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Genomic Characterization and Evolutionary Analysis of a Novel IBV Strain (HH06) Isolated in Northeastern China

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ABSTRACT

The spike (S) glycoprotein is a key virulence factor for coronaviruses (CoVs), and CoV variants are categorized through S gene analysis. In this study, we present the phylogenetic analysis of a newly isolated infectious bronchitis virus (IBV) strain in relation to available genome and protein sequences, based on network analysis, multiple sequence alignments, selection pressures, and evolutionary fingerprinting techniques, specifically in the People's Republic of China. We selected 111 CoV strains for analysis: Alphacoronaviruses (Alpha-CoVs; n = 12), Betacoronaviruses (Beta-CoVs; n = 37), Gammacoronaviruses (Gamma-CoVs; n = 46), and Deltacoronaviruses (Delta-CoVs; n = 16). Phylogenetic clustering showed that SARS-CoV-2 and SARS-CoVs grouped with Bat-CoVs and MERS-CoV within Beta-CoVs (C). The IBV HH06 strain from Avian-CoVs closely resembled Duck-CoV and partridge S14, LDT3 (from teal and chicken hosts). In contrast, Beluga whale-CoV (SW1) and Bottlenose dolphin-CoVs, of mammalian origin, were more distantly related but still fell under the Gamma-CoVs category with Avian-CoVs. Motif analysis revealed conserved domains in the S protein, which showed similarity within the same phylogenetic group but varied across different viral lineages. A recombination network tree demonstrated that SARS-CoV-2, SARS-CoV, and Bat-CoVs, while branching separately, shared common clades. MERS-CoVs from camel and human sources clustered into a distinct group but remained closely linked to SARS-CoV-2, SARS-CoV, and Bat-CoVs. In contrast, HCoV-OC43, of human origin, clustered with bovine CoVs but was more distantly related to other human-origin viruses like SARS-CoV-2 and SARS-CoV. These findings underline the ongoing genetic recombination and evolutionary processes of CoVs, which could maintain them as potential threats to both veterinary and human health.

Keywords: Molecular epidemiology, Zoonotic transmission, Coronaviruses, Infectious bronchitis virus, Evolution

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Introduction

Coronaviruses (CoVs) are RNA viruses that primarily infect the respiratory systems of domestic and wild birds and mammals, including humans. They belong to the Orthocoronavirinae subfamily in the Coronaviridae family [1, 2], and are divided into the genera Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus [3]. CoVs are enveloped viruses, characterized by a helical-symmetry nucleocapsid and spike-shaped projections. Their genome consists of a positive-sense single-stranded RNA of 26–32 kilobase pairs, encoding structural proteins such as the spike (S) glycoprotein (composed of S1 and S2 subunits), envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein [4–6]. The S glycoprotein is crucial for viral

attachment and entry into host cells, contributing to the virus's virulence [7, 8]. The S protein is highly mutable and evolves quickly, with mutations in certain amino acids altering its antigenic properties, leading to the emergence of new viral strains [9]. Variations in the S1 subunit by 20–50% are typically associated with different serotypes, though even minor changes (2% or 10–15 amino acid alterations) can generate distinct serotypes of infectious bronchitis virus (IBV) [10, 11]. This evolutionary process is critical in the emergence of new viral strains, with specific amino acid positions being conserved for maintaining protein function and structure [12, 13]. Thus, identifying these conserved regions can provide insights into the evolutionary pressures and functional significance of the CoVs S protein.

IBV, the first coronavirus discovered by Schalk and Hawn in 1931 in North Dakota [14], was followed by the identification of related CoVs in various animals. The first human CoV, associated with the common cold, was isolated in the 1960s [15, 16]. In recent decades, pandemics caused by SARS-CoV and MERS-CoV have led to significant mortality [17, 18]. The emergence of SARS-CoV-2 in late 2019 marked the onset of a new global pandemic [19]. CoVs are known for their wide tissue tropism, primarily targeting the respiratory system, but they can also infect the gastrointestinal and reproductive systems [20, 21]. Avian-CoVs from the Gammacoronavirus genus cause infectious bronchitis in poultry, leading to respiratory, renal, and reproductive diseases, with significant economic impact on broilers (weight loss) and layers (reduced egg production) [10, 22]. While chickens are considered the primary hosts of IBV, infections have also been reported in turkeys (Turkey-CoV) [23], pheasants (Pheasant-CoV) [24], and other bird species like ducks (Duck-CoV) [25], peafowl (PeF-CoV) [26], and pigeons (Pi-CoV) [27]. These strains are typically less pathogenic, but the host range for IBV can extend to swans, mallards, and other waterfowl, showing symptoms similar to those caused by M41, 793B, and QX lineages [24].

