Duck Circovirus in Northern Vietnam: Genetic Characterization of the Complete Genome and Epidemiological Analysis of the Causative Agent

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Research Article

Keywords: Duck circovirus, Genetic characterization, Genotype, Positive selection, Vietnam

Posted Date: March 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1412543/v1

Abstract

In the present study, tissue samples collected from a total of 130 commercial ducks collected from clinically suspected flocks and diseased birds in six provinces of northern Vietnam were tested for DuCV infection. The DuCV genome was detected in 56 out of 130 (43.08%) duck samples by PCR. Of 38 tested farms, 26 (68.42%) were positive for the DuCV genome. The rate of the DuCV genome detection in ducks at 3–4 (54.17%) is significantly higher ($p<0.05$) than that of <3 (32.43%) and >7 (33.33%), insignificantly higher than that of 5–7 (43.33%) ($p=0.11$) weeks of age. Length of the six Vietnamese DuCV genomes obtained ranged from 1,988 to 1,995 nucleotides. Among the six Vietnamese DuCV genomes, nucleotide identities were from 83.24% to 99.69%. Phylogenetic analysis of the complete genomes indicated that the DuCVs circulating in northern Vietnam were divided into two main genotypes I and II, and several subgenotypes. The Vietnamese DuCVs were closely related to the Chinese, Taiwanese, and Korean strains. One positive selection site of capsid protein was detected.

Full Text

Duck circovirus (DuCV) was first reported in a six-week-old duck flock in Germany in 2003 [1]. Afterward, DuCV infection was reported in several countries in the world, such as U.S., Hungary, South Korea, Poland, Taiwan, and China [1–6]. Indicators in affected ducks are developmental and feathering disorders, growth retardation, and low body weight [1]. Li *et al*. reported that there were local lesions in the spleen, thymus and bursa of Fabricius (BF) observed in ducks infected with DuCV [7]. In addition, DuCV targets and causes lymphocyte depletion, necrosis, and histiocytosis in the BF, which results in immunosuppression in infected ducks [1]. Therefore, infected ducks are easily infected with secondary infections as *Riemerella anatipestifer, Escherichia coli*, *Pasteurella multocida*, Duck hepatitis virus, or novel goose parvovirus [6, 8–10]. Recently, DuCV has been reported to serve as a causative agent of primary sclerosing cholangitis in natural and reproductive cases [11].

DuCV belongs to the genus Circovirus, which is a member of the family *Circoviridae*. DuCV is icosahedral, non-enveloped, and approximately 15–16 nm in diameter. The viral genome contains a circular single-stranded DNA with approximately 1,9 kb in length [12], which consists of two main open reading frames (ORFs) that encode the structural capsid (Cap) protein and two non-structural proteins, respectively [12, 13]. The Cap protein has been reported to play a critical role in the viral virulence in the hosts [5, 12]. The two non-structural proteins, which are encoded by ORF1 and ORF3, serve as viral replication (Rep) [12] and inducer of apoptosis [13].

Phylogenetic analyses of the complete genome or full-length ORF1 or ORF2 sequence indicated that DuCV is divided into two genetically different genotypes (I and II), and five sub-genotypes, Ia, Ib, Ic, IIa, and IIb [14]. Recently, sub-genotypes Ic and IId have been reported to [formatting] emerge on duck farms in China [15, 16]. Previous studies demonstrated the presence of intersubtype recombination, which may indicate the appearance of novel sub-genotypes [14, 15, 17].

Our preliminary study of DuCV infection indicated that 22 out of 31 (70.97%) duck tissue samples collected in several duck farms in Hanoi city, northern Vietnam were positive for the DuCV genome by polymerase chain reaction (PCR) [18]. The result suggested that DuCV infection might be at high prevalence in northern Vietnam. Based on phylogenetic analyses of the partial ORF1, we tentatively divided the three DuCV strains into a similar genetic group [18]. To date, a lack of the Vietnamese DuCV strains in GenBank and systemic analysis were performed to elucidate the genetic characteristics of the Vietnamese DuCV strains. In this study, we investigated the presence of DuCV in northern Vietnam, and furthermore, conducted molecular characterization of identified DuCV strains based on the complete genome.

