EXISTENCE OF GENE 5 INDICATES CLOSE GENOMIC RELATIONSHIP OF TURKEY CORONAVIRUS TO INFECTIOUS BRONCHITIS VIRUS

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Summary. – A segment of genomic RNA extending from the 3′-end of the membrane (M) protein gene to the 5'-end of the nucleocapsid (N) protein gene of Turkey coronavirus (TCV) was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The primers were derived from the corresponding sequences of Infectious bronchitis virus (IBV). The PCR products were cloned and sequenced and their nucleic acid structure and similarity to the published sequences of IBV were analyzed. Gene 5 containing two overlapping open reading frames (ORFs), 5a and 5b, was localized between M and N genes of TCV. The overall nucleotide sequences of the amplified regions from TCV isolates shared 88.4% to 91.8% similarity to the corresponding region of IBV strains. The consensus transcription-associated sequence of IBV, CTTAACAA, was highly conserved in the TCV genome with regard to nucleotide sequence and location in terms of the initiation codons of the genes 5 and N. The similarities between the predicted amino acid sequences of ORFs 5a and 5b of TCV isolates and the homologous genes of IBV strains were 85.4% to 94.0%. The results indicate the existence of gene 5 in the genome of TCV and a close relatedness of the TCV gene 5 to the IBV gene 5 in location and nucleotide sequence.

Key words: avian infectious bronchitis virus; turkey coronavirus; genomic relatedness, gene 5

Introduction

Outbreaks of turkey enteritis associated with TCV (Turkey coronavirus, species *Turkey coronavirus*, genus *Coronavirus*, family *Coronaviridae*) have caused serious economical losses in the turkey industry in Indiana, North Carolina and other states in the USA for the last several years. Although the economical importance of this disease has been recognized for decades (Nagaraja and Pomeroy, 1997), the structure and organization of TCV genome remains poorly determined. Nevertheless, development of strategies to prevent, control, and treat the disease requires in-depth understanding of the molecular biology of TCV.

Coronaviruses (members of the family *Coronaviridae*) are pleiomorphic enveloped spherical particles surrounded by a fringe of 20-nm long club-shaped spikes. The diameter of a coronaviral particle varies from 50 nm to 150 nm. The coronavirus genome is a positive single-stranded capped RNA with a polyadenylated 3′-end.

Complete sequence of the genomic RNA has been determined for IBV (Infectious bronchitis virus, species *Infectious bronchitis virus*, genus *Coronavirus*, family *Coronaviridae*) (27,569 nucleotides; Boursnell *et al.*, 1987), Murine hepatitis virus (MHV, species *Murine hepatitis virus*, genus *Coronavirus*) (31,092 nucleotides; Lee *et al.*, 1991),

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Abbreviations: BCV = Bovine coronavirus; cDNA = complementary DNA; DEPC = diethylpyrocarbonate; EDTA = ethylenediamine tetraacetic acid; FIPV = Feline infectious peritonitis virus; HCV = Human coronavirus; IBV = Infectious bronchitis virus; M = membrane; MHV = Murine hepatitis virus; N = nucleocapsid; ORF = open reading frame; PCR = polymerase chain reaction; S = spike; TAS = transcription-associated sequence; RT = reverse transcription, reverse transcriptase; TBE = Tris-borate-EDTA; TCV = Turkey coronavirus; TGEV = Transmissible gastroenteritis virus

Table 2. Sequence pair distances for coding region of ORF 5a amino acids of three TCV isolates and three IBV strains

Viruses	Percent identity/Percent divergence					
	540[a]	517[b]	404[c]	CU[d]	KB[e]	Beaud[f]
540[a]	–	100.0	88.1	94.0	91.0	88.1
517[b]	0.0	–	88.1	94.0	91.0	88.1
404[c]	13.0	13.0	–	88.1	92.5	88.1
CU[d]	6.2	6.2	13.0	–	91.0	88.1
KB[e]	9.6	9.6	7.9	9.6	–	91.0
Beaud[f]	13.0	13.0	13.0	13.0	9.6	–

[a]540 = TCV isolate from Indiana, USA.
[b]517 = TCV isolate from Indiana, USA.
[c]404 = TCV isolate from Virginia, USA.
[d]CU = CU-T2, a German strain of IBV. GenBank Acc. No. U49858.
[e]KB = KB8523, a Japanese strain of IBV. GenBank Acc. No. M21515.
[f]Beaud = Beaudette, a US strain of IBV. GenBank Acc. No. AJ311317.

