectable with the limited numbers of samples tested (Li et al, 2010).

Cycloviruses in farm and wild animals

Consensus *rep* PCR was also used to screen meat samples (muscle tissue) of chicken, beef, goat, sheep, and camel from Nigeria and Pakistan and of pork and beef from the US (Li et al, 2011; Li et al, 2010). Cyclovirus *rep* sequences were detected in 22 of 40 (55%) Nigerian chicken samples, 7 of 51 (14%) Pakistani and Nigerian beef samples, 3 of 27 (11%) camel samples from Nigeria and 8 of 73 (11%) Pakistani and Nigerian goat and sheep samples. Inverse PCR from animal tissues for full genome sequencing necessitated pre-amplification using in vitro rolling circle amplification with Phi29 polymerase and random primers (Li et al, 2011). The animal tissue derived cyclovirus genomes had characteristics (Table 1) similar to those from human and chimpanzee feces, except that the nonamer sequence of a chicken cyclovirus (5'-TAATACTAA-3') and bovine cyclovirus (5'-TAATACTAG-3') differed from that of other cycloviruses. The *rep* gene of another cyclovirus (CyCV-PK beef23) was also interrupted by a 169 bases intron. A cyclovirus from goat had a genome with 99% identity to CyCV-PK beef23 from a cow (Li et al, 2011). When the partial cyclovirus *rep* sequences generated by consensus PCR from large numbers of animals tissues and from human and chimp feces were phylogenetically compared, only a small subset of sequences from farm animals clustered with those from human or chimp feces (Li et al, 2011). Minor overlaps was therefore observed between the numerous cyclovirus species found in Pakistani and Nigerian human feces and those found in the meat samples from the same countries (unlike in the US where only PCV is found by consensus PCR in human feces) indicating the likely circulation of distinct set of cyclovirus species in human versus farm animals.

Cycloviruses have also been identified in the feces of bats from the US and China (Li et al, 2010; Ge et al, 2011). The detection of cyclovirus in a bat muscle tissue also supports the likelihood of viral replication in these wild mammals rather than simply passage of virus from ingested food through the digestive track (Li et al, 2011). A new clade of circoviruses (based on partial genome sequences), distinct from those in birds and pigs, were also identified in the feces of Chinese bats as well as more divergent bat *rep* sequences that phylogenetically fell outside the *Circoviridae* clade (Ge et al, 2011; Li et al, 2010).

Can circovirus or cyclovirus infect humans ?

There is presently little evidence that PCV1 or PCV2 can infect humans despite frequent exposures to these viruses on pig farms or through pork consumption (9/13 or 69% of US bought pork tested was PCV DNA positive) (Li et al, 2010). Human exposure may have also occurred through the use of a licensed live attenuated oral human rotavirus vaccine containing PCV1 DNA (Victoria et al, 2010) shown in a pig cell culture to be infectious

(McClenahan et al, 2011) although with greatly reduced infectivity (Baylis et al, 2011). The excellent safety record of this extensively tested and efficacious rotavirus vaccine also supports a lack of PCV1 pathogenicity for human (Curns et al, 2010). No PCV DNA was found by PCR after screening more than 1000 samples from various tissues of both healthy and immunosuppressed humans (Hattermann et al, 2004), in plasma samples from 18 xenotransplantation recipients of pig islet cells (Garkavenko et al, 2004) or by consensus PCR in 200 human plasma samples (Li et al, 2010). PCV2 was reported in a colon biopsy from an ulcerative colitis patient, although contamination with PCV2 from stool is difficult to exclude (Bernstein et al, 2003). One study reported the presence of weakly cross-reactive anti-PCV antibodies in sera of humans, cows and mice (Tischer et al, 1995), while another reported a lack of PCV antibodies in cows and horses (Ellis et al, 2001). Productive infection with PCV did not occur in a variety of human cell lines inoculated with PCVs (Hattermann et al, 2004) although a more recent study showed productive infection with PCV1 of a human hepatocellular carcinoma cell line (Beach et al, 2011). The lack of detection of high affinity antibodies and absence of nucleic acid detection in human plasma or tissue indicates that human infection with PCV either does not occur or is uncommon, despite the frequent detection of PCV in the stool of pork-consuming US residents (Li et al, 2010). PCV detection in human stool likely reflects its transit through the human gut without enteric viral replication.