Tissue samples from a total of 130 2- to 8-week-old broiler ducks were obtained from a total of 38 duck farms with slightly increased mortality, according to the owners' personal reports and local veterinarians in Hanoi (HN), Haiduong (HD), Thainguyen (TN), Bacgiang (BG), Thaibinh (TB), and Hungyen (HY) cities/provinces of northern Vietnam. Tissue samples collected during April to October 2021 were used in this study. From each farm involved in this study, two to six ducks displaying depression, diarrhea, poor performance, and weakness were selected for sample collection. None of the farms involved in this study had previously used the DuCV vaccine. From each duck, tissue samples, including lung, liver, spleen, heart, intestine, brain, and bursa of Fabricius were collected and pooled into one tube to make individual pooled samples. Each pooled tissue sample was homogenized in phosphate-buffered saline as a 10% homogenate.

DNA was extracted from the homogenized samples using Viral Gene-spin™ Viral DNA/RNA Extraction Kit (iNtRON Biotechnology, Seoul, Korea). PCR for the DuCV genome detection was conducted using a pair of primers [6]. The thermal condition is as follows: 94 ^{o}C for 5 min, 35 cycles of 94 ^{o}C for 45 s, 45 ^{o}C for 30 s, 72 ^{o}C for 30 s, followed by 72 ^{o}C for 10 min. PCR product of 230 bp was electrophoresed on 1.5% agarose gel and visualized under UV light.

The complete genome sequence of the Vietnamese DuCV was amplified by PCR using four pairs of primers [19] and self-designed. PCR products of 886, 996, 505, and 802 bp in size, respectively, were separated on 1.5% agarose gels and purified by GeneClean® II Kit (MP Biomedicals, Santa Ana, CA, USA). Nucleotide sequencing was performed in 1st BASE, Malaysia.

The Clustal W multiple alignment tool [20] in BioEdit v.7.2.5 [21] was used to align and analyze the nucleotide sequences and deduced amino acid (aa) sequences derived from DuCV. Homology in nucleotide and a sequences was examined by the GENETYX v.10 software (GENETYX Corp., Tokyo, Japan) and compared with other publicly-available sequences using the BLAST program. A maximum likelihood method with the best fit model (Tamura 3 parameter) of nucleotide substitutions and bootstrap value of 1,000 replicates was used to construct the phylogenetic tree based on the complete genome, Rep (879 bp), Cap (774 bp), and ORF3 (297 bp) gene sequences of six currently identified Vietnamese and 44 foreign DuCV strains (Suppl. Table 1) using MEGA6 software [22]. The viral complete genomes obtained in this study have been deposited into GenBank under accession numbers OM176552 to OM176557.

Three DuCV strain sequences from the current study and six other sequences from GenBank were used to identify recombination events using RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, Phyl-Pro, LARD, and 3Seq methods implemented in Recombination Detection Program (RDP) version Beta 4.97 [23] with default settings. The algorism that showed $p < 0.05$ was regarded as reliable. Elaboration of evolutionary selection profiles was performed by following the Fast Unconstrained Bayesian AppRoximation (FUBAR) method (http://www.datamonkey.org/) [24].

Fisher's exact test was used to identify significant differences in the rate of the DuCV genome detection between age groups. A value of $p < 0.05$ was considered statistically significant.

