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Faecal virome of healthy chickens reveals a large diversity of the eukaryote viral community, including novel circular ssDNA viruses

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Abstract

This study is focused on the identification of the faecal virome of healthy chickens raised in high-density, export-driven poultry farms in Brazil. Following high-throughput sequencing, a total of 7743 *de novo*-assembled contigs were constructed and compared with known nucleotide/amino acid sequences from the GenBank database. Analyses with BLASTX revealed that 279 contigs (4 %) were related to sequences of eukaryotic viruses. Viral genome sequences (total or partial) indicative of members of recognized viral families, including *Adenoviridae*, *Caliciviridae*, *Circoviridae*, *Parvoviridae*, *Picobirnaviridae*, *Picornaviridae* and *Reoviridae*, were identified, some of those representing novel genotypes. In addition, a range of circular replication-associated protein encoding DNA viruses were also identified. The characterization of the faecal virome of healthy chickens described here not only provides a description of the viruses encountered in such niche but should also represent a baseline for future studies comparing viral populations in healthy and diseased chicken flocks. Moreover, it may also be relevant for human health, since chickens represent a significant proportion of the animal protein consumed worldwide.

INTRODUCTION

Chickens are major source of animal protein for human consumption worldwide. Feed conversion and poultry productive performance are strongly dependent on the gut microbiota and on adequate functioning of the gastrointestinal tract [1]. In view of this, over the past several years, great efforts have been made to characterize the bacterial population in the chicken gut [2–5]. However, despite the interdependence between bacterial and viral contents in the gut, remarkably little is known about the intestinal virome of poultry [6, 7].

High-throughput sequencing has allowed unprecedented advances in the characterization of complex microbial communities [8, 9]. Such technology, allied to constantly improving methods of metagenomic analysis, has the potential to circumvent the limitations of conventional virological methods, such as the limited ability to replicate most viruses *in vitro*, unsuccessful PCR amplification of viral genomes or failure of antibodies to recognize unknown viruses [10].

Using such an approach, researchers have explored the diversity of the faecal virome from different species at a rapidly increasing rate [10–13]. In turkeys, great strides have

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Abbreviations: AdV, adenovirus; AGV2, avian gyrovirus 2; CAV, chicken anaemia virus; ChCV, chicken calicivirus; ChPBV, chicken picobirnavirus; CRESS-DNA, circular Rep-encoding ssDNA; FAdV, fowl adenovirus; GyV4, gyrovirus 4; PBV, picobirnavirus; RV, rotavirus; SiV, sicinivirus.

Selected complete or partial genome sequences were submitted to GenBank, and the following accession numbers were obtained: fowl adenovirus RS/BR/2015/1 to 53, KY053058 to KY053110; CAV_RS/BR/15, KY024579; GyV4_RS/BR/15, KY024580; AGV2_RS/BR/2015, KY039279; chicken calicivirus RS/BR/2015, KY120883; chicken smacovirus RS/BR/2015/1, KY086298; chicken smacovirus RS/BR/2015/2, KY086301; chicken smacovirus RS/BR/2015/3, KY086300; chicken smacovirus RS/BR/2015/4, KY086299; chicken stool-associated gemycircularvirus RS/BR/2015, KY056250; chicken stool-associated circular virus_RS/BR/2015, KY056251; galliform aveparvovirus 1RS/BR/2015, KY069111; chicken picobirnavirus strain RS/BR/2015/1, KY123114; chicken picobirnavirus strain RS/BR/2015/2, KY123115; chicken picobirnavirus strain RS/BR/2015/3, KY123116; sicinivirus RS/BR/2015/1, KY069112; sicinivirus RS/BR/2015/2, KY069113; chicken megrivirus RS/BR/2015/1, KY086293; chicken megrivirus RS/BR/2015/2, KY086292; chicken proventriculitis virus RS/BR/2015/1, KY086297; chicken proventriculitis virus RS/BR/2015/2, KY086296; chicken proventriculitis virus RS/BR/2015/3, KY086295; chicken proventriculitis virus RS/BR/2015/4, KY086294; rotavirus A RS/BR/2015/1 to rotavirus A RS/BR/2015/21, KY069090 to KY069110; rotavirus D strain RS/BR/2015/1 to rotavirus D strain RS/BR/2015/22, KY069068 to KY069089.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

been made in the discovery, description and characterization of a multitude of intestinal viruses [14]. As such, a better characterization of the intestinal virome of healthy chickens would be expected to contribute toward increasing productivity by promoting better efficiency of feed conversion, decreasing the possibility of occurrence of disease and, ultimately, increasing food safety by minimizing potential risks to public health [15, 16].

In this study, metagenomic analyses were performed on the faecal virome of healthy, 3- to 5-week-old broilers. The birds were raised in export-driven commercial flocks in Southern Brazil under fairly standard rearing conditions. Sequence data on purified viral nucleic acids extracted from stools were generated in an Illumina MiSeq platform. Similar proportions of viruses of eukaryotes and prokaryotes were identified in samples; however, this report focuses on the description of viral genomes of eukaryotes from chicken faecal samples.

RESULTS AND DISCUSSION

Overview of sequence data

A total of 541 988 paired-end sequence reads with an average of 146.1 nt were generated. Sequence reads were *de novo* assembled and compared against a non-redundant database using BLASTX and BLASTN programmes. Among 7743 assembled contigs, 44.5 % had no similarity to any sequences in GenBank, in agreement with previous findings in human stool samples [10, 17]. Approximately 4 % (279) of the sequences showed similarity to known eukaryotic viral sequences with an E-value cut-off of 10^{-3}. Fig. 1(a) presents the taxonomic classification and the relative amount of the putative viral genomes assembled from the samples examined.

Summary of the findings on the faecal virome of healthy chickens

Fifty-seven percent of the sequences with best BLASTX hits corresponding to eukaryotic viruses were related to DNA viruses of the families *Adenoviridae*, *Circoviridae* and *Parvoviridae*. In addition, a variety of unclassified circular Rep-encoding ssDNA (CRESS-DNA) viral genomes were identified.

Regarding RNA viruses, the identified viral genomes could be assigned to families *Caliciviridae*, *Picobirnaviridae*, *Picornaviridae* and *Reoviridae* (Fig. 1b). Table S1 (available in the online Supplementary Material) presents a summary of the eukaryotic viral sequences detected in the present study.

Next, a brief report on the major groups of viruses identified in the faecal virome of healthy chickens is presented.

DNA genomes

Fowl adenoviruses

Adenoviruses (AdVs) are non-enveloped, icosahedral viruses with a DNA genome consisting of a double-stranded molecule with 26 to 45 kbp [18]. AdVs have been identified in a wide range of vertebrate species, including amphibians, birds, fishes, mammals and reptiles [19]. AdVs that infect chickens are named fowl AdVs (FAdVs). These are currently classified in the genus *Aviadenovirus*, comprising five species (FAdVs A–E) and 12 serotypes (FAdV-1 to 8a and 8b to 11) [20]. In the current study, 53 contigs representing partial genome sequences with high similarity to FAdVs were identified, ranging from 208 to 1217 bp in length (GenBank nos. KY053058 to KY053110).