Numerous cyclovirus species were found in the feces of children from developing countries (Pakistan, Nigeria, Tunisia) which were for the most part distinct from other cyclovirus species amplified from the muscle tissues of farm animals from the same countries (Li et al, 2010) (Figure 1). The presence of what appears to be human-restricted cyclovirus species in the stool of children from developing countries (based on the still limited human and animal sampling) may therefore reflect enteric replication of some cyclovirus species in humans while other cyclovirus species may replicate in farm animals. Although contamination from other sources cannot be definitely excluded in the case of cyclovirus DNA detected by PCR from market bought meat samples the detection of different cyclovirus species in the muscle tissue of various farm animal species and of a bat support the likelihood that cycloviruses are able to replicate in multiple mammalian hosts (Li et al, 2011; Li et al, 2010; Li et al, 2010). Human cyclovirus infection awaits more definitive confirmation through viral DNA detection in human tissues (free of fecal matter contamination), detection of cyclovirus-specific antibodies, and in vitro cyclovirus replication in human cells.

Circoviruses in fish

A circovirus was recently described in Barbel fry fishes (*Barbus barbus*) from a Hungarian fish farm showing high fry fish mortality (Lorincz et al, 2011). No cause of high fish mortality was found after testing for typical fish pathogens (adenovirus, herpesvirus, iridovirus), parasites by light microscopy, bacteria by isolation, and toxic substances by cell culture tests (Lorincz et al, 2011). Using a consensus PCR approach circovirus sequences were detected in dead fish fries and the rest of the genome was then amplified by inverse PCR. Phylogenetically, the *Barbel circoviruses* (BaCV) clustered with circoviruses (Figure 1) with the same nonamer atop the stem loop as in PCV1, and in non-anseriformes avian circoviruses (TAGTATTAC). The nonamer sequence of BaCV therefore differed from that of the anseriformes avian circoviruses with which the Barbel fry fishes share their aquatic environment. It therefore seems unlikely that BaCV represents a passive contamination of fishes with ingested avian circoviruses shed in bird droppings. PCR prevalence studies indicated that nearly half the Barbel fries tested were positive in several organs, most commonly the spleen and liver while other fresh water fish tested were PCR negative. The role of BaCv in the high fry fish mortality remains unknown but may represents the first example of circovirus replication in a fish.

Cycloviruses in dragonfly

Even more unexpected was the identificaton by metagenomics of a cyclovirus genome in the abdomens of dragonflies captured in the South Pacific (Rosario et al, 2011). The *rep* of the dragonfly cycloviruses (DfCyV) genomes exhibited characteristics typical of cycloviruses and phylogenetically clustered with them (Figure 1) sharing 60–68% amino acid similarity with the Rep of mammalian cycloviruses, an evolutionary distance no greater than that seen amongst mammalian cycloviruses. Whether the cycloviruses infected the dragonflies, were consumed by dragonflies, which feed on small insects, or were from another source is unknown. Cyclovirus detection in non-blood feeeding insects does indicate that these viruses may infect some invertebrates. If cyclovirus replicate in insects, it is also conceivable that the nucleic acids sequences detected in the feces of bat (Ge et al, 2011; Li et al, 2010) or human (Li et al, 2010) may be from insects-contaminated food although the detection of cycloviruses in muscle tissues of non-US farm animals and a bat (Li et al, 2011; Li et al, 2010) argues in favor of genuine and common cyclovirus infection of mammals.