Table 3. Sequence pair distances for coding region of ORF 5b amino acids of three TCV isolates and three IBV strains

Viruses	Percent identity/Percent divergence					
	540[a]	517[b]	404[c]	CU[d]	KB[e]	beaud[f]
540[a]	–	98.8	87.8	86.6	85.4	85.4
517[b]	1.2	–	89.0	87.8	86.6	86 6
404[c]	13.3	11.9	–	90.5	90.5	88.1
CU[d]	14.8	13.3	10.2	–	96.4	94.0
KB[e]	16.3	14.8	10.2	3.7	–	95.2
Beaud[f]	16.3	14.8	13.0	6.2	4.9	–

[a]540 = TCV isolate from Indiana, USA.
[b]517 = TCV isolate from Indiana, USA
[c]404 = TCV isolate from Virginia, USA.
[d]CU = CU-T2, a German strain of IBV. GenBank Acc. No. U49858.
[e]KB = KB8523, a Japanese strain of IBV. GenBank Acc. No M21515.
[f]Beaud = Beaudette, a US strain of IBV. GenBank Acc. No. AJ311317.

comparison of sequence distances for ORFs 5a and 5b is summarized in Tables 2 and 3, respectively. The similarity scores between the TCV isolates and IBV strains within the ORF 5a amino acid sequence ranged from 88.1% to 100%. The two Indiana isolates of TCV (540 and 517) shared the highest similarity (100%). The similarity scores between the Virginia isolate (404) of TCV and the two remaining TCV isolates and the IBV strains CU and Beaud were 88.1%, while that between the isolate 404 and the IBV strain KB8523 was 92.5 %. The similarity scores between the IBV strain Beaud and the IBV strain CU.

The similarity score between the TCV isolates and IBV strains tested within 5b amino acid sequence ranged from 85.4% to 98.8%. The two Indiana isolates of TCV (540 and 517) shared the highest similarity (98.8%). The similarity scores between the Virginia isolate (404) of TCV and the isolates 540 and 517 were 87.8% and 89.0%, respectively. The lowest similarity was observed between the TCV isolate 540 and IBV strains KB8523 and Beaud (85.4%). The similarity scores among IBV strains ranged from 94.0% (CU-T2 and Beaud) to 96.4% (CU-T2 and KB8523).

Phylogenetic analysis

The phylogenetic relationship among nucleotide sequences in the amplified regions of TCV isolates 540, 517, and 404 and IBV strains CU-T2, KB8523, and Beaud is shown in Fig. 4. The phylogenetic analysis indicated that the TCV isolates may be grouped within the same genomic lineage and the IBV strains within a separate cluster.

Discussion

The present study demonstrated the existence of gene 5 in TCV genome. The location of gene 5 between the 3′-end of M gene and the 5′-end of N gene and the structure of gene 5 containing two overlapping ORFs, 5a and 5b, were consistent with that of IBV. In addition, the nucleotide sequences of the amplified fragments of gene 5 within M and N genes and amino acid sequences encoded by ORFs 5a and 5b had high similarity to the corresponding sequences of IBV. These results extend our previous findings (Akin *et al.*, 2001) that two avian coronaviruses, TCV and IBV, are highly related genetically at the N gene and gene 5 level.

The presence of gene 5 between the 3′-end of M gene and the 5′-end of N gene is a unique feature of avian coronaviruses, TCV and IBV, and is not found in any other mammalian coronaviruses as determined to date. There is only a small stretch of non-coding nucleotide sequences between the 3′-end of M gene and the 5′-end of N gene of other coronaviruses: 9 nucleotides for BCV(GenBank Acc. No. AF058943) and HCV (GenBank Acc. No. AF304460), 12 nucleotides for TGEV (GenBank Acc. No. AF104420), 12 nucleotides for Feline infectious peritonitis virus (FIPV, species *Feline coronavirus*, genus *Coronavirus*) (GenBank Acc. No. A22378), and 14 nucleotides for MHV (GenBank Acc. No. J02252). This distinct feature of genome structure of TCV implied that TCV has a relatively close evolutionary relationship with IBV.