In this study, samples were obtained from ducks with weakness and poor growth on farms where slightly increased mortality was observed in ducks at 2–17 weeks of age. The DuCV genome was detected in 56 out of 130 (43.08%) samples tested. Among provinces/cities, HN and TN indicated the highest rates (54.17% and 53.33%, respectively), which were significantly higher than those of BG and HY (40%) ($p \leq 0.05$), HD (33.33%) ($p < 0.01$), and TB (19.05%) ($p < 0.001$) (Table 1). Of the 38 suspected farms, 26 farms were positive for the DuCV genome (Table 1). The proportion of DuCV-positive ducks at 3–4 weeks of age was 54.17% (26/48), which was significantly higher than those at below 3 weeks (32.43%) ($p = 0.002$) and above 7 weeks (33.33%) ($p = 0.003$) of age. The proportion was also higher than that at 5–7 weeks of age (43.33%) ($p = 0.11$) (Table 2). The flock levels ranged from small (< 500 ducks), medium (500–1,000 ducks), and large (> 1,000 ducks) flocks. The positive rates of the DuCV genome at small, medium, and large flocks were 66.67%, 73.33%, and 64.28%, respectively. There were insignificant differences in DuCV-positive rates among flock sizes ($p > 0.05$).

The complete genome of the Vietnamese DuCV obtained in this study ranged from 1,988 to 1,995 nucleotides (nt) in length. The different length of the Vietnamese DuCV complete genomes were due to deletion and insertion mutations in the non-protein-coding region of the viral genome. Neither deletion nor insertion mutations were found on the protein-coding region of the viral genome.

For genetic characterization, six DuCV-positive samples from ducks obtained at different locations farmed in HN, HD, TN, BG, TB, and HY were randomly selected for viral genome sequencing. Complete genome sequences from the six Vietnamese strains were aligned and compared with other referenced sequences retrieved from GenBank. The nt identity ranged from 83.24–99.69% among the six Vietnamese DuCV strains obtained in this study. Among these, the highest nt identity was found between Vietnam/VNUA–HN47/2021 and Vietnam/TB61/2021 (99.69%) while the lowest was between Vietnam/VNUA–HY40/2021 and Vietnam/VNUA–HD89/2021 (83.24%). Comparing the viral complete genomes from the six Vietnamese DuCV strains in this study and those sequences abroad, six Vietnamese strains showed nt identity of 99.54% (Vietnam/VNUA–HN47/2021 vs China/HZ170301/2017), 99.44% (Vietnam/VNUA–TB61/2021 vs China/HZ170301/2017), 98.44% (Vietnam/VNUA-HY40/2021 vs South Korea/KC851821.1/D12-KD-028/2–12), 99.24% (Vietnam/VNUA–TN85/2021 vs China/MF627688.1/JSPX03E/2016), 99.04% (Vietnam/VNUA–HD89/2021 vs

China/HG52019.1/Fujian/2010), and 97.98% (Vietnam/VNUA–BG135/2021 vs Taiwan/DQ166838.1/TC4/2002).

Phylogenetic analysis based on the DuCV complete genome indicated that the Vietnamese DuCV strains we obtained in northern Vietnam were divided into two genotypes (I and II) and three sub-genotypes (Ia, Ib, and Ic) with high supporting bootstrap values (Fig. 1A). Phylogenetic trees of the full-length ORF1, ORF2, and ORF3 sequences also supported that the Vietnamese DuCV strains belonged to genotypes I and II (Figs. 1B–D). Genotype I was represented by four strains, while the remaining two strains were found to belong to genotype II. The six Vietnamese DuCV strains were clustered with those of DuCV strains from China, Taiwan, and South Korea.