Phylogenetic evidence for cross-species transmission of circovirus and cycloviruses

Detection of viral DNA in feces may reflect either passive passage through the gut without replication or actual enteric replication in that host. When circovirus or cyclovirus DNA can be PCR amplified from tissues, it is more likely to reflect viral replication in some tissue of that host species. The International Committee for the Taxonomy of Viruses suggested criteria for circovirus species demarcation of genome nucleotide identities of less than 75% and Cap protein amino acid identities of less than 70% (Tood et al, 2005). Based on these distance criteria 3 species, Columbid circovirus (CoCV), PCV2, and cyclovirus species 2, were recently found in the tissues of more than one host species. PCV2 sequences were detected in tissues of 13/20 mice and 5/21 rats collected from two PCV2-infected pig farms, but not in rodent samples collected from areas outside these pig farms (Lorincz et al, 2010). Sequence analysis showed that the PCV2 sequences from rodents were similar to those from pigs at the two farms, belonging to the PCV2b genogroup with low levels of nucleotide differences (Lorincz et al, 2010). Low level PCV2b replication could also be demonstrated in inoculated mice (Cságola et al, 2008; Deng et al, 2011). A report of PCV2 infection in cows found PCV2 DNA in 6/100 lung tissue samples from cows with respiratory disease and in 4/30 aborted bovine fetuses (Nayar et al, 1999). A recent study in Germany showed 5/25 calves with a fatal hemorrhagic disease syndrome contained PCV2 while 1/8 non-hemorrhagic disease syndrome cases were positive (Kappe et al, 2010), although the role of PCV2 in bovine disease has been questioned (Willoughby et al, 2010). Using consensus *rep* PCR screening, PCV2 sequences were also detected in 5/19 store bought beef samples from the USA and the full-length genomes from three USA beef specimens shared 99% nucleotide identity with PCV2 (Li et al, 2011). Phylogenetically, all recently identified PCV2 bovine strains clustered with the PCV2b genotypes (Olvera et al, 2007; Guo et al, 2010), while a decade old publication reported a PCV2a genotype in cows (Nayar et al, 1999).

Circoviruses closely related to Columbid circovirus (CoCV) from pigeons were also detected in muscle tissues of 8 out of 40 chickens from Nigeria (Li et al, 2011). The chicken circovirus genome was 92% identical to that CoCV (Mankertz et al, 2000; Todd et al, 2001; Todd et al, 2008) and the Rep and Cap proteins shared 93% and 98% amino acid similarity with CoCV, respectively. Closely related circovirus species can therefore be found in both pigeons and chickens.

While many of the newly identified species of cycloviruses were amplified from muscles tissues of single farm animal host species, one particular cyclovirus species (cyclovirus species 22) was found in the tissues of goats as well as cows (CyCV-PKgoat21 and CyCV-PKbeef23) (Li et al, 2011). Based on more limited *rep* sequencing multiple mammalian farm animals species were also infected with cyclovirus species 2 (Li et al, 2010).

Detection of closely related viruses in the muscle tissues of different animal hosts (rather than in their feces) therefore includes PCV2 in pigs, rodents, and cattle, CoCV in pigeon and chicken and CyCV-PK goat21/beef23 in goat and cow. Whether these reports reflect “dead end” infections without further transmission in a new host species, or the ability of these viruses to establish ongoing transmission in multiple host species will require further molecular epidemiology studies. These results do provide some preliminary evidence that cross-species transmission of circovirus and cyclovirus have occured. Confirmation in the form of direct cyclovirus inoculation followed by rising viral titers and sero-conversion, as well as observation of cross-species transmission between species in natural or farm settings will further substantiate evidence for such viral zoonoses.