One of the characteristic features of coronavirus replication is the synthesis of a 3′-coterminal nested set of polycistronic subgenomic mRNAs by a discontinuous transcription mechanism. Several conserved TAS have been identified for different coronaviruses proximal to the

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540     CAGTGGCTTGCTAAGTGTGAACCAGACCACTTGCCTAAAGATATATTTGT   50
517     ..................................................   50
404     .......................T.....G...G.GGTG...CC.G....   50
CU      ..................................................   50
KB      .........................................C........   50
Beaud   ..................................................   50

540     GTGCACACCGGATAGACGTAATATCTATCGTATGGTGCAGAAATACACTG  100
517     ..................................................  100
404     ..C...T...C......C.C..G.A.G..T....................  100
CU      T..T.......................C.................T....  100
KB      T......G..........C..C.....C......................  100
Beaud   T..T.......................C.................T....  100

540     GTGATCAAAGCGGAAATAAGAAAAGGTTTGCTACATTTGTCTATGCAAAG  150
517     ..................................................  150
404     ....C.............................G.....A.........  150
CU      ....C..................C.T........G...............  150
KB      ....C............................................A  150
Beaud   ....C.............................G...............  150

540     CAGTCAGTAGACACCGGCGAGCTAGAAAGTGTAGCAACGTCTGGAAGTAG  200
517     ..............T...................................  200
404     ...G..........T.......................AGGA........  200
CU      ...........T..T.......................AGGA..G.....  200
KB      .....G........T.......................AGGA........  200
Beaud   ...........T..T.......................AGGA........  200

540     CCTTTACACATAAATGTGTGTGTGTAGAGAGTATTTAAAATTATTCTTCA  250
517     ..................................................  250
404     .............................G....................  250
CU      T...............................................T.  250
KB      T...............................................T.  250
Beaud   T...............................................T.  250

540     ATAGTGCCTCTATTTTAAGAGCGCGGAAGAGTATTTGTTTTGAGGATATT  300
517     ..................................................  300
404     .......T..........................................  300
CU      ........................AT..T......AT.............  300
KB      G..........G.........T..AT.........AT.............  300
Beaud   ....C......G............AT..........A...........C.  300

540     AATATAAATCCTTTTTGTTTTGCACTCTCTTTGCAAGAGTTATTATTTAA  350
517     ..................................................  350
404     ............C....................G................  350
CU      ............-.C......AT.........T......C..........  349
KB      ............C.......CAT.........T.................  350
Beaud   ............C........AT......C..T......C.....-----  345

540     GCAACAGTTTTTCCTTTCTTTTGTTTGGAAGAAAGTTGTTGTTAATGGTG  400
517     ...............................................A..  400
404     .......G.......C................................C.  400
CU      AA............AC........GCCA..A.CT................  399
KB      AA............AC........GCCA..A.CT................  400
Beaud   .............................................C....  351
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Fig. 2

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540     TAGAATTCCAAGTAGAAAATGGAAAAGTCCACT- ACGAAGGAAATCCAAT 449
517     . . . . . - . . . . . 449
404     . . . . . - . . . . . C. . CG. 449
CU      . . ACC. . T. . . . . T. . . . . T. . . T. . . . . CA. . . G. 449
KB      . . ACC. . T. . . . . T. . . . . T. . . - . . . . . A. . . . . 449
Beaud   . TACC. . T. . . . . T. . . . . T. . . - . . . . . CA. . . G. 400

540     CTTCCAAAAAGGTTGTTGTAGTTTGTGGTCCGATTATAAGAAAGATTAGA 499
517     . . . . . T. . . . . 499
404     T. . T. . . . . A. C. . . . . C. . . . . G. . . . . A. 499
CU      T. . . . . AA. . . . . A. . . . . 499
KB      . . . . . A. . . . . 499
Beaud   T. . . . . GA. . . . . A. . . . . - - - - - - 445

