

Genetic diversity of pigeon circovirus in Hungary

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Abstract Pigeon circoviruses (PiCV) had been identified worldwide and are responsible for immune suppression and a variety of diseases collectively referred to as young pigeon disease syndrome. Samples from racing pigeons were collected throughout Hungary and analyzed for the presence of PiCV by polymerase chain reaction. The capsid protein coding gene was amplified from ten PiCVs of different origins, and compared with known PiCV sequences. The results indicated that PiCV was highly variable, the viruses formed five distinct genetic groups. Differences of the 3' end of the gene suggested the possibility of genetic recombination among these groups.

Keywords Pigeon circovirus · Phylogenetic analysis · Capsid gene

Pigeon circovirus (PiCV) belongs to the *Circovirus* genus of the *Circoviridae* family comprising of small non-enveloped viruses with single stranded circular DNA genomes of 1.7–2.5 kilobases (kb). The average PiCV genome size is 2.0 kb with minor variations in length among different

isolates. Viral genomes within the *Circovirus* genus are characterized by two major open reading frames (ORF), namely ORF1, encoding the replication associated protein (Rep), and ORF2 in opposite orientation, coding for the only structural protein of the virion—the capsid protein (Cap). PiCV is responsible for a complex and variable syndrome affecting mostly young pigeons, aptly named young pigeon disease syndrome (YPDS) [1]. The disease is generally characterized by a wide range of clinical signs, such as lethargy, poor race performance, weight loss, respiratory diseases, diarrhoea, and other non-characteristic findings [2]. According to histopathology examinations, lymphocyte depletion can be observed in the primary and secondary lymphoid tissues, a common feature of circovirus infections, suggesting that PiCV affected birds are immune suppressed. Secondary infections because of the limited immune responses are common which is a major feature of PiCV infection and greatly determines the clinical signs and pathological lesions.

Circovirus infection of pigeons was first described in the USA in 1993 [3], but according to a subsequent retrospective study the infection and YPDS had already been present in pigeon flocks earlier [2]. By now, PiCV infection has been identified in diseased pigeons worldwide: South Africa [4], Australia and Canada [2], Northern Ireland [5], England [6], Germany [7], Italy [8], France [9], Belgium [10], China [11], and Taiwan [12]. Aside from a single report of PiCV in the Czech Republic [13], the presence of the virus in Central or Eastern Europe, or sequences from these regions have not been reported yet. Here we report a survey of the incidence of PiCV in Hungarian pigeon flocks, together with the phylogenetic analysis of ORF2 of the newly identified and the published PiCV sequences.

Organ tissues (mostly livers and spleens) and blood serum samples were collected from 116 healthy pigeons of

Nucleotide sequence data reported in this article are available in the GenBank database under the accession numbers: JF330089–JF330098.

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different ages in 16 racing pigeon flocks (5–10 birds/flock) of different regions in Hungary during the year 2010. Tissues were homogenized as described previously [14] and the viral DNA from tissue homogenates and sera was extracted with the Viral Gene-spin™ DNA/RNA Extraction Kit (Intron Biotechnology Inc., Korea) as recommended by the manufacturer. For the primary detection of PiCV DNA, a primer pair (PiCF 5'-CGCGCGAGACTT CAGTGAGA-3' and PiCR 5'-TGTTGATCCGCCGGAAGAG-3') specific for a conserved region of ORF1 of known PiCVs (between 500 and 920 bases of the genome) was designed using the Primer3 program [15]. Polymerase chain reaction (PCR) was carried out as a regular amplification procedure in 50 µl reaction volume containing 2 µl of purified sample DNA, 5 µl GreenTaq buffer (Fermentas, Lithuania), 1 µl dNTP (1 nmol), 0.5 µl of each primer (25 pmol), and 1 unit of the DreamTaq Green DNA polymerase (Fermentas, Lithuania). The PCR conditions were: preheating at 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by a final extension step of 72°C for 7 min. The amplicons were detected by agarose-gel electrophoresis.