Of the 53 contigs which could be related to FAdVs, only one corresponded to the genomic region coding for part of the *hexon-associated protein VIII* gene, often used for

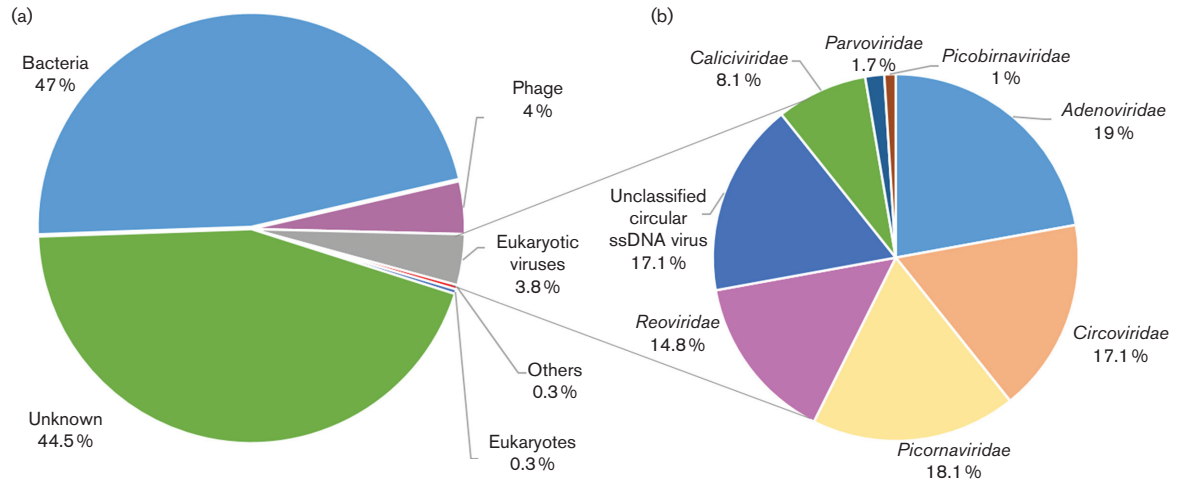

Fig. 1. Putative classification of viral genome sequences detected in pooled stool samples of healthy chickens. Percentages express relative amounts of viral genomes assigned to each particular family (or unclassified) within the total number of contigs obtained. (a) Overview of the *de novo*-assembled Illumina reads. (b) Percentages of eukaryotic viral contigs distributed in different viral families.

phylogenetic analyses [21]. As such, this was the only genomic segment that could be compared with previously reported counterparts. The deduced, partial aa hexon sequence obtained here, named FAdV RS/BR/2015/52, clusters with FAdV-D type 9 (GenBank NC_000899) (Fig. S1).

The presence of FAdVs in faeces of healthy broilers is expected, since FAdV infections are ubiquitous and tend to be asymptomatic in chickens [22]. However, severe disease has been associated to some FAdVs types, especially in young or immunocompromised birds [19, 23, 24], as well as inclusion body hepatitis and hydropericardium in specific pathogen-free chickens [21]. From the findings reported here, it is possible that FAdV RS/BR/2015/52, most likely a type FAdV-D species, may infect healthy birds with no apparent association with disease, since the chickens showed no clinical signs at the time of sampling.

Chicken gyroviruses

Gyroviruses are small non-enveloped DNA viruses with icosahedral symmetry [25]. Such viruses possess circular, single-stranded genomes of ~2.3 kb in length and are classified in the genus *Gyrovirus* within the family *Anelloviridae* [26]. In the current study, the full genomes of gyrovirus 4 (GyV4) and chicken anaemia virus (CAV), as well as a near-complete genome of avian gyrovirus 2 (AGV2), were identified.

The GyV4 full-length genome (named GyV4_RS/BR/15, GenBank KY024580) is 2035 nt long. This genome contains a 518-nt-long non-translated region displaying an average of 55.4 % GC content and a polyadenylation signal (AATAA). Two overlapping ORFs were predicted in the same genome strand (putative VP1, 352 aa long; putative VP2, 217 aa long) (Fig. 2a). A BLASTP search on VP1- and VP2-predicted aa sequences revealed similarities of 99 % and 100 %, respectively, to previously described counterparts in GyV4. Phylogenetic analyses of the *VP1* gene showed that GyV4_RS/BR/15 clustered closely with GyV4 detected in human stool specimens and in chicken meat for human consumption (Fig. 2b) [27]. GyV4 has also been reported in faecal specimens from ferrets, indicating possible dietary sources from consuming infected birds [28, 29]. Therefore, it seems likely that GyV4 may, in fact, be a virus of avian origin, which was incidentally detected in the intestinal contents of other carnivore species, such as reported in the previous studies mentioned above.

The complete CAV genome reported here (strain CAV_RS/BR/15, GenBank KY024579) is 2298 nt in length with a 475-base-long non-translated region and 59.6 % of GC content. The genome organization showed typical features of previously reported CAV genomes, with three overlapping ORFs putatively coding for VP1 (1350 nt), VP2 (651 nt) and VP3 (366 nt) (Fig. 2c). The degree of sequence similarity among CAV_RS/BR/15 and previously reported CAV genomes ranged from 95 % to 100 %. In comparison to the reference CAV genome (accession no. NC_001427), CAV_RS/BR/15 has a 21-nt deletion located within the transcription regulatory region. Such deletion seems associated to a better efficiency to replicate in MDCC-MSB1 cells [30]. However, it is still unclear whether such alteration might affect viral pathogenicity [31, 32]. Phylogenetic analyses based on the VP1 nucleotide sequences cluster CAV_RS/BR/15 into CAV group D, together with isolates from China, USA, Argentina, Japan, Australia and Malaysia (Fig. 2d) [33]. Moreover, the CAV sequence reported here is closely related to the Argentinean isolate 'CAV-10' (accession no. KJ872513), suggesting that both might have a common origin.

The AGV-2-related reads generated a contig (2224 nt) with 98 % to 100 % aa similarity to the previously reported AGV2 sequence (GenBank NC_015396) (Fig. 2e). Phylogenetic trees constructed with basis on the partial VP1 nt sequences indicated that the AGV2 reported here (AGV2 RS/BR/2015, GenBank KY039279) clusters along with AGV2 sequences previously detected in chickens in this same region (Southern Brazil) [34] and in Africa [35] (Fig. 2f). As for GyV4, AGV2 and a number of other gyroviruses have been identified in faeces of humans and ferrets, most likely consequent to the consumption of birds [28, 36]. Although AGV2 has been previously reported in chicken's sera, tissues and feather shafts [34, 37, 38], this is the first report on the identification of AGV2 genomes in the intestinal contents of chickens.

Highly divergent circovirus-like genome sequences

Eukaryotic viruses with circular ssDNA genomes represent 'minimal' viral elements, often with less than 6 kb and encoding a maximum of six proteins [39]. As a result, most ssDNA viruses are highly dependent on the host's replicative machinery and require a conserved replication initiator protein (Rep) for its replication [40]. Viral metagenomic studies revealed a highly diverse population of novel eukaryotic CRESS-DNA viruses in faeces of a number of species, including chimpanzees [41], pigs [12], rodents [42], bats [43, 44], bovines [45], dromedaries [46], humans [47] and caribous [48]. In this study, six full-length genomes of novel CRESS-DNA viruses were identified. Four of these genomes shared 56 % to 94 % aa similarity to the Rep protein of smacoviruses recently reported in faeces from human and non-human primates [49]. Thus, to facilitate description, these four genomes were provisionally named here 'chicken-associated smacoviruses' (ChSmCV RS/BR/2015 from 1 to 4, GenBank KY086298 to KY086301).