Cases of ingestion and excretion without viral replication, as seen for PCV infected pork eaten in the US, may have been detected in the feces of a Nigerian child excreting CoCV/ChickenCV, possibly reflecting consumption of chicken meat (Li et al, 2010). Similarly a circovirus genome (CsaCV-chimp17) related to raven circovirus (RaCV) (Rep protein 80% similar) was detected in the feces of several wild African chimpanzees (Li et al, 2010) (Figure 1). Considering that birds and eggs are occasionally eaten by wild chimpanzees, this avian circovirus-like genome may have originated from ingested bird meat or eggs or even from chimpanzee consuming plants soiled by infected bird feces. Another example of cyclovirus with a likely dietary source may be cyclovirus species 2, found in the tissues of several farm animal species, but also in the stool of Pakistani, Tunisian, and Nigerian children (Li et al, 2010).

Rep sequences identified in animal genomes and environmental samples

Besides circoviruses and cycloviruses, other more divergent *rep* sequences were also identified using high-throughput sequencing and/or consensus PCR in the feces of human (Li et al, 2010), chimp (Blinkova et al, 2010; Li et al, 2010), bats (Li et al, 2010; Ge et al, 2011), rodents (Phan et al, 2011), pigs (Shan,T et al, 2011), blood fed mosquitoes (Ng et al, 2011), within an algeal cell (Yoon et al, 2011), and in environmental samples of seawater, reclaimed waters, sewage, and soil (Rosario et al, 2009; Rosario et al, 2009; Blinkova et al, 2009; López-Bueno et al, 2009; Kim et al, 2008). Our knowledge of the diversity of small circular ssDNA genomes containing rep proteins has therefore greatly expanded although for most cases the actual cellular host remains undetermined.

To determine the relationship of these *rep*-containing small circular DNA genomes, a phylogenetic tree was created based on the Rep protein using Bayesian analysis as previously described (Ng et al, 2011) (Figure 2). Phylogenetically, the *rep* sequences of these genomes falls outside of the *Circoviridae* clade with very deep branches, likely indicating the existence of multiple viral families with distinct tropisms possibly infecting a wide range of eukaryotes. In the case of the *rep*-containing small circular DNA genome amplified from a single algae cell purified by fluorescence-activated cell sorting, its marine protist host was identified as a *picobiliphyte* indicating that these genomes can replicate in algae (Yoon et al, 2011). The closest rep relatives of this algae virus were *rep* from plant nanoviruses and from ocean metagenome data (Yoon et al, 2011).

When genomes are derived from fecal or environmental samples, the cellular host cannot be deduced a priori and viruses released from infected plants, insects, protozoa, or bacteria may

account for some of the *rep*-containing small circular DNA genomes in feces or environmental samples. Purification of viral particle associated nucleic acids (using filtration to remove bacteria-sized particles and digestion with nucleases to remove non-viral capsid protected nucleic acids) reduces the possibility that these *rep*-containing genomes reflect plasmid DNA (Victoria et al, 2009). The detection of *rep* genes on small circular DNA genomes also makes it unlikely that they originated from viral genomes integrated in cellular host chromosomes. For example novel small circular DNA genomes were detected in the feces of wild chimpanzees and called chimpanzee stool associated circular genome viruses (ChiSCV) (Blinkova et al, 2010). Although replication of these viruses in primate cells is conceivable these genomes may also reflect a previously unknown plant virus family based on closest (although still highly divergent) similarity to the algae virus and plant nanoviruses (16–22% rep amino acid identity) (Blinkova et al, 2010) (Figure 2). The recent characterization of *rep* inserted in the chromosomes of various eukaryotes including protozoans (Giardia intestinalis and different species of Entameoba), diatoms, algae, and placozoans (the simplest of all non-parasitic multi-cellular animals), were even more closely related to ChiSCV than nanovirus *rep* (Liu et al, 2011). While the cellular origin of ChiSCV remains unknown it is also conceivable that it was produced by parasites in the gut of chimpanzees.