Fifteen out of the 16 flocks, altogether 66 out of the 116 tested pigeons were positive for PiCV, suggesting that the virus was prevalent in Hungary. The 420 bp ORF1 amplicons were sequenced and compared with each other. The analysis showed that this area of the genome was conserved, with only limited sequence variations. The positive samples were grouped according to their origins and ORF1 sequence data. Based on these, ten different groups of viruses of identical characteristics could be established. Complete ORF2 amplicons were generated for PiCV representative strains of these groups using the primer pair PiCVcapF (5'-GGAATTC-AGCGACCGCAATGC-3', *EcoRI* restriction site underlined) and PiCVcapR (5'-CGCGGATCC-ATGAGGAGGCGGAGATTC-3', *BamHI* restriction site underlined). The PCR conditions were as described previously in the study, but the elongation step was extended to 90 s. The amplicons were cloned using the pBacPak9 plasmid (Clontech, USA) linearized with *BamHI* and *EcoRI* restriction endonucleases (Fermentas, Lithuania). Purified DNA, two distinct clones for each amplicon, were sequenced (Macrogen Inc., Korea) with the BAC1 (5'-AACCATCTCGCAAATAAATA-3') and BAC2 (5'-ACGCACAGAATCTAGCGCTT-3') sequencing primers. The lengths of the ORF2 sequences were either 813 or 822 nucleotides including the stop codons (Fig. 2). Putative Cap proteins were predicted by the MEGA 5 software [16] and aligned using the BioEdit software [17]. The sizes of the predicted proteins were 270 and 273 amino acids (aa). Among ten complete PiCV ORF2 sequences determined, two of the ORF2 sequences (PiCV-H1 and PiCV-H9), although collected at distant locations, were completely

identical with each other. The sequences of parallel clones of the amplicons were identical, except for one sample in which nucleotide (nt) differences between the two clones were detected at two positions: adenine/cytosine at nt 43 and cytosine/thymine at nt 164. A third clone was also sequenced for this sample and contained cytosine in both positions. But the third clone differed from the other two sequences at nt 194 where thymine was changed to cytosine. The mutation at nt 43 was silent, but the described changes at nt 164 and 194 changed the aa sequence of the putative Cap protein at positions aa 55 (threonine to isoleucine) and aa 65 (phenylalanine to serine), respectively. Although the possibility of a mixed infection could not be ruled out, as sequence diversity was identified in multiple clones, they are unlikely caused purely by PCR amplification, the genetic diversity of PiCV population in that flock could also be generated in vivo by an unknown mechanism. For the three clones in this case, only the consensus sequence was registered in GenBank and included in the phylogenetic tree. The ten Hungarian PiCV ORF2 sequences were compared with the 15 complete PiCV ORF2 sequences available in the GenBank database. Two partial sequences from the Czech Republic also in GenBank were omitted from the phylogenetic analysis of complete ORF2s, but were used for general comparisons with the matching portions of full ORF2s. The BioEdit software [17] was used for the generation of sequence identity matrices. Phylogenetic tree was constructed with the neighbor-joining method of the MEGA 5 software [16] using 1,000 bootstrap values. According to the phylogenetic tree (Fig. 1) and the sequence identity values (Table 1), PiCVs formed five distinct genetic groups (A–E). In an earlier study, Todd et al. [18] defined two main clusters of PiCV although with a lower number (12) of sequences included in the study. Based on the origin of PiCV sequences, group A of the current study contained European, North-American, and Asian viruses. The two partial PiCV sequences from the Czech Republic also belonged to this group. Groups B and C were only comprised of European PiCVs, whereas group D contained five of the PiCVs of this study together with sequences identified in China. A small but markedly distinct group (E) was formed by only two viruses originating from North-America and Australia. The nucleotide and amino acid identity values within and among the groups are shown in Table 1.

The identity among the groups was between 73.5 and 84.3% based on the nt sequences and 71.6–86.9% according to the aa sequences. Within the groups the identity was higher than 92% (nt) and 96% (aa), except for group C where the aa identity was lower (94.8%), and group E where both the nt and the aa identities were below 90%. These values are somewhat lower than those of other findings [11, 18]. It seems likely that groups C and E will

Fig. 1 Phylogenetic grouping of ORF2 nucleotide sequences of PiCVs. The viruses are represented by the name of the country of detection and the GenBank code. The Hungarian viruses are indicated in *bold*. The optimal tree with the sum of branch length = 0.97096328 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches

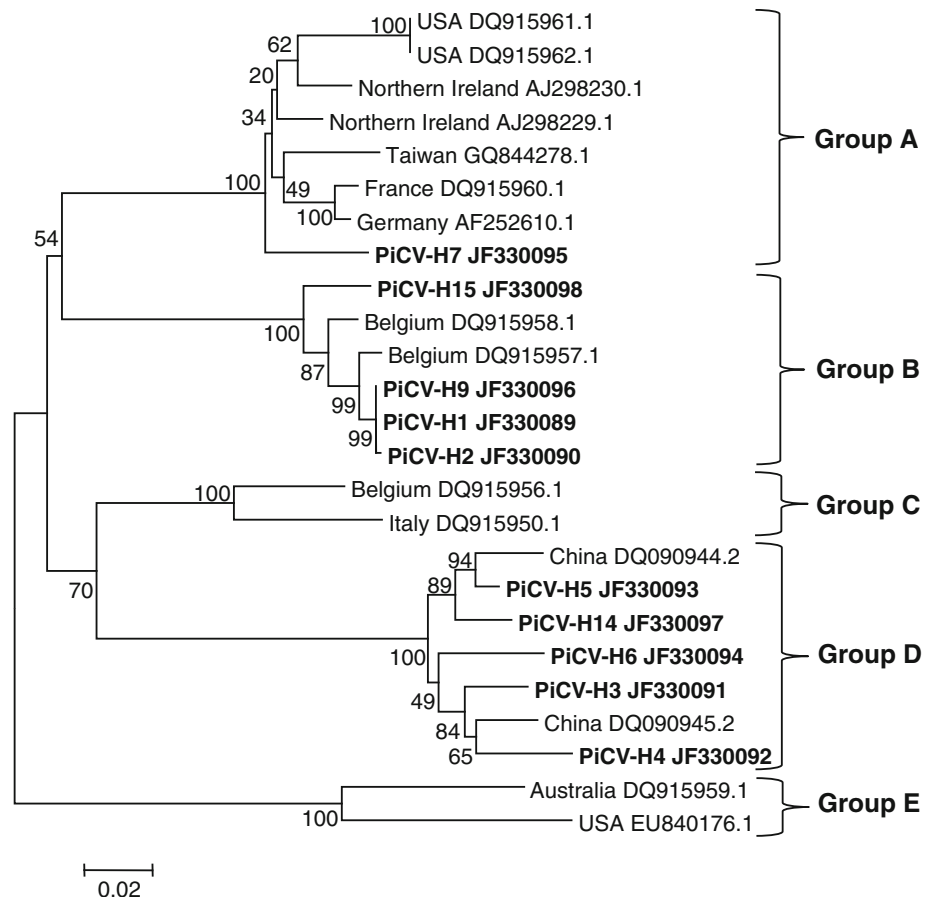


Table 1 Identity values within and among the established PiCV genetic groups

Groups	A (%)	B (%)	C (%)	D (%)	E (%)
A	92.8–100 ^a 97.0–100 ^b	81.9–84.3 ^a	79.3–83.9 ^a	76.2–79.4 ^a	75.5–77.3 ^a
B	83.8–85.3 ^b	96.0–100 ^a 97.4–100 ^b	80.1–82.2 ^a	77.8–79.7 ^a	75.4–78.0 ^a
C	82.6–86.9 ^b	80.9–83.9 ^b	92.4 ^a 94.8 ^b	79.2–81.8 ^a	74.2–75.9 ^a
D	76.8–79.7 ^b	76.6–78.4 ^b	80.3–83.2 ^b	93.1–97.3 ^a 96.7–99.6 ^b	73.5–76.3 ^a
E	74.1–77.0 ^b	75.0–77.2 ^b	73.0–76.3 ^b	71.6–72.7 ^b	88.4 ^a 89.7 ^b