One of the other two CRESS-DNA viral genomes identified showed 52 % similarity to the Cap protein of the caribou faeces-associated gemycircularvirus (GenBank NC_024909) and was named 'chicken stool-associated gemycircularvirus RS/BR/2015' (ChSGV RS/BR/2015, GenBank KY056250). The sixth full-length CRESS-DNA identified here was named 'chicken stool-associated circular virus RS/BR/2015' (ChSCV RS/BR/2015, GenBank KY056251) and displayed 42 % similarity to the Rep protein of the rodent stool-associated circular genome virus (GenBank JF755404). All of these six sequences vary in size from 2143 to 4668 nt and contain at least two

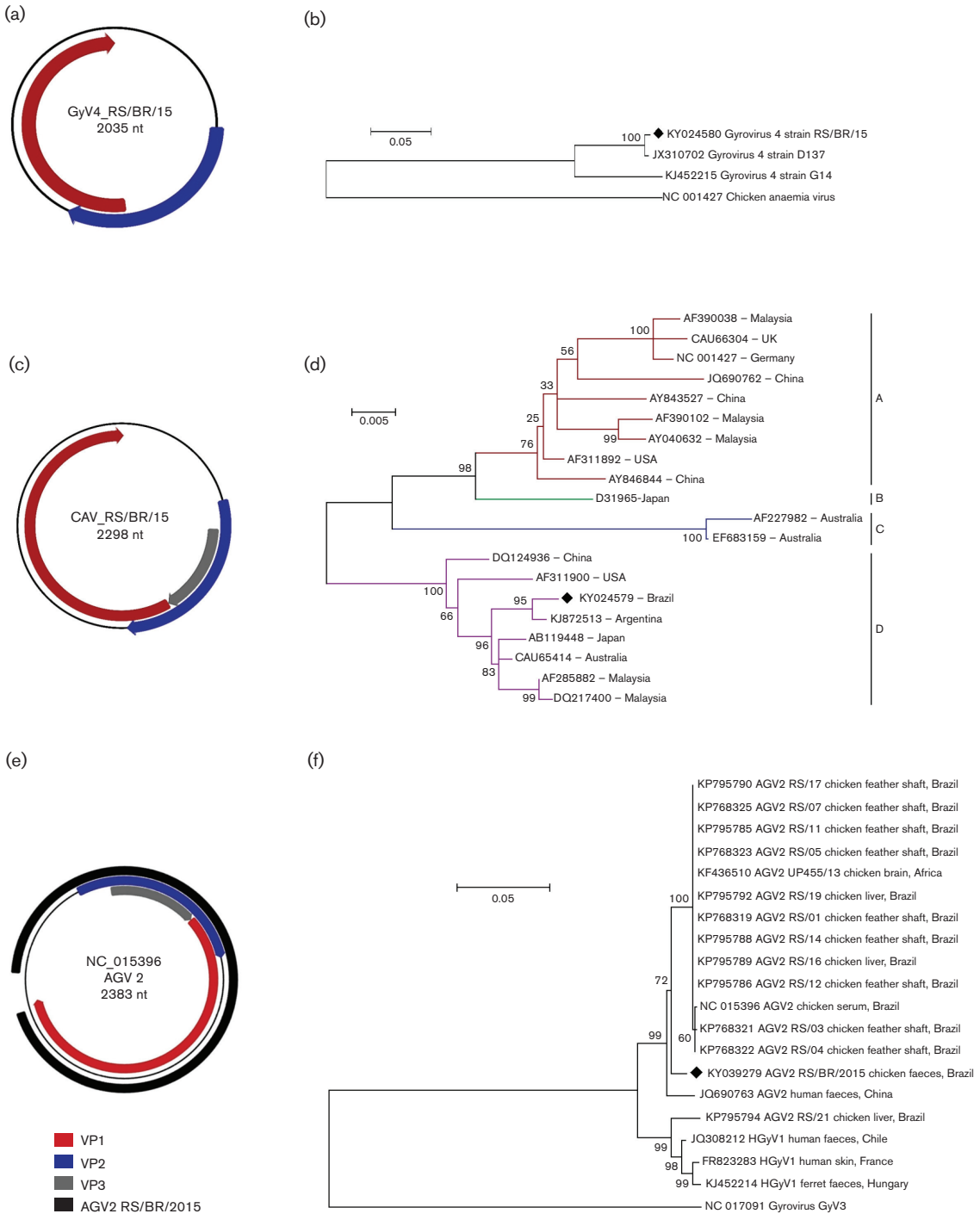

Fig. 2. Putative genome organization and phylogenetic analyses of chicken gyroviruses. (a) Genomic organization of GyV4. (b) Phylogenetic tree based on GyV4 VP1 nucleotide sequences. (c) Genomic organization of CAV. (d) Phylogenetic inferences based on CAV VP1 nucleotide sequences. (e) Schematic representation of the reference genome of AGV2 (NC_015396). The black bar represents the AGV2 genome identified in this study. (f) Phylogenetic tree generated with VP1 nucleotide sequences. Phylogenetic trees were constructed by neighbour joining with a 1000 bootstrap replicates. Sequences identified in this study are highlighted by black diamonds.

ORFs arranged in opposite orientations and encoding two putative proteins: a replicase (204–327 aa) and a capsid protein (305–326 aa) (Fig. 3a). Conserved nonamers at the top of predicted stem-loop structures (Fig. 3b) and two rolling-circle replication motifs II (xHxH) and III (YxxK) were detected in all six genomes.

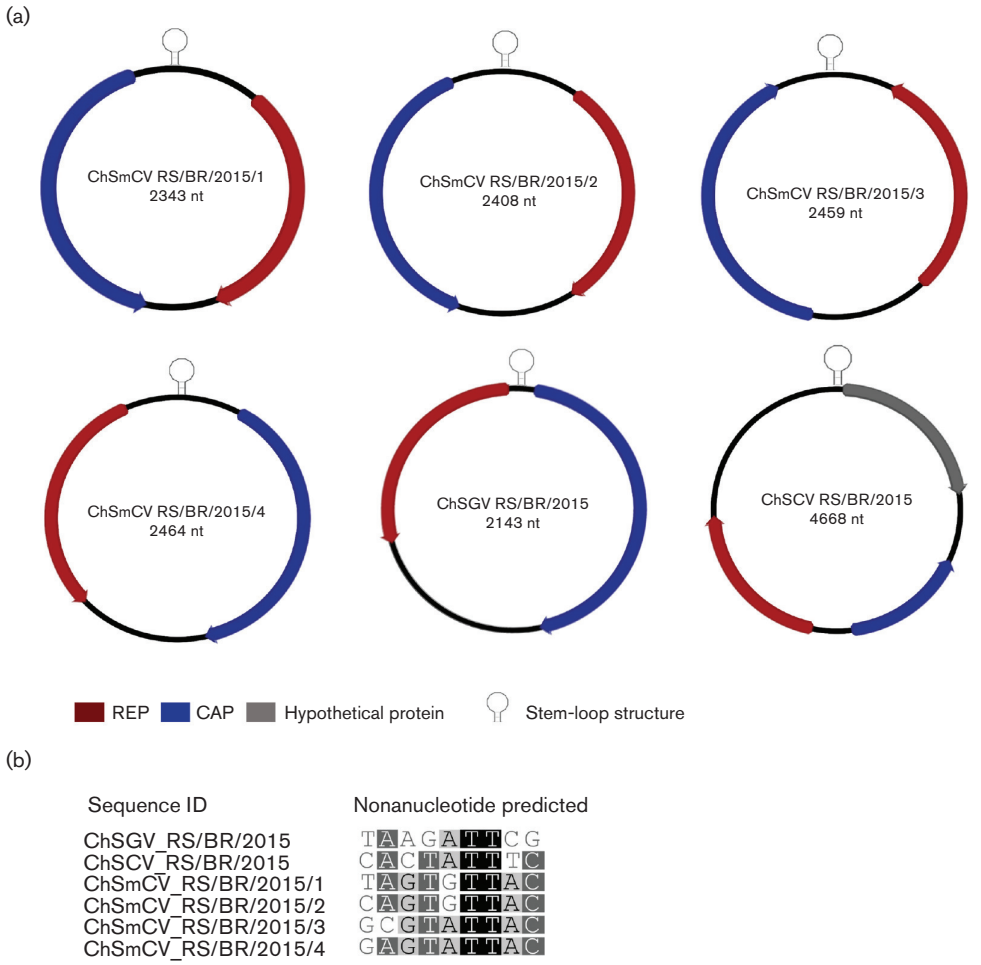

Fig. 3. Novel CRESS-DNA viruses identified in faeces of chickens. (a) Putative genome organization. REP, replication-associated protein gene; CAP, capsid protein gene; hypothetical proteins refer to putative ORFs with no known function. (b) Conserved nonamers identified at the top of predicted stem-loop structures.