Human feces also contains numerous *rep* sequences only distantly related to those of the *Circoviridae* (Victoria et al, 2009). The lack of close sequence similarity of these *rep* with those of viruses with known hosts preclude drawing firm conclusions about their cellular hosts although human cells cannot yet be excluded. In bat feces *rep* sequences that fell in multiple groups outside the *Circoviridae* clade were also detected (Li et al, 2010; Ge et al, 2011). In the feces of wild rodents, Rep related sequences were the most common virus-related sequences detected, ahead of dicistroviruses and densoviruses both considered restricted to insect hosts (Phan et al, 2011). Considering the high-level of insect viral sequences it is possible that some of the new Rep encoding genomes also originate from insects eaten by rodents. The rodent fecal Rep encoding genomes were highly variable in size ranging from 1.1 to 3.8 kb and contained one to two rep homologues and from 2 to 8 other discernable ORF. Their variable stem loop nonamers were located either 5’ or 3’ of their rep ORF. One group of *rep* sequences clustered phylogenetically with those in the genome of the ubiquitous Giardia intestinalis (Figure 2), indicating that these small circular genomes, while shed in rodent feces, may actually replicate and be released from common gut protozoan parasites (Phan et al, 2011).

Feces from piglets on a high-density farm also contained *rep*-containing small circular DNA genomes (Shan,T et al, 2011). These genomes ranged from 2.8–3.9 Kb and phylogenetically their *rep* clustered together with the *rep* integrated in the Entamoeba histolytica genome (~33% identity). Relatives of *E. histolytica*, such as *E. polecki* and *E. suis*, are common pig-infecting protozoas. The *rep*-containing small circular DNA genomes in pig feces may therefore originate from common gut protozoan parasites (Shan,T et al, 2011).

Environmental metagenomics has also revealed a large collection of highly variable *rep*-containing small circular DNA genomes (Rosario et al, 2009; Kim et al, 2008). Both marine and reclaimed waters (the end product of waste water treatment), as well as soil contained multiple, deep-branched, *rep*-containing small circular DNA genomes of unknown cellular origins (Kim et al, 2008; Rosario et al, 2009).

A recent systematic search of NCBI databases by Liu et al using Rep sequences of circoviruses, geminiviruses and nanoviruses has also revealed a very wide spread distribution of *rep*-related genes integrated on the chromosomes of mammals, frogs, lancelets, crustaceans, mites, gastropods, roundworms, placozoans, hydrozoans, protozoans,

land plants, fungi, algae, and phyoplasma bacterial and their plasmids (Liu et al, 2011). In this study the cycloviruses *rep* gene were most closely related to those in the germ line of parasitic mites (Liu et al, 2011). Integration in the chromosomes of these Arachnids reflects past infections of mites with cyclovirus-related viruses. The recent detection of cyclovirus DNA in the abdomen of dragonflies (Rosario et al, 2011) may be related considering that mites are frequent ectoparasites of dragonfly. Whether some cylcoviruses, like numerous arboviruses, are able to switch host from arthropods to mammalian hosts (in whose muscle tissues their DNA can also be detected) remains to be demonstrated.

Sequence related to circovirus *rep* were also identified on the chromosomes of various mammals including cats, dogs, giant pandas, two-fingered sloth, and opposum (Liu et al, 2011) indicating that circoviruses once replicated in ancestors of modern day mammals and marsupials. Circovirus-like *rep* were also seen in the germline of lancelets (small eel-like primitive vertebrates), as well as on the chromosomes of a frog and a gastropod reflecting the wide host range that circovirus-like *rep*-containing genomes once had. Further sampling of the viral populations in diverse eukaryotic hosts will be needed to determine which host species are still subject to infection with *rep*-encoding small circular ssDNA viruses.

Conclusions

The genomes of numerous circoviruses, cycloviruses and other *rep*-containing small circular ssDNA viruses have been recently characterized revealing a very high level of genetic diversity. The detection of an new clade of circoviruses in bats (Ge et al, 2011) demonstrates that pigs and boars are not the only circovirus-susceptible mammals and likely portend the detection of circoviruses in more mammals. The characterization of cycloviruses in the tissues of multiple mammals (Li et al, 2011; Li et al, 2010) also attests to the still only partially characterized genetic diversity within the Circoviridae family. Given the rapidly expanding known host range and genetic diversity of circoviruses and cycloviruses, frequent human exposure through food or animal feces, and possible cross-species transmission between other mammals and birds, it seems probable that some circovirus and/or cyclovirus species will eventually be shown to replicate in humans. The pathogenicity, if any, of the newly identified circoviruses and cycloviruses in mammals remains to be determined.