540     ATAATTAAGCCACCAACTACACTTATTTTTAAAAAGGGCGTTTTAGCTTA 549
517     . . . . . 549
404     TGG. . . . . A. . . . . T. . ACT. . . . . C. . G. . T. . G. . . . . G. . . . . 549
CU      . . . . . T. . . . T. . . . . T. . GA. . . . . TT. . . 549
KB      . . . G. . . . . T. . . . . T. . GA. . . . . TT. . . 549
Beaud   . . . . . G. A. . . . . T. . . . . T. . GA. . T. . . . . CT. A. 495

                    5a
540     TAAATGCTTAACAAATACGGACGATGAAATGGCTGACTAGTTTTGGAAGA 599
517     . . . . . 599
404     C. . . AA. . . . . 599
CU      C. . . C. . . . . T. . . . . 599
KB      C. . . C. . . . . C. . . . . 599
Beaud   C. . . AA. . . . . 545

540     GCGGTTGTTTCATGTTACAAAGCACTACTATTAACTCAACTTAGAGTGTT 649
517     . . . . . 649
404     T. A. . . A. . . . G. . C. . T. . . . C. . . . . T. A. . . . . 649
CU      . . A. . . A. . . . . T. . . . C. . . . . T. A. . . . . 649
KB      . . A. . . A. . . . . T. . . . C. . . . . T. A. . . . . 649
Beaud   . . A. . . A. . . . T. . . . T. . . T. C. . . . . 595

540     AGATAGGTTAATTTTAGATCACGGACCAAAACGCGTTTTAACGTGTAGTA 699
517     . . . . . 699
404     . . . . . C. . . . . G. . . AC. . . . . GC. . 699
CU      . . . . . C. . . . . 699
KB      . . . . . C. . . . . G. . . 699
Beaud   . . . . . T. CT. . . . . 645

540     GGCGAGTGCTTTTGTTTCAGTTAGATTTAGTTTATAGGTTGGCGTTTACG 749
517     . . . . . 749
404     . . . . . AG. . . . A. . . . . GA. . . . . A. A. . . . 749
CU      . . . . . C. C. . . . . 749
KB      . A. . . . . C. AG. G. . A. . . . . C. . . . . A. . . . 749
Beaud   . . . . C. . . . . AG. . . . A. . . . . A. . . . 695

                    5b
540     CCCACCCAACCGCTGGTATGAATAA- - - - - - AGATAATCCTTTTCGCAGA 793
517     . . . . . - - - - - - . . . . . 793
404     . . . . . T. . . . . TAGTAA. . C. . . . . 799
CU      . . . . . T. . . . . TAGTAA. . . . . G. . 799
KB      . . . . . T. . . T. . . . . TAGTAA. . . . . G. . 799
Beaud   . . . . . T. . . . . C. . . . . TAGTAA. . . . . G. . 745
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Fig. 2

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540     GCAATAGCAAGAAAAGCGCGAATTTATCTGAGAGAAGGATTAGATTGTGT   843
517     ..................................................   843
404     ...........................T....G.................   849
CU      .............................T....................   849
KB      .............................T....................   849
Beaud   .................T................................   795

540     TTACTTTCTTAACAAAGCAGGACAAGCAGATCCTTGCCCCGAGTGTACCT   893
517     ..................................................   893
404     ..........G.......G...........G.....T..T.T........   899
CU      ..............................G.....T....C........   899
KB      ..............................G.....T....C........   899
Beaud   ..............................G.....T....C........   845

540     CTCTGGTATTCCGAGGGAAAATTTGTGAGGAACACATAAATAATAATAAT   943
517     ..................................................   943
404     .C..A.......A.........C...............T...........   949
CU      ....A.......A........C............................   949
KB      ....A.......A........C................C.......C...   949
Beaud   ....A.......A........C................C...........   895
                  N gene
540     CTTTTGTCATGGCAAGCGGTAAGGCTACTGGGAAAACAGACGCTCCGGCG   993
517     ...............................A..................   993
404     ...............................A..G........C..A...   999
CU      ...........C.............A.....A..G........C..A...   999
KB      ................T........A.....A..G........C..A...   999
Beaud   ......................A..AG....A...........C..A...   945