Deduced aa sequences of Rep and Cap proteins of the Vietnamese DuCV strains were compared (Fig. 2). Six major variable regions were found in Cap protein of the current Vietnamese DuCV strains, which included residues 3 to 15 (3G/R-4R/S-12G/S/A-15K/R), 31 to 64 (31A/G-47N/H-55N/S-56Q/G-64K/R), 104 to 124 (104T/Q-106N/S/G-109F/Q-118M/I-124I/V), 143 to 159 (143V/I-152K/R-159S/A), 177 to 213 (177V/I-183V/I-187I/T-193E/Q-194E/Q-194T/G-195T/S-196K/T-197Y/H), and 232 to 238 (232T/A-235V/D/E-236N/D/E-237A/G-238Q/K (Fig. 2). Of the six Vietnamese DuCV sequences, four sequences had a single specific aa substitution without sharing to each other at residues 12S, 23L, 183L, 195S, 196T, 197H in Cap protein and 260H and 283S in Rep protein. Two aa substitutions in Cap protein at residue 12S and 55N of the Vietnamese DuCV strains (Vietnam/VNUA–HN47/2021 and Vietnam/VNUA–TB61/2021) were unique.

Recombination analyses revealed no putative recombination event ($p > 0.05$) was found among the current six DuCV strains using RDP 4 software. Analyses of natural selection profiles of the Vietnamese DuCV sequences indicated that 89 sites of Cap protein and 49 sites of Rep protein were found to be under negative selection (Suppl. Tables 2 and 3). However, only one positive selection was found at residue 106 of Cap protein (Table 3).

Understanding DuCV infections is important as they cause immunosuppression in infected hosts.. In our previous report, we recorded that a high positive rate (70.97%) of DuCV infection was found among ducks farmed in Ha Noi city in northern Vietnam [18]. To date a lack of the Vietnamese DuCV genome sequences is found in GenBank, and additional sequence data of the complete genome is necessary to further characterize and understand the evolution of DuCV strains. To our knowledge, this is the first study to characterize nucleotide sequences of the complete genome of the Vietnamese DuCV strains.

In this study, the DuCV genome was detected in 43.08% of duck samples collected in northern Vietnam which is higher than that of the previous study [9, 25], but lower than our previous report [18]. However, samples used in this study were collected from ducks with poor performance rather than being randomly collected from a duck flock. It has been reported that the rate of DuCV infection varies among countries, such as Hungary (84%) [6], Taiwan (38.2%) [26], China (33.29%) [10], South Korea (21.8%) [27], and the U.S. (6.06%) [5]. This variation could be attributed to differences in sampling size, time, location, and sensitivity of detection methods used. DuCV was detected in all sampled areas. In addition, a high

positive rate (68.42%) of the DuCV genome was detected at farm level, suggesting that DuCV is distributed widely in northern Vietnam. In northern Vietnam, duck production systems include millions of farms (personal communication), which range from small- to large scale operations.. Therefore, 38 tested farms obtained is representative of a small sample. Interestingly, sampling sites were at regions that accounted for 21.17% and 52.81% of the total number of ducks in Vietnam and the northern part (25 cities/provinces), respectively [28]. Therefore, it may be noted that this sampling was representative for the source population in term of DuCV detection. In the current study, we found a high rate of DuCV-positive in Vietnamese ducks at 3–4 weeks of age (54.17%), suggesting that 3–4-week-old ducks may be more susceptible to DuCV infection [10].

In the present study, the Vietnamese DuCV genomes had three ORFs 1, 2, and 3, which were similar to those of other members of DuCV family as described previously [13, 29]. Regarding genotyping, two genotypes (I and II) and subgenotypes (Ia, Ib, Ic, IIa, and IIb) of DuCV were classified based on full-length ORF1 or ORF2 sequences [14]. Later reports found novel subgenotypes of DuCV based on analyses of the complete genomes [15, 16]. In the present study, the Vietnamese DuCV strains belonged to both genotypes I and II. In addition, several subgenotypes of genotype I were also obtained among Vietnamese DuCV strains, suggesting multiple origins of DuCV strains in the country. Therefore, additional DuCV sequence data is required for better understanding the molecular diversity of DuCV strains worldwide.