Avian-CoVs exhibit high potential for genetic recombination during replication [28], and genetic techniques are vital for understanding the relatedness of different microorganisms and their evolutionary mechanisms [29, 30]. The recent COVID-19 pandemic has emphasized the zoonotic potential of CoVs [31, 32], making it essential to study their genetic origins, evolution, and transmission pathways to prevent future outbreaks. In this study, we explored the phylogenetic relationship of a newly isolated IBV strain using genome and protein sequence analysis, incorporating phylogenetic, recombinant network, multiple sequence, selection pressure, and evolutionary fingerprinting methods.

Materials and Methods

Chicken embryos

For virus isolation and titration, 9–11-day-old specific-pathogen-free (SPF) chicken embryos were obtained from the Experimental Animal Center at Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, People's Republic of China.

Isolation and identification of IBV HH06

The IBV strain HH06 (GenBank accession MH181793.1) was recovered from Hy-Line chickens showing symptoms of infectious bronchitis at a farm in Northeastern China. The strain was preserved at the Veterinary Pathology Laboratory, Northeast Agricultural University, as per the method described by Ren *et al.* [33]. To isolate the virus, it was passaged three times in the allantoic cavities of 9-day-old SPF embryonated chicken eggs (ECE). Characteristic signs of IBV such as embryo stunting, hemorrhages, and dwarfing were observed [34]. The 50% embryo infectious dose (EID50) was determined by inoculating 10-fold serial dilutions into 9-day-old ECE, as outlined in previous studies [35].

RNA extraction and RT-PCR

Total RNA was extracted from allantoic fluid using TRIzol reagent (TaKaRa, Dalian, China) following the manufacturer's instructions. Reverse transcription was performed using a Qiagen RT-PCR kit. The reaction mixture included 8 µL DEPC-treated water, 4 µL 5×RT-buffer, 1 µL dNTP, 1 µL Oligo (dT), 5 µL RNA, 0.5 µL m-MLV, and 0.5 µL RNase. For detection, IBV-N primers (189 bp) [Sense: CAAGCTAGGTTTAAGCCAGGT; Antisense: TCTGAAAACCGTAGCGGATAT] [36] were used. The thermal cycle conditions were: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55.7°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes and

held at 4°C. PCR products were analyzed using 1% agarose gel electrophoresis and visualized by UV trans-illumination (Bio-Best 140E, SIM, USA) [37].

Cloning and sequencing

PCR products were purified using the Gel Extraction Mini Kit (Omega, USA), and ligated into the pMD18-T cloning vector (TaKaRa, Japan). These were then transformed into competent E. coli JM109 cells (Beijing TransGen Biotech, PRC). Recombinant plasmids were confirmed by PCR and restriction enzyme digestion, with conditions matching the previously described amplification protocol. Three positive clones were cultured and sequenced by Shanghai Sang-gong Biological Engineering Technology & Services Co., Ltd (Shanghai, China).

Genetic, phylogenetic, motif analysis, and sequence alignment

To assess genetic relationships, 111 CoV sequences from the Coronaviridae family were selected, including the IBV HH06 strain (GenBank MH181793.1). These were aligned using ClustalW, and a phylogenetic tree was constructed using the maximum-likelihood method (JTT model) in MEGA 7.0, with 1,000 bootstrap replicates (https://www.megasoftware.net) (40). Sequences from GenBank, representing the four main CoV genera, were included in the analysis. Motif analysis was carried out using the MEME suite (https://meme-suite.org/meme/tools/meme). The S protein sequences of 31 CoVs from different hosts and genotypes were aligned using ClustalW in MEGA 7.0, and conserved amino acids were highlighted using the GeneDoc program [38].

Recombinant network analysis

The possibility of recombination events was analyzed by constructing a network tree from protein sequences of IBV HH06 and 110 reference CoVs using the SplitTree 4.13.1 software (http://www.bio-soft.net/tree/SplitsTree.htm) [39].

Selection pressure analysis

Codon sites under selection pressure were analyzed using the SELECTION online tool (https://selecton.tau.ac.il/). The ω ratio was adjusted for codon sequences of CoV proteins, and selection pressures were measured using a maximum-likelihood test based on Bayesian inference [40]. The results were visualized with color scales to indicate different types of selection.

Evolutionary fingerprinting

We utilized the Evolutionary Fingerprint (EFP) model to visualize evolutionary patterns as probability distributions. This method allows for comparison of distributions in a manner that minimizes the effects of variations in dataset size and evolutionary divergence. The EFP analysis was carried out using the DataMonkey classical tool (https://www.datamonkey.org/) [41], on the aligned CDS sequences of selected CoVs, including SARS-CoV-2, SARS-CoV, MERS-CoV, and IBV.