The phylogenetic tree constructed based on the Rep-deduced aa sequences allowed clustering of ChSmCV RS/BR/2015/1 along with the turkey stool-associated circular ssDNA viruses [50], while ChSmCV RS/BR/2015/2 to 4 clustered separately, along with smacoviruses detected in human faeces [47] (Fig. 4). Additional phylogenetic analyses showed that ChSCV RS/BR/2015 clusters along with the 'stool-associated circular virus' reported in faeces of dromedaries and rodents [42, 46]. The ChSGV RS/BR/2015 genome clustered more closely to members of the proposed family *Genomoviridae* (previously named *Gemycircularvirus* group) [51]. The first member of the newly described *Genomoviridae* family was isolated from a plant-pathogenic fungus [52] and later reported in sewage, faeces and cerebrospinal fluids from unexplained cases of child encephalitis [48, 53–55].

CRESS-DNA viruses have been detected in the intestinal contents of a broad range of asymptomatic and diarrhoeic animals, including humans [41, 45, 56–59]. Except for the similar sizes and genome organization, little is known about their origin, tropism and biology [50]. Yet, such viruses may be major virome components in most terrestrial and aquatic environments [53]. It is possible that CRESS-DNA viruses in faeces might be related to ingested food or infections of simbiont organisms present in the gastroenteric tract as protozoa, fungi or bacteria [47, 49].

Galliform aveparvovirus

Galliform aveparvovirus 1 (also named chicken parvovirus, ChPV) are small, naked, icosahedral ssDNA viruses, classified in the *Aveparvovirus* genus of the family *Parvoviridae* [60]. Here, a near-complete genome of ChPV comprising the full coding sequence was identified, named ChPV RS/BR/2015 (GenBank KY069111). The assembled contig is 4615 nt long and comprises the full coding region of the ChPV genome (Fig. 5a). The genome architecture of ChPV RS/BR/2015 reveals three ORFs typical of members of the *Aveparvovirus* genus. The first ORF (694 aa,

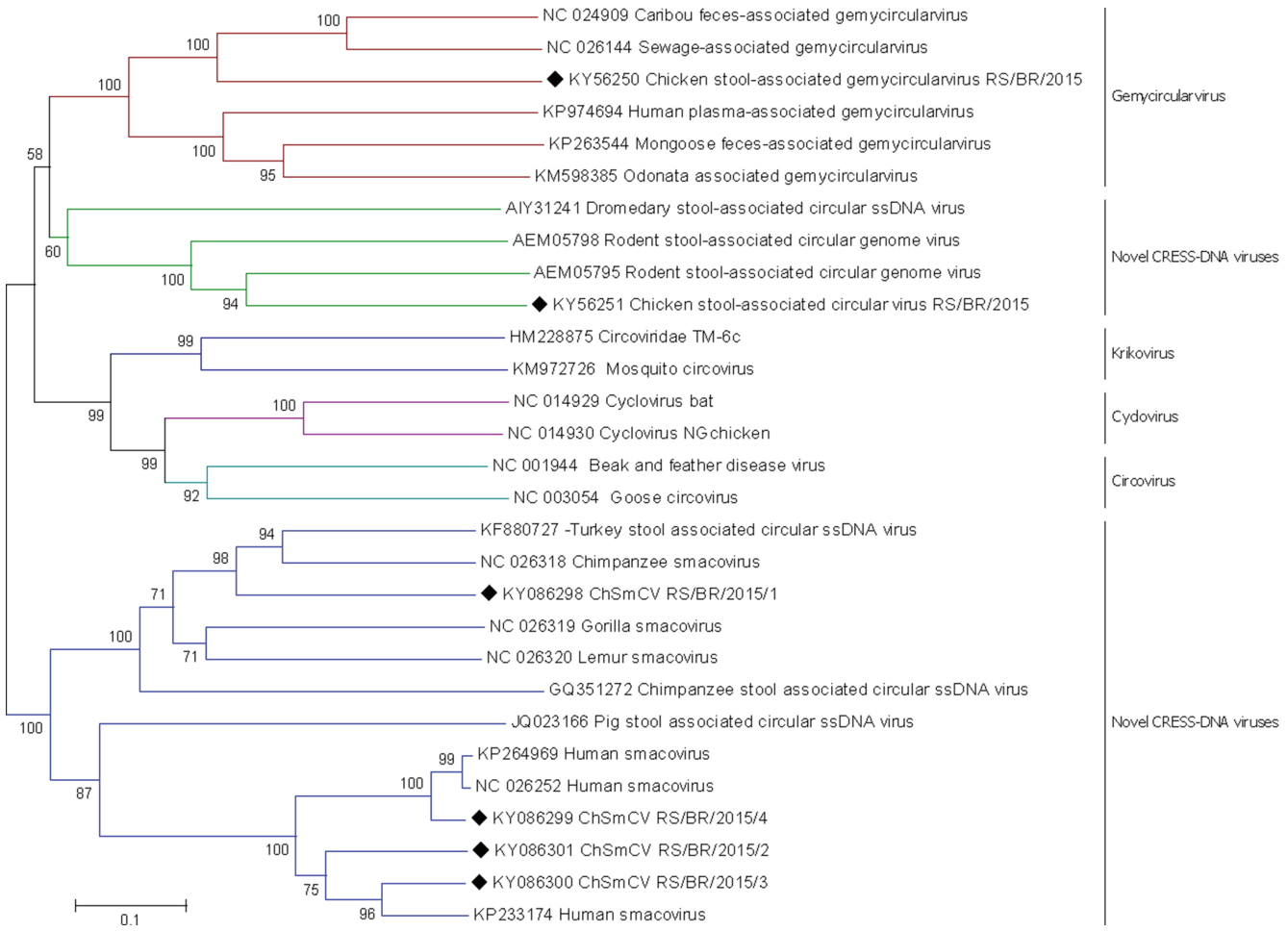

Fig. 4. Phylogenetic analysis of chicken CRESS-DNA viruses identified in faeces of chickens. Phylogenetic inferences based on Rep amino acid sequences were carried out by neighbour joining with a 1000 bootstrap. Sequences identified in this study are highlighted by black diamonds.

78.3 kDa) encodes a putative non-structural (NS) protein with 99 % aa similarity to the prototype sequence ABU-P1 (GenBank NC_024452). The second ORF in ChPV RS/BR/2015 genome shows 87 % aa identity with the NS protein (NP1) of ABU-P1. The third predicted ORF displays 95 % aa similarity to its counterpart in ABU-P1 and encodes two putative capsid proteins VP1 (675 aa, 76.5 kDa) and VP2 (536 aa, 60.4 kDa), believed to be translated from ORF3 by alternative splicing [61].