Recombination is one of the evolutionary processes which has been reported in DuCV from China based on sequence data of complete genomes. Inter-subtype recombination might generate new DuCV strains or novel subgenotypes. In addition, the recombination regions could occur on both protein-coding and non-protein-coding regions of the viral genome [14, 15, 17]. Unfortunately, no recombination events have been found among the six DuCV strains obtained in this study. This is due to the fact that the low number of the DuCV genome may affect the results of recombination analysis. Additional complete genome sequences of the DuCV are needed to further study the evolutionary characteristics of DuCV strain in Vietnam.

Virulence of DuCV strains seemed to be evaluated by the Cap protein and apoptin, which operated the apoptotic action. Wang *et al*. [30] suggested that three of six variable regions at residues 3–15, 104–124, 232–238 on Cap protein, might contribute as epitope differences. However, all aas of these regions were not found under positive selection, which was similar to analysis of Zhang *et al*. [14]. The only one aa (residue 106) of the variable region (104–124) of the Vietnamese DuCVs indicated under positive selection, which differed from that of aa residues 104 and 109 [14]. Xing *et al*. [13] demonstrated that ORF3 plays a critical role in pathogenesis and immunosuppression. However, aas under positive selection were not detected on Rep protein among the Vietnamese DuCVs, while ORF3 is completely within Rep protein. In contrast, Grenfell *et al*. [31] noted that virulence genes are under positive selection.

In summary, DuCV-positive rate was detected at a high rate among duck flocks of young individuals in northern Vietnam. Phylogenetic and molecular analyses of the complete genome revealed that the six Vietnamese DuCV strains belonged to genotypes Ia, Ib, Ic, and II. This is the first report on characterization

of DuCV strains circulating in the country based on the complete genomes. No recombination event has been detected among the Vietnamese DuCV strains. Only one aa on Cap protein was found to be under positive selection. Further investigation should be conducted to understand the possible evolutionary mechanisms acting on DuCV and improve the control of DuCV infection in duck production across Vietnam.

Declarations

Acknowledgments. We also thank Dr. Kristen Kelli Coleman at Duke University and Dr. Amanda Farrell at Harvard School of Medicine for English edits and proofreading of our manuscript.

Author contributions. GTHT, VPL, and HVD designed the research. HDV, GTHT, VPL, VNB, DQT, and TDD performed the research. GTHT, NTM, TTTV, and HVD contributed to sample collection. GTHT and HVD wrote the manuscript. All the authors read and approved the final manuscript.

Funding. No funding was received.

Conflict of interests. Giang Thi Huong Tran, Ngan Thi Mai, Vuong Nghia Bui, Tung Duy Dao, Dai Quang Trinh, Tra Thi Thu Vu, Van Phan Le, and Hieu Van Dong declare that they have no conflict of interest.

Ethical approval. This article does not contain any studies with human or animal subjects performed by any of the authors.

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Tables

Table 1 Detection of the duck circovirus genome in ducks in different regions in northern Vietnam

Province/city	No. of collected samples	No. of gene positive samples/(%)	No. of flocks	No. of gene positive flocks/(%)
Hanoi	48	26/(54.17)	11	9 (81.81)
Haiduong	6	2/(33.33)	2	1 (50)
Thainguyen	15	8/(53.33)	3	2 (66.67)
Bacgiang	30	12/(40)	11	8 (72.72)
Thaibinh	21	4/(19.05)	7	4 (57.14)
Hungyen	10	4/(40)	4	2 (50)
Total	130	56 (43.08)	38	26/(68.42)

Table 2 Detection of the duck circovirus genome in the field duck samples according to age and the number of flocks and flock size

Criteria	Weeks/Number of ducks	No. of samples	No. of gene positive samples/(%)
Age	< 3	37	12 (32.43)
	3 – 4	48	26 (54.17)
	5 – 7	30	13 (43.33)
	> 7	15	5 (33.33)
Flock size	< 500	29	10/(34.48)
	500 – 1,000	64	31/(48.44)
	> 1,000	37	15/(40.54)

Table 3 Positive selection in the duck circovirus capsid protein of the current six Vietnamese DuCV strains