For some of the *rep*-bearing circular genome of uncertain origin, candidate hosts can be proposed based on sequence similarities. In a manner similar to the circovirus genome found in several mammalian germ lines (Liu et al, 2011)(Katzourakis and Gifford, 2010; Belyi et al, 2010) the detection of rep sequences integrated in the genomes of two parasitic protozoa (Gibbs et al, 2006) presumably reflect past viral replication in these single cell eukaryotes. The detection in pig and rodent feces of small circular DNA genomes encoding Rep phylogenetically related to those integrated in protozoa genomes may therefore reflect fecal shedding of viruses replicating in parasites in these mammalian guts (Shan,T et al, 2011; Phan et al, 2011).

The recent description of viruses closely related to mammalian and avian circoviruses and cyclovirues in fish and insects indicates that the *Circoviridae* family either predates the divergence of these animals and underwent relatively few genetic changes as their hosts diverged or that cross species transmissions allowed members of the *Circoviridae* family to bridge very wide host species barriers. Members of the picornavirus-like superfamily of ssRNA viruses infect all the major branches of eukaryotic life leading to the hypothesis that the viral order picornavirales predates the radiation of their current hosts (Koonin et al, 2008). In a similar manner the identification of viral-like *rep* gene in the genome of eukaryotic hosts spanning the entire range of cellular complexity (Unikonts, Chromalveolates, Excavates, and Plantae) and as part of the rolling circle amplification

machinery of some bacterial plasmids also suggest that this group of viral DNA genomes has very deep evolutionary roots predating the emergence and radiation of eukaryotes.

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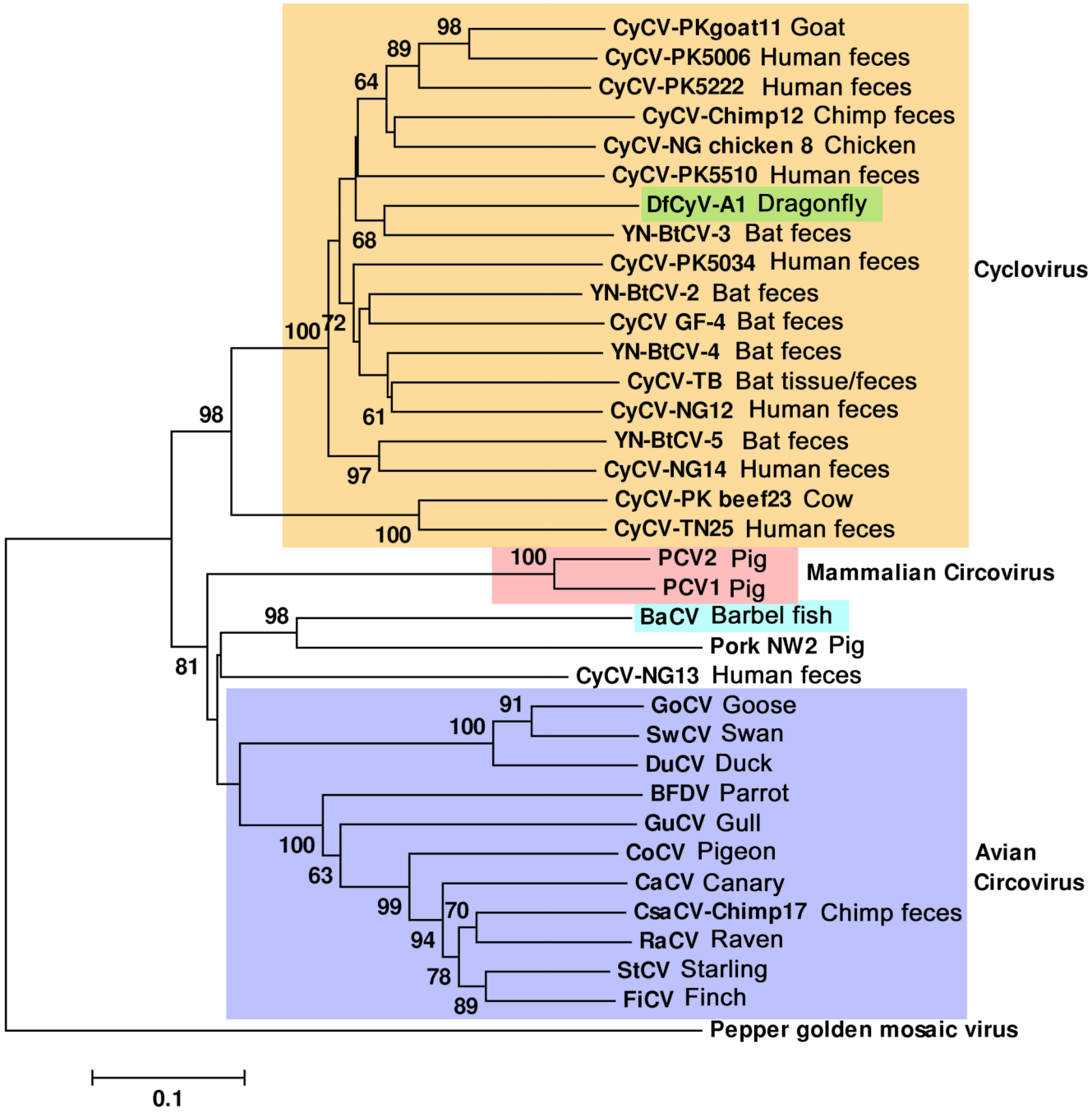