Phylogenetic analyses based on the nt sequences of the full coding region reveal that ChPV RS/BR/2015 is closely related to galliform aveparvovirus 1 IPV (KU569162), another member of the *Aveparvovirus* genus detected in Southern Brazil. Both formed a distinct cluster from other chicken and turkey parvoviruses previously described (Fig. 5b). Although ChPV has been associated with malabsorption syndrome in broilers, this virus has been detected in both diseased and healthy chickens [62]. In this study, ChPV RS/BR/2015 genome was identified in apparently healthy birds at the time of sampling, which is in agreement with the findings cited above.

To date, few complete ChPV genome sequences are available. The near-complete, full coding sequence of ChPV RS/BR/2015 reported here is an additional source of information for expanding our knowledge on the biology of ChPV.

RNA genomes

Chicken caliciviruses

Caliciviruses are small (27–36 nm), non-enveloped, icosahedral viruses with a linear ssRNA genome [63]. Currently, five genera within the family *Caliciviridae* are recognized by the ICTV: *Nebovirus*, *Norovirus*, *Lagovirus*, *Sapovirus* and *Vesivirus*. In addition, five unclassified caliciviruses (atlantic salmon calicivirus, 'bavovirus', 'nacovirus', 'recovirus' and 'valovirus') have been proposed to form new genera [64, 65]. Here, the complete genome of a new chicken calicivirus (ChCV RS/BR/2015, GenBank KY120883) was identified. Its genome is 8176 nt long (excluding the poly-A tail),

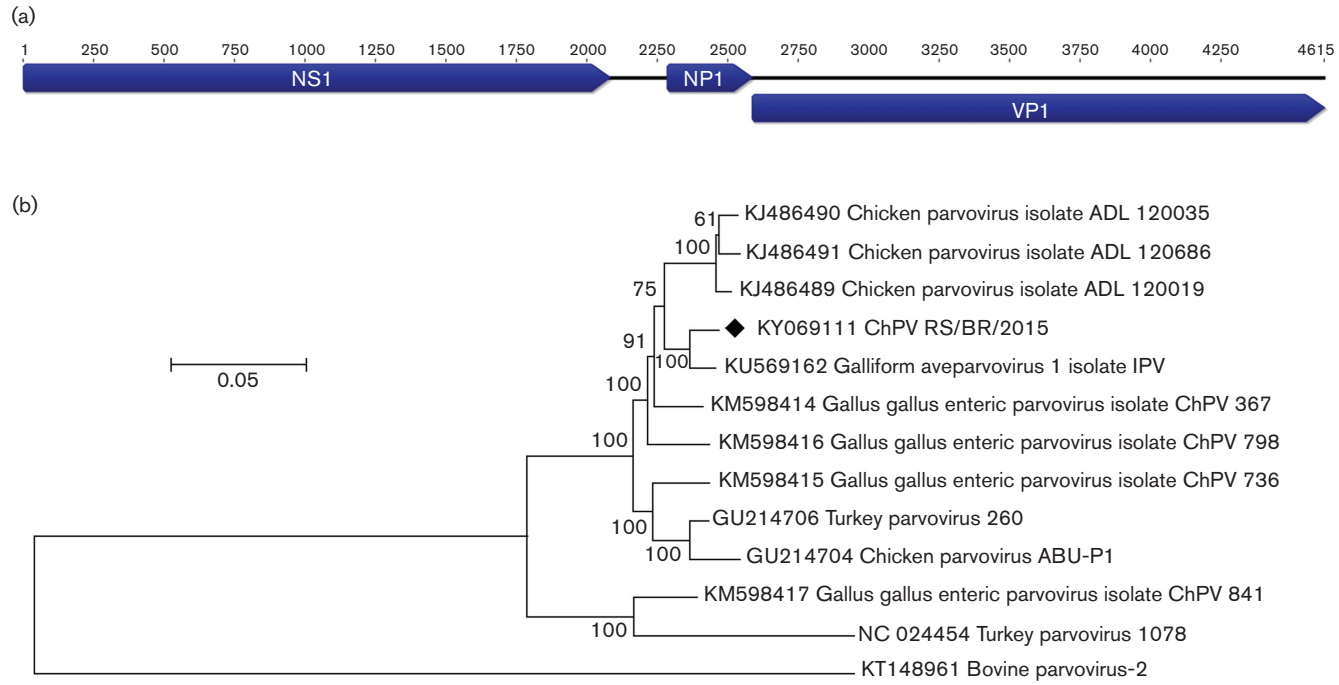

Fig. 5. Genome organization of the putative protein coding region of ChPV RS/BR/2015 and phylogenetic comparison with other parvoviruses. (a) Representation of the complete genomic organization of ChPV RS/BR/2015. The sequence length (4615 nt) corresponds to the full protein coding genome region. (b) Phylogenetic inferences based on nucleotide sequences of the entire coding region were carried out by neighbour joining with a 1000 bootstrap. Black diamond highlights ChPV RS/BR/2015.

with 54 % GC content, in line with mammalian and avian caliciviruses previously reported [66]. The genome architecture of ChCV RS/BR/2015 reveals two main ORFs (Fig. 6a). The predicted ORF1 (2313 aa, 249.5 kDa) encodes a putative polyprotein which is 96 % similar to its counterparts in a previously published ChCV (accession no. KM254171). Some aa motifs typically conserved in CVs' polyproteins were identified, including the NTPase motif GXPGXGKT at position 345, the protease motif GDCGXP at position 1176 and the RNA-dependent RNA polymerase (RdRp) motifs DYSKWDST, GLPSG and YGDD at positions 1457, 1512 and 1560, respectively. The second ORF (287 aa, 30.4 kDa) reveals high similarity (96.1 %) with the *VP2* gene of ChCV strain V0021/Bayern/2004 (GenBank HQ010042). The *VP2* gene encodes a structural protein whose function has not yet been determined [67, 68].

Phylogenetic analyses of ChCV RS/BR/2015 performed using the RdRp aa sequence confirmed that such genomes clustered along with other ChCVs in the proposed *Bavovirus* genus (Fig. 6b). The creation of a new genus within the *Caliciviridae* has been proposed by others to accommodate such viruses [65, 69]. All previously reported ChCV nt sequences deposited at GenBank originated from Germany, Netherlands and Korea. The identification of ChCV RS/BR/2015 in Brazil suggests a widespread distribution of these viruses.

Picobirnaviruses

Picobirnaviruses (PBVs) are small, non-enveloped viruses with bisegmented dsRNA genomes with approximately 4.2 kbp [70]. The large genome segment (S1) encodes the capsid protein, while the small segment (S2) encodes the RdRp. PBVs are highly variable and have been classified in two major groups (GI and GII) based on the S2 genomic sequence [71]. In the present study, three contigs related to PBV genomes were identified (Fig. 2a). One of these, chicken PBV (ChPBV) RS/BR/2015 1 (264 nt, GenBank KY123114), corresponds to a portion of the *capsid* gene and shares 41 % similarity with the equivalent region on the dromedary PBV genome (GenBank KM573789). The other PVB-related sequence, ChPBV RS/BR/2015 2 (258 nt, GenBank KY123115), also matches a portion of the *capsid* gene, displaying 41 % similarity to the homologous region on the porcine PBV genome (GenBank KF861771). The third PBV-related contig, ChPBV RS/BR/2015 3 (GenBank KY123116), shares the highest aa similarity (77 %) with the *RdRp* gene of human PBV (GenBank GQ915028).

Phylogenetic analyses of ChPBV are usually based on the conserved region of the *RdRp* gene [72]. The only PBV-related contig obtained here, which comprised a portion of the *RdRp* gene, named ChPBV RS/BR2015_3, clustered along with members of PBV genogroup II (Fig. 2b).