Site	α	β	β – α	Prob [α > β]	Prob [α < β]	BayesFactor [α < β]
106	3.241	37.584	34.343	0.021	0.953	44.352

Figures

Fig. 1

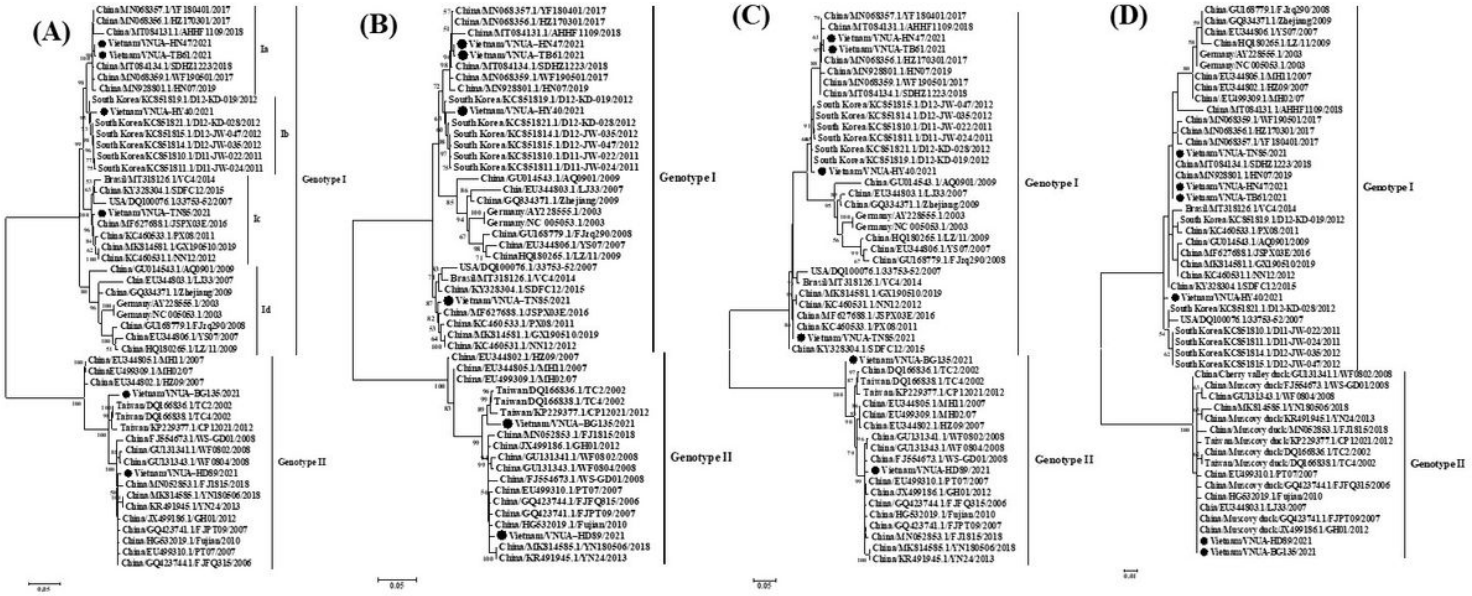

Figure 1

Phylogenetic tree of (A) the complete genome, (B) Rep, (C) Cap, and (D) ORF3 sequences of the Vietnamese DuCV strains compared with those available in GenBank. GenBank sequences are indicated by the country name/accession number. The maximum likelihood method in MEGA6 software was used to establish the phylogenetic tree (1,000 bootstrap replicates). Number at each branch point indicates bootstrap values ≥50% in the bootstrap interior branch test. The current Vietnamese strains are indicated by circles. Two major genotypes were identified and designated as I and II.