540     CCAGTCATCAAACTAGGAGGACCAAAGCCACCTAAAGTTGGTTCTTCTGG  1043
517     ..................................................  1043
404     ..................................................  1049
CU      ..........................A.......................  1049
KB      ..................................................  1049
Beaud   ........T.................A...........C...........   995

540     AAGTGTGTCTTGGTTTCAAGCAATAAAAGCCAAGAAGCTAAATTCACCTC  1093
517     ....................T..................T..........  1093
404     ..A..CA...........................................  1099
CU      ..A..CA...........................................  1099
KB      ..A..CA...........................................  1099
Beaud   ..A..CA..............................T.....A......  1045

540     CGCCCAAGTTTGAAGGTAGCGGT                             1116
517     .......................                             1116
404     .......................                             1122
CU      .A..T..T...............                             1122
KB      .A.TT..............T...                             1122
Beaud   .......................                             1068
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Fig. 2

Nucleotide sequences of the gene 5 region of the PCR amplified fragments of the three TCV isolates and their similarity to those of the three IBV strains

The positions of missing nucleotides are marked by (-), while the positions of identical nucleotides are marked by (.). Putative initiation codons are underlined in bold. Putative stop codons are marked by regular lines drawn above them. The conserved TAS sequence CTTAACAA is boxed. For the abbreviations of virus isolates or strains see Tables 1–3.

(a)

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540     MKWL T S F GRAVVS CYKAL L L T QL R VL DRL I  L DHGP KRVL T   40
517     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   40
404     . . . . . . . . . S . I . . . . . . . . . . . . . . . . . . . . . . . . T . .   40
CU      . . . . . I . . . . . I . . . . . . . . . . . . . . . . . . . . . . . . . . .   40
KB      . . . . . . . R . . . I . . . . . . . . . . . . . . . . . . . . . . . . . . .   40
Beaud   . . . . . . . . . . . I . . . . S . . . . . . . . . . . . . . . . . . L L . . . .   40

540     C S R R V L L F QL DL V Y R L AF T P T QP L V.          65
517     . . . . . . . . . . . . . . . . . . . . . . . . .       65
404     . A. . . . . V. . . . I . . . . Y. . . . S . . .        65
CU      . . . . . . . . . . . . . . S . . . . . . . . S . . .   65
KB      . G. . . . . V. . . . . . . . . . Y. . . . S . . .      65
Beaud   . . . . . . . V. . . . . . . . . . Y. . . . S . A.      65
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(b)

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540     MN- - KDNP F RRAI  ARKARI  YL RE GL DCVYF L NKAGQADP CP E   40
517     . . - - . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   40
404     . . NS . A. . . . . . . . . . . . . . . . . . . . . . . D. . . . . E. . . V   42
CU      . . NS . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . E. . . A   42
KB      . . NS . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . E. . . A   42
Beaud   . . NS . . . . . . G. . . . . . . . . . . . . . . . . . . . . . . . . E. . . A   42

540     C T S L VF RGKI  CEEHI  NNNNL L S WQAVRL L GKQT L RRQS S N.   80
517     . . . . . . . . . . . . . . . . . . . . . . . . . . . . E. . . . . . . . . . .   80
404     . . . . . . Q. . . . . . . . Y. . . . . . . . . . . . . ER. . PQ. . . . . .   82
CU      . . . . . . Q. . T. . . . . . . . . . . . C. . . . Q. ER. . PQ. . . . . .   82
KB      . . . . . . Q. . T. . . . . H. . . . . . . . V. . Q. ER. . PQ. . . . . .   82
Beaud   . . . . . . Q. . T. . . . . H. . . . . . . . . . . KQ. E. . . PQ. . . L. .   82
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Fig. 3

Amino acid sequences of ORFs 5a (a) and 5b (b) of three TCV isolates and their similarity to those of three IBV strains

The positions of missing amino acids are marked by (-), while the positions of identical nucleotides are marked by (.). For the abbreviations of virus isolates or strains see Tables 1–3.