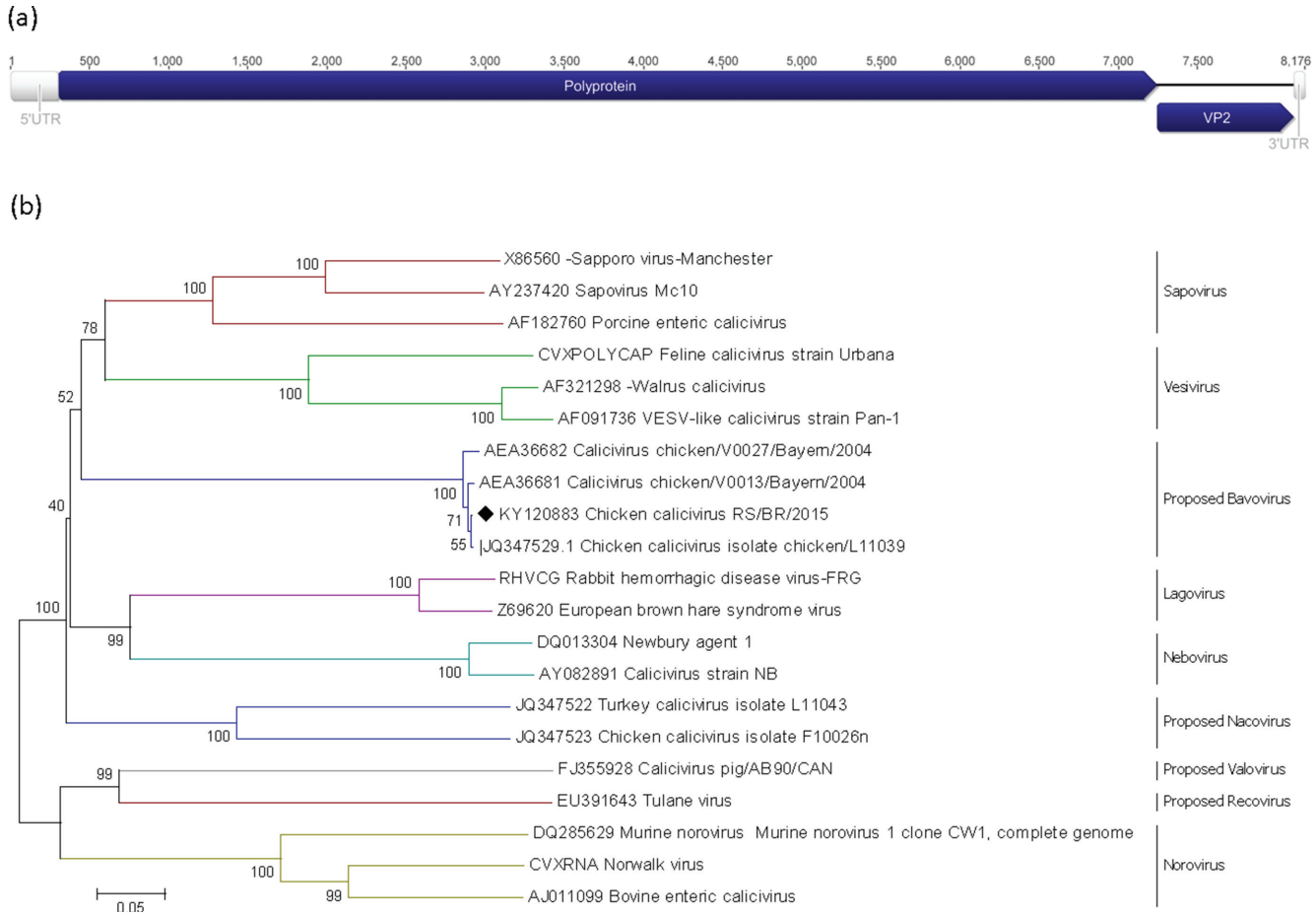

Fig. 6. Putative genome organization and phylogenetic analysis of ChCV strain RS/BR/2015. (a) The genome contains two main predicted ORFs: ORF1 spans almost the complete genome; ORF2 is located at the 3′ end of the genome. (b) Evolutionary relationships based on RdRp aa sequences of ChCV strain RS/BR/2015 with members of the family *Caliciviridae*. Neighbour-joining analyses were performed with 1000 bootstrap replicates. The calicivirus genome introduced here (ChCV RS/BR/2015) is labelled with a black diamond.

PBVs have been detected in faecal samples from both asymptomatic and diarrhoeic or immunocompromised individuals [70]. However, no definite association of PBV with pathogenicity has been drawn [14, 71]. In this study, PBV partial genomic segments were identified in faeces of chickens with no signs of illness, as have other studies [72]. These findings suggest that PBVs are part of the normal intestinal virome of chickens. Further studies should be conducted to investigate the role of PBVs in the intestinal microbiota [46].

Divergent chicken picornavirus genomes

Picornaviruses are non-enveloped viruses with a single-stranded, positive-sense RNA genome. The family *Picornaviridae* currently comprises 31 genera, although new genera are constantly being created [26, 73]. In the last decade, viral genomes of at least eight different genera have been identified in birds: *Avihepatovirus, Avisivirus*, *Gallivirus*, *Megrivirus*, *Oscivirus*, *Passerivirus*, *Sicinivirus* and *Tremovirus*, as well as an unassigned genus comprising phacovirus and chicken picornaviruses 2 and 3 [74]. Nevertheless, little is known about the diversity of picornaviruses in poultry [75]. Here, the near-complete genomes of two novel siciniviruses (SiVs) (genus *Sicinivirus*) and partial sequences which seem related to members of genus *Megrivirus* were identified.

The SiV-related genomes detected in the present study (SiV_RS/BR/2015 1 and 2) reveal a genomic organization common to picornaviruses, with a single large ORF flanked by untranslated regions at both 5′ and 3′ ends, plus a poly (A) tail. The SiV sequence, named RS/BR/2015 1 (GenBank KY069112), is 9253 nt long, with 53.8 % GC content. The predicted large ORF of the genome encodes a putative polyprotein precursor of 2861 aa (314.2 kDa) and shares 85 % aa similarity with SiV A (GenBank NC_023861.1). The second SiV genome identified here, SiV_RS/BR/2015 2 (GenBank KY069113), is 9128 nt in length, with a 53.7 % GC content. A long ORF was predicted, which encodes a 2868 aa (315.2 kDa) putative protein, with 85 % aa similarity to that of SiV A (GenBank NC_023861.1).

The potential protease cleavage sites on the putative polyprotein were mapped by aligning SiV_RS/BR/2015 1 and 2 and other published SiV nucleotide sequences (Fig. 7a). The two SiV genomes reported here share the same layout of predicted cleavage sites and conserved aa motifs as the previously described 2C helicase (GPPGCGKS; DDVGQ), 3C protease (QFKDL; GLCG) and 3D RdRp (KDELR, GGNPSG, YGDD and FLKR) [75, 76].

The $3D^{pol}$ region was used to evaluate the evolutionary relationship between SiV_RS/BR/2015 1 and 2 and other picornaviruses (Fig. 7b). The clustering of SiV_RS/BR/2015 1 and 2 in the phylogenetic tree reveals a close relationship between these two viruses.