Figure 1.

Phylogenetic analysis of circovirus and cyclovirus species based on the complete amino acid sequence of the Rep protein using the neighbour-joining method with p-distance and 1000 bootstrap replicates. The bar represents 10% estimated genetic divergence. The GenBank accession numbers of the Rep sequences of viruses used in the phylogenetic analyses are in Table 1. The Geminivirus pepper golden mosaic virus (U57457) was used as the outgroup.

Figure 2.

(A) Genome organization of a subset of representative Rep-encoding genomes from rodent feces. (B) Phylogenetic analysis using Bayesian inference (MrBayes) as described (Ng et al, 2011) of the complete Rep from circovirus-like genomes identified in feces from mammals (red), plants (green), environmental samples and an algae (blue), and unicellular eukaryotes (yellow). Numbers above each node represent posterior probabilities of the Bayesian analysis. Branch lengths are based on the number of inferred amino acid substitutions, as indicated by the bar (0.3 substitutions per amino acid position).

Table 1

Genome organization of reported circovirus and cyclovirus species

Circo/cyclo-virus species	Isolation source	Genome (nt)	Rep (aa)	Cap (aa)	5' Intergenic region (nt)	3' Intergenic region (nt)	Nonanucleotide motif	Accession no.
PCV1	Pig tissue	1759	312	233	82 (1724–46)	36 (986–1021)	TAGTATTAC	AY660574
PCV2	Pig tissue	1768	314	233	83 (1736–50)	38 (996–1033)	AAGTATTAC	AY424401
DuCV	Duck BF tissue	1991	292	257	110 (1929–47)	228 (927–1154)	TAGTATTAC	DQ100076
GoCV	Goose BF tissue	1821	293	250	132 (1762–72)	54 (955–1008)	CAGTATTAC	AJ304456
CoCV	Pigeon BF tissue	2037	317	273	90 (1988–40)	171 (995–1165)	TAGTATTAC	AF252610
RaCV	Raven feather/blood	1898	291	243	86 (1848–35)	204 (912–1115)	TATTATTAC	DQ146997
SwCV	Swan organ tissue	1785	293	251	107 (1726–47)	40 (930–969)	TAGTATTAC	EU056310
BFDV	Parrot skin	1993	299	244	126 (1975–107)	232 (1008–1239)	TATTATTAC	AF071878
GuCV	Gull BF tissue	2035	305	245	207 (1928–99)	172 (1018–1189)	TAGTATTAC	DQ845074
FiCV	Finch BF tissue	1962	291	249	29 (1962–28)	307 (905–1211)	GAGTATTAC	DQ845075