Fig. 2

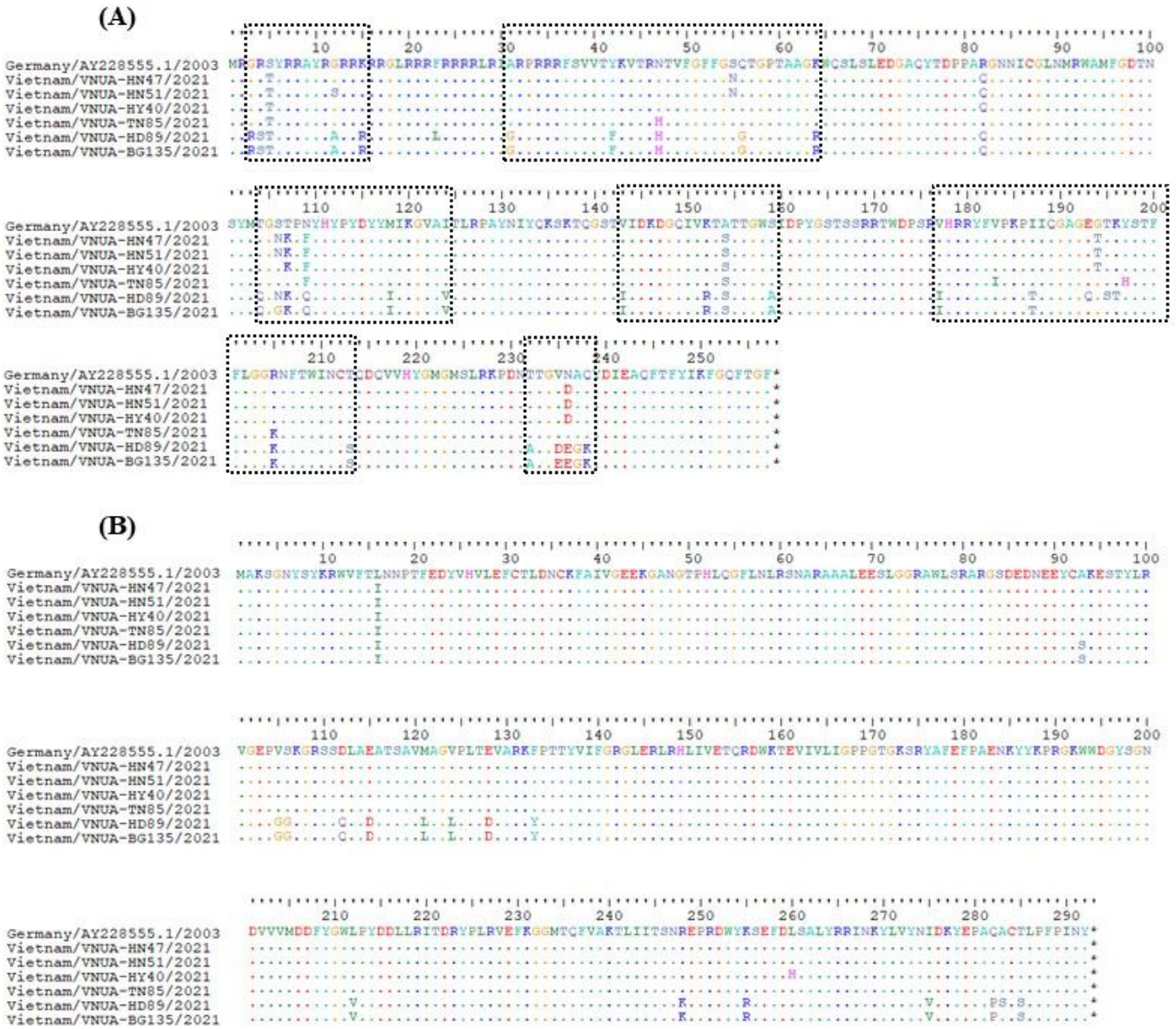

Figure 2

Amino acid substitutions in (A) Cap and (B) Rep proteins of the Vietnamese DuCV strains. Variable regions on Cap protein are indicated as boxes.

Supplementary Files

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