In addition to SiVs, other six picornavirus-related sequences were detected, which corresponded to 'chicken megrivirus' (ChMV) and 'chicken proventriculitis virus' (ChPrV), both classified in the genus *Megrivirus* [77]. Two contigs, named ChMV RS/BR/2015 1 and 2 (GenBank KY086293 and KY086292, respectively), showed 96 % to 100 % deduced aa similarity to one of

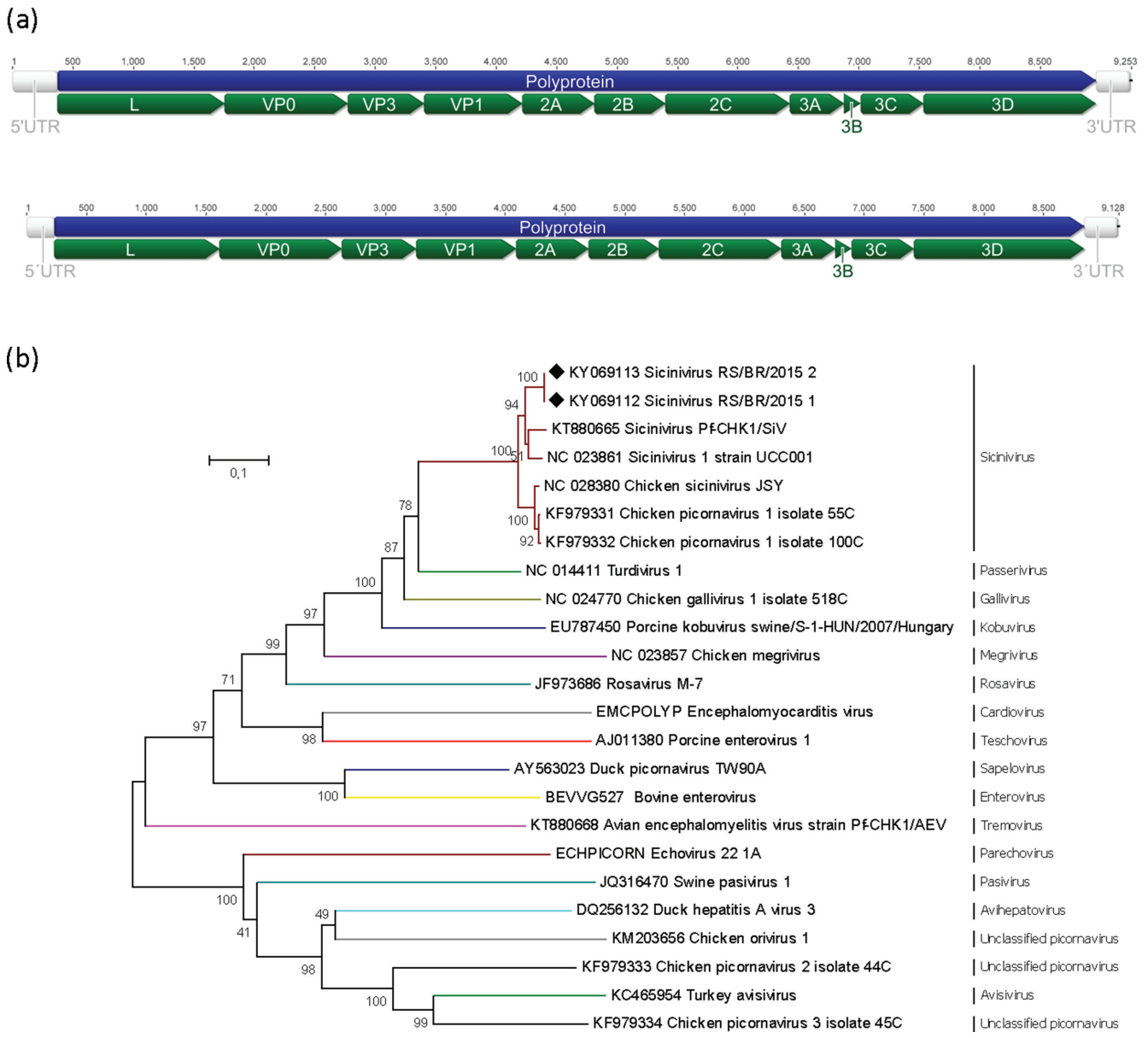

Fig. 7. Putative genome organization and phylogenetic analysis of SiVs RS/BR/2015 1 and 2. (a) Genome organization and predicted cleavage proteins in the polyprotein of SiV RS/BR/2015 1 and 2. (b) Phylogenetic tree generated with basis on the 3D (RdRp-coding) aa sequences, constructed by neighbour joining with 1000 bootstrap replicates. SiV RS/BR/2015 1 and 2 identified in this study are labelled with black diamonds.

the previously published ChMVs (GenBank KF961186). Another four contigs (ChPrV RS/BR/2015 1 to 4, GenBank KY086294–KY086297) shared the highest aa similarity (86–100 %) with ChPrV Korea/03 (GenBank KJ690629) (Fig. 8a). Phylogenetic trees based on the P1 and P2 coding regions, which encode the capsid protein and NS proteins, confirmed that the sequences identified in the current study are closely related to members of the genus *Megrivirus* (Fig. 8b, c).

The wide diversity of picornaviruses in faeces of healthy chickens identified in current study is strongly suggestive of the possibility of recombination events between viruses [74, 78]. However, this remains to be investigated in the future.

Rotaviruses

Rotaviruses (RVs) are non-enveloped viruses classified in the genus *Rotavirus* of the family *Reoviridae* [79]. The RV genome is composed of 11 segments of dsRNA which encodes six structural proteins (VP1–4, VP6 and VP7) and six NS proteins (NSP1–6) [80]. Based on the antigenic characteristics of VP6 protein, RVs have been subdivided into eight serological species (A–H) [81]. In this study, 43 RV-related contigs were identified, ranging in size from 231 to 1589 nt. Among these, 22 shared a high aa similarity (94–100 %) to group D RVs (accession no. KY069068–KY069089), whereas 21 contigs showed 93 % to 100 % identity with group A RVs (accession no. KY069090–KY069110). Phylogenetic analyses based on partial VP6