initiation codon of the first ORF for each particular subgenomic mRNA. The consensus sequences of the TAS sites are CT(T/G)AACAA for IBV, ATC(T/C)AAAC for BCV, AACTAAAC for TGEV, AATC(T/C)A(A/T)AC for MHV, and AACTAAAC for FIPV (Spaan *et al.*, 1988; Stirrups *et al.*, 2000). The distance between TAS and the first ORF is different in each subgenomic mRNA of different coronaviruses. For example, the distance between the canonical IBV TAS 3′-end to the initiation codon of the first ORF is 465 nucleotides for theRNA-dependent RNA polymerase gene, 52 nucleotides for the spike protein gene, 23 nucleotides for gene 3, 77 nucleotides for the M protein gene, 9 nucleotides for gene 5, and 93 nucleotides for the N protein gene (Stirrups *et al.*, 2000). Both the TAS nucleotide sequences and the distance between the TAS 3′-end and the initiation codon of the first ORF are suggested to play an important role in the transcription of mRNAs. However, the intergenic consensus TAS of TCV has never been reported before. As shown in the present study, the TAS sequences of TCV and IBV were found highly conserved. The distance between TAS and the initiation codon of gene 5 and that between TAS and the initiation codon of N gene of TCV were also similar to those of IBV.

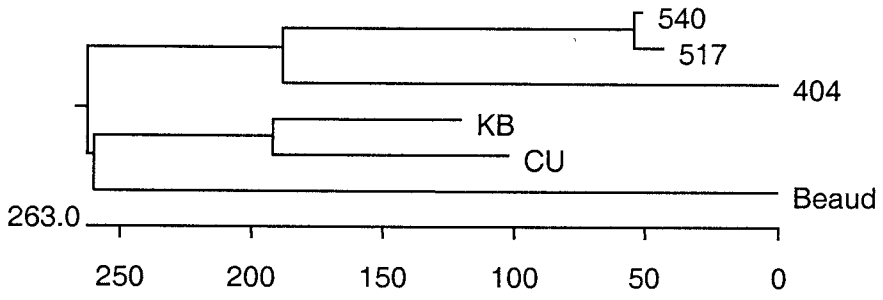

Fig. 4

Phylogenetic analysis based on the PCR amplified fragment containing the gene 5 region demonstrating relationships among three TCV isolates and three IBV strains

The similarity scores were calculated by the Lasergene Program using the unweighed pair-group method by arithmetic averages. The horizontal distances are proportional to the number of nucleotide differences. For the abbreviations of virus isolates or strains see Tables 1–3.

The predicted proteins of ORFs 5a and 5b were of small size (less than 10 K). The functions of these gene products are not known. Several ORFs encoding non-structural proteins have been recognized in coronavirus genomes (Boursnell *et al.*, 1987; Lee *et al.*, 1991; Herold *et al.*, 1993; Eleouet *et al.*, 1995). The number, nucleotide sequence, and gene order of these ORFs varied markedly among different coronaviruses. It is speculated that these genes were inserted into different sites of coronavirus genomes due to the RNA recombination-prone discontinuous transcription mechanism and were not essential for virus replication and pathogenesis. However, the sequence analysis made in the present study indicated that both nucleotide sequences and location of gene 5 ORFs and the consensus TAS of TCV are highly conserved similarly to those of IBV. Given such a highly conserved sequences and structures within avian coronaviruses, gene 5 may play an important role in the pathogenesis of coronavirus diseases in avian species.

It has been reported that M and N genes of TCV were by more than 98% similar to those of BCV (Verbeek and Tijssen, 1991). However, several earlier attempts to amplify these genes of TCV using primers derived from BCV sequences have not been successful (Akin *et al.*, 2001). In line with our previous findings, the presence of gene 5 located upstream of N gene of TCV as shown in the present study does not support the idea of a close relationship between TCV and BCV. There are only 9 nucleotides between M and N genes of BCV. On the contrary, the results of the present study provide further evidence that TCV and IBV may be closely related. The phylogenetic analysis based on N gene made in our previous study (Akin *et al.*, 2001) indicated that the TCV isolates can be clustered within the same genomic lineage as the IBV isolate KB8532. The phylogenetic analysis based on the amplified region made in the present study showed that the TCV isolates can be grouped within the same genomic lineage while the IBV strains including KB8532 can form a separate cluster. Analysis of other genomic segments of the TCV genome will improve our understanding of the genomic relationship of TCV to other coronaviruses as well as molecular nature and pathogenesis of turkey coronavirus infection.

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