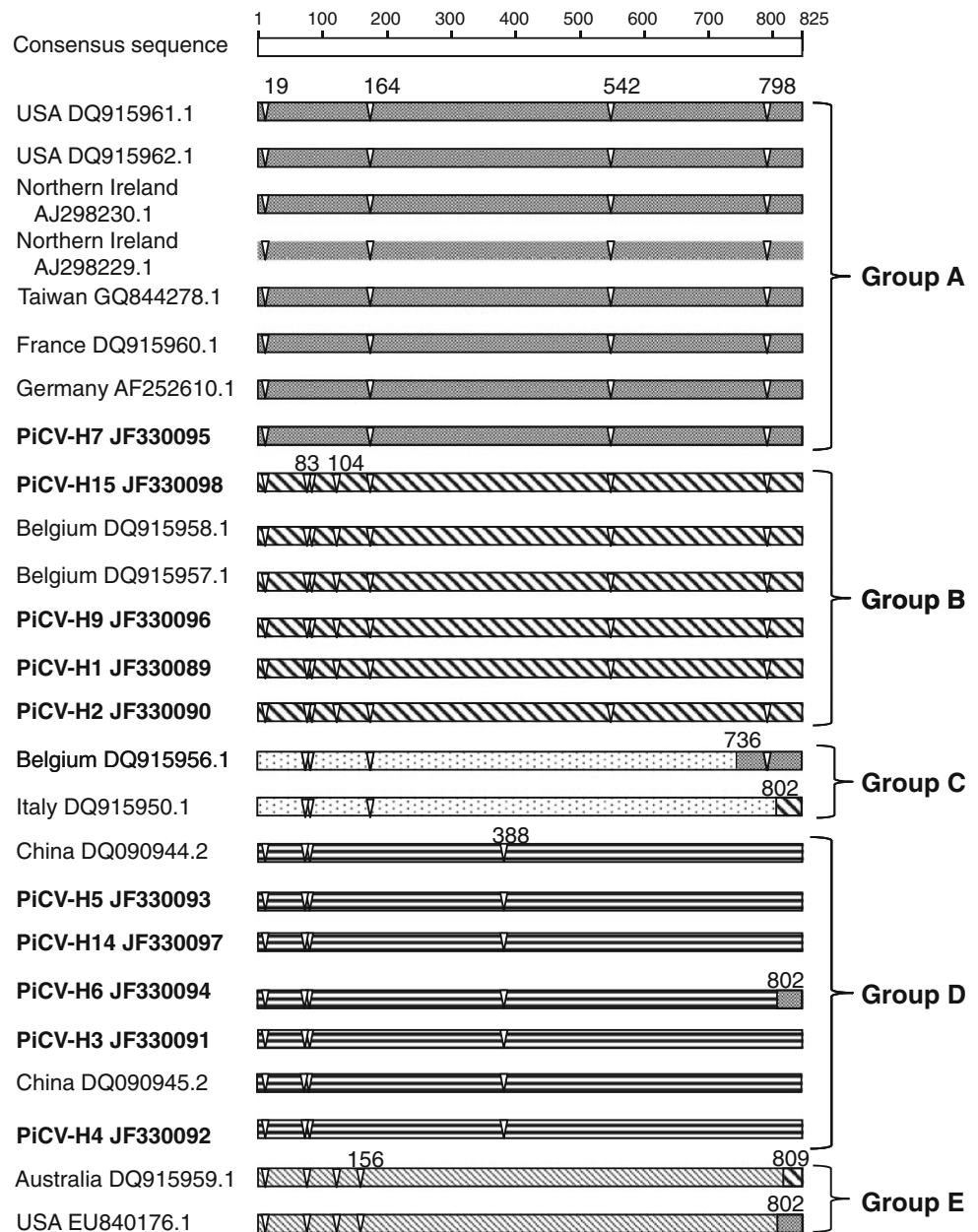
^a Nucleotide identity

^b Amino acid identity

be divided into further genetic groups or subgroups if more sequences become available, as the sequences in these groups showed higher variability than the viruses of the other three groups. The sequence alignment also indicates that the 3' end of ORF2 of the two group C viruses were different from each other (Fig. 2). The corresponding end of the PiCV ORF2 sequence from Italy [19] was similar to that of group B viruses, but in case of the PiCV identified in Belgium [20] it was similar to group A sequences. Such differences could also be observed for the 3' end of ORF2 in group E viruses, where the sequence from

North-America was similar to group A 3' ends, whereas this area of the PiCV from Australia resembled group B viruses and the group C sequence from Italy. Change of the end portion of a group D virus (PiCV-H6) ORF2 to group A sequence was also detected. These observations suggest the possibility of recombinations among different PiCVs, similarly to those described for porcine circoviruses [14, 21, 22]. Also, based on analogy with porcine circovirus type 2, where the carboxy-terminal end of the Cap protein is important for antibody induction [23], the variability of the given region in PiCV ORF2 affect the

Fig. 2 Size and nucleotide sequence differences of PiCV ORF2 genes. Viruses are labeled as in Fig. 1. Bars indicating the Cap genes are filled according to the phylogenetic group they occupy. The upper bar represents the consensus of the known PiCV ORF2 sequences; nucleotides are numbered from 1 to 825. Empty arrowheads indicate the location of deleted (compared to the consensus sequence) nucleotide triplets. The result of possible recombination events of the 3' end in members of groups C–E are shown by the shading corresponding to the donor sequences



interactions between the virus and host immune system. The majority of the differences in the nucleotide and predicted amino acid sequences of the PiCV ORF2s were located near the amino-terminal end where deletions (or additions) of triplets of nucleotides were detected, and the mosaic structures seen at the C-terminal end of ORF2 are probably resulted from recombination events among the groups C–E (Fig. 2). The significance of triplet differences and recombination is unknown but both may have impact on the immune response directed to the capsid protein. The deletions and insertions of nucleotide triplets in ORF2 did not cause frame shift mutations in amino acid sequence, but may affect the Cap protein structure which has yet to be determined.

Pigeon circovirus infections are not among the ones of major economic importance like paramyxovirus and herpesvirus infections [24], but considering the high value of the individual racing pigeons and the losses during races (up to 90–100%) due to the infection, they may have serious impact on this sport and its traditions. The most obvious way of YPDS prevention and of improving the quality of the affected flocks would be the introduction of PiCV vaccination, but the vaccine efficacy based on these findings might be influenced by the presence of divergent viruses.

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