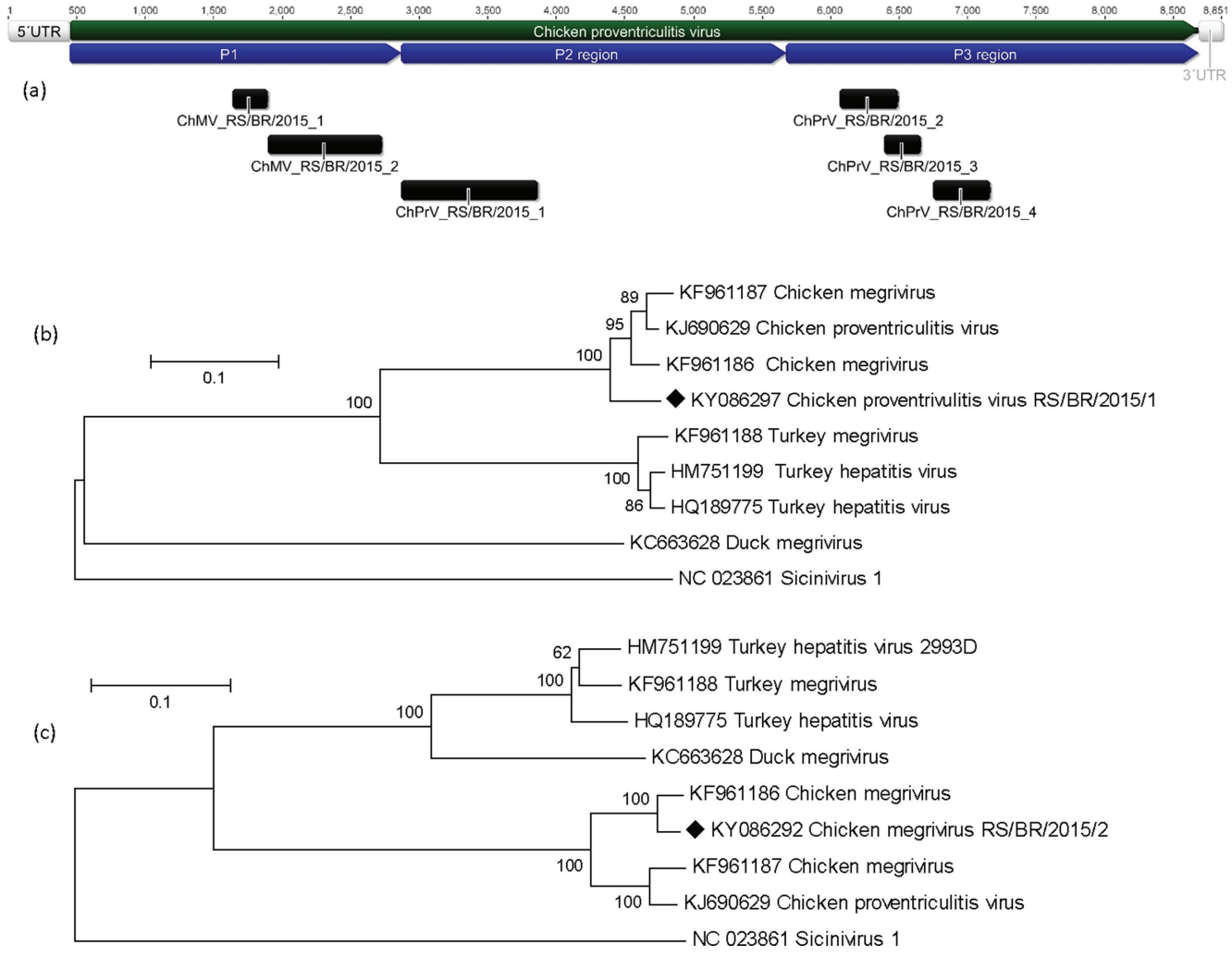

Fig. 8. Phylogenetic analysis of chicken megriviruses. (a) Schematic representation of the genome of megriviruses (ChMV) using chicken proventriculitis virus (ChPrV) as model (~8.8 kb-KJ690629). Black bars represent the contigs identified in the present study. (b) Phylogenetic tree based on the deduced aa sequences of contig ChMV_RS/BR/2015_2 (in a). (c) Phylogenetic tree based on deduced aa sequences of contig ChPrV_RS/BR/2015_1 (in a). Neighbour-joining analyses were performed with 1000 bootstrap replicates. Sequences identified in this study are highlighted with black diamonds.

deduced aa sequences confirmed the putative serogrouping of such viral genomes within groups A and D (Fig. S3).

RVs have been associated to severe diarrhoea in young animals in various species [82]. Group A RVs have often been detected in chickens, humans and other mammals, whereas RV-D are only detected in avian species [83]. Zoonotic transmission, as well as possible recombination/reassortment events, has been demonstrated between human and animal RVs [84, 85]. Infections with distinct groups of RVs, as identified in this study, might represent potential sources for recombination/reassortment events that may contribute to the emergence of additional viral genotypes.

Concluding remarks

The ability to characterize the complexity of the gut microbiome of different species has deeply improved over the past decade, thanks to advances in high-throughput sequencing. By utilizing culture-independent methods, metagenomics has provided relevant information about the composition and diversity of the viral communities in the hosts. In this study, metagenomic analyses of faecal samples of healthy, commercially reared, export-ready chickens revealed a virome constituted by a wide range of viruses representative of known families, as well as a number of novel viruses, most of them with small circular DNA genomes. In this study, the brief description of the genomes of viruses encountered in the faecal virome of healthy chickens presented here is expected to provide a baseline for future studies comparing viral populations of healthy and diseased flocks.

METHODS

Faecal samples

Intestinal contents were collected in four commercial chicken-producing farms in the state of Rio Grande do Sul, Brazil. Birds were produced under standard rearing conditions in commercial flocks, following the protocols of biosafety conditions usually used for export quality flocks. During the year 2015 (May to October), faecal samples were collected from five healthy chickens per house, from birds whose age ranged from 3 to 5 weeks old. Faecal contents were obtained directly from the intestinal tract of euthanized animals and frozen at −80 °C until processing. All procedures were approved by the Commission of Ethics on Animal Use of the Veterinary Research Institute Desidério Finamor (CEUA – IPVDF, no. 21/2014).

Viral nucleic acid purification and Illumina sequencing

Samples were pooled, resuspended in 10 volumes of PBS (pH 7.2) and vigorously vortexed for 5 min. Subsequently, they were centrifuged at 3000 ***g*** for 30 min at 4 °C and filtered through a 0.45 µm filter (Millipore) to remove bacterial cell-sized particles and other particulate debris. The supernatants were centrifuged on a 25 % sucrose cushion at 150 000 ***g*** for 4.5 h at 4 °C (in a Sorvall AH629 rotor), and the pellet containing viral particles was treated with a mixture of 2 µl DNase (2 U μl^{-1}, Turbo DNase; Ambion), 5 µl RNase A (20 mg ml^{-1}; Invitrogen) and 0.5 µl benzonase (25 U μl^{-1}; Novagen) at 37 °C for 2 h to digest susceptible nucleic acids [12]. Viral DNA was then extracted using a standard phenol–chloroform protocol [86]. Viral RNA was extracted with TRIzol LS (Ambion) following manufacturer's instructions.

Viral DNA was enriched by multiple displacement amplification using φ29 DNA polymerase [87]. Viral RNA was converted in cDNA libraries using a SeqPlex RNA amplification kit (Sigma Aldrich) [88]. The DNA products resulting from such enrichment protocols were pooled in equimolar amounts and purified using a Genomic DNA clean and concentrator (Zymo Research). The quality and quantity of the DNA were assessed by spectrophotometry (L-Quant; Loccus Biotechnology) and fluorometry (Qubit; Invitrogen), respectively. The DNA libraries were further prepared with 50 ng of purified MDA/SEqPlex DNA using a Nextera DNA sample preparation kit and sequenced using an Illumina MiSeq instrument (2×150 paired-end reads with the Illumina v2 reagent kit).

Data assembly and processing

The quality of generated sequences was evaluated using FastQC. The sequences with bases having a Phred quality score <20 were trimmed with the aid of Geneious software (version 8.1.7). The paired-end sequence reads were assembled into contigs with SPAdes 3.5 [89]. All assemblies were confirmed by mapping reads to contigs generated by SPAdes using Geneious version 8.1.7 software. The assembled contigs and singlet sequences were examined in search for similarities to known sequences with BLASTX software. Sequences with E-values of $\leq 10^{-3}$ were classified as likely originating from a eukaryotic virus, bacteria, phages, eukaryote or unknown, based on the taxonomic origin of the sequence with the best E-value. ORF predictions and genome annotations of the complete near-full-length genomes were performed with the aid of Geneious software. Gene and protein comparisons were performed with BLASTN and BLASTP programmes.

Reconstruction of phylogenetic trees

Sequences representative of known adeno, calici, circo, CRESS ssDNA, gyro, parvo, picorna, picobirna and RVs were obtained from GenBank and aligned with the sequences identified in the present study with CLUSTALX software version 2.0 [90]. Phylogenetic trees were generated by the neighbour-joining method with a p-distance model. The confidence levels of the tree branch nodes were obtained by analysis of 1000 bootstrap replicates. Analyses were made on MEGA6 [91]. The GenBank accession numbers of the viral sequences used in the phylogenetic analyses are shown on tree figures.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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