StCV	Starling organ tissue	2063	289	276	79 (2021–36)	283 (907–1189)	CAGTATTAC	DQ172906
CaCV	Canary organ tissue	1952	290	250	77 (1907–31)	249 (905–1153)	TATTATTAC	AJ301633
CsaCV-chimp17	Chimp feces	1935	291	232	198(1772–34)	162 (911–1072)	CAGTATTAC	GQ404851
BaCV	Barbel tissue	1957	319	214	99 (1906–47)	253 (1008–1260)	TAGTATTAC	GU799606
CyCV1-PK5006	Human feces	1723	278	219	230 (1516–22)	–	TAATACTAT	GQ404844
CyCV-PK5222	Human feces	1740	279	218	247 (1516–22)	–	TAATACTAT	GQ404846
CyCV-PK5510	Human feces	1759	280	219	271 (1679–190)	–	TAATACTAT	GQ404847
CyCV-PK5034	Human feces	1780	277	218	293 (1691–203)	–	TAATACTAT	GQ404845
CyCV-Chimp12	Chimp feces	1747	280	220	255 (1515–22)	–	TAATACTAT	GQ404850
CyCV-NG14	Human feces	1795	286	230	245 (1707–156)	–	TAATACTAT	GQ404855
CyCV-TN25	Human feces	1867	286	222	160 (1762–54)	6(1087–1092)	TAATACTAT	GQ404857
CyCV-NG12	Human feces	1794	281	218	284 (1691–180)	7(1027–1033)	TAATACTAT	GQ404850
CyCV-NG13	Human feces	1699	307	221	105 (1622–27)	4(952–955)	TAGTATTAC	GQ404856
CyCV-NG chicken 8	Chicken tissue	1760	278	222	258 (1525–22)	–	TAATACTAA	HQ738643
CyCV-PK goat11	Goat tissue	1751	278	231	222 (1552–22)	–	TAATACTAT	HQ738636
CyCV-PK beef23	Cow tissue	1838	280	212	180 (1711–52)	7 (1065–1071)	TAATACTAG	HQ738634
CyCV-TB	Bat tissue/feces	1703	278	225	192 (1535–23)	–	TAATACTAT	HQ738637
CyCV-GF4	Bat feces	1844	281	227	326 (1747–228)	–	TAATACTAT	HM228874

Circo/cyclo-virus species	Isolation source	Genome (nt)	Rep (aa)	Cap (aa)	5’ Intergenic region (nt)	3’ Intergenic region (nt)	Nonanucleotide motif	Accession no.
YN-BtCV-2	Bat feces	1771	276	219	284 (1675–187)	–	TAATACTAT	JF938079
YN-BtCV-3	Bat feces	1743	277	223	236 (1655–147)	1 (982)	TAATACTAT	JF938080
YN-BtCV-4	Bat feces	1741	279	223	229 (1535–22)	–	TAATACTAT	JF938081
YN-BtCV-5	Bat feces	1818	286	222	292 (1719–192)	–	TAATACTAT	JF938082
DfCyV-A1	Dragonfly abdomen	1741	309	224	158 (1666–82)	–	TAATACTAT	HQ638069

Note: 1. Nucleotide position 1 was set at the residue “A” at position 8 of the nonamer sequence for all genomes contained in this table. 2. Bursa of Fabricius tissue: BF tissue.