Analysis of evolutionary diversification

The neutrality test was performed using maximum likelihood calculations of the dN-dS ratio with the HyPhy software embedded in MEGA 7.0, employing the Nei-Gojobori method [42]. Gaps and incomplete data were excluded from the analysis. The evolutionary history of CoVs' S gene was reconstructed using the Kimura 2-parameter model for maximum likelihood inference, and a phylogenetic tree was constructed using MEGA 7.0 software [43]. Genetic sequences were compared for similarity using ClustalW. We also estimated synonymous (s) and non-synonymous (n) substitutions for each codon, along with the corresponding synonymous (S) and non-synonymous (N) sites. These estimations were based on joint maximum likelihood reconstructions under the Muse-Gaut model of codon substitution and the Tamura-Nei model for nucleotide substitution [41]. The tree topology was automatically computed to estimate maximum likelihood values. The dN-dS test statistic is used to detect codons under positive selection, where dS refers to synonymous substitutions per site (s/S) and dN indicates non-synonymous substitutions per site (n/N). A positive dN-dS value suggests an excess of non-synonymous substitutions, and the likelihood of rejecting the null hypothesis of neutral evolution (p-value) was calculated [44]. A p-value of < 0.05 was considered significant. Normalized dN-dS values, calculated using the total substitutions in the tree, enable cross-comparison of different datasets. Maximum likelihood calculations of dN and dS were

performed using the HyPhy software. This analysis involved 110 nucleotide sequences, with 2,625 positions in total after excluding gaps. The evolutionary analysis of diversifying selection also incorporated various approaches to identify episodic diversifying selection at individual codon sites. The MEME model (Mixed-effects model evolution) was applied using Markov Chain Monte Carlo (MCMC) to combine fixed effects and detect positive selection at individual branch sites, ensuring robustness against model misspecification [45]. The MEME fitting used the MG94xREV codon model with the GTR nucleotide model for initial estimates. Selection pressure was evaluated using two parameters, $\beta- < \alpha$ and $\beta+$ for each site, with an alternative model for each site's variability in substitution rates [40]. The likelihood ratio test (LRT) based on a $\chi 2$ distribution was used to assess statistical significance, with p-values < 0.05.

Results

Isolation, identification, and confirmation of IBV HH06

The IBV HH06 strain was successfully isolated using 9–11-day-old SPF embryonated chicken eggs (ECE). Morphological changes such as dwarfism, hemorrhages, and congestion were observed in the embryos. Allantoic fluid was harvested, and the presence of IBV HH06 was confirmed using RT-PCR with IBV-N specific primers (189 bp).

Genetic, phylogenetic, and motif analysis of the S Protein

A phylogenetic tree **(Figure 1)** was created from the amino acid sequences of the S glycoprotein to examine the genetic relationship among four main CoV genera (Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus), representing both mammalian and avian viruses. The study included SARS-CoV-2, SARS-CoV, and MERS-CoV from mammals, alongside the avian IBV HH06 strain. IBV HH06 clustered with the GI-19 genotype (QX-type) of Avian-CoVs, showing close relation to Duck-CoV DK/CH/HN/ZZ2004 (GenBank AEO86768.1) and partridge S14 (GenBank AAT70772.1), as well as LDT3 (GenBank AAU14248.1), which infect teal and chicken hosts. Other Avian-CoVs like Ph-CoV strains ph/China/I0710 (GenBank QDA76255.1) and PSH050513 (GenBank AAZ85066.1) were also grouped closely. This suggests that Avian-CoVs evolve within hosts and between genotypes. The S protein sequences of Beluga whale-CoV SW1 (GenBank ABW87820.1) and Bottlenose dolphin-CoVs (37112-1, GenBank QII89019.1) were distantly related to Avian-CoVs but still clustered within the Gammacoronavirus group. A separate cluster of Delta-CoVs, including sparrow, quail, and porcine CoVs, was found to group with Feline-CoVs and Canine-CoVs. This created a separate cluster of both mammalian and avian CoVs. Another group included human CoVs like HCoV-OC43, while murine, equine, and rodent CoVs formed a different cluster. MERS-CoVs, belonging to Beta-CoVs, were closely related to both SARS-CoVs and Bat-CoVs. The S gene was identified as the primary determinant of serotype and tissue tropism in different virus strains. Conserved motifs in the spike protein were identified via MEME **(Figure 2)**, with ten motifs in total, which were cross-referenced and confirmed using the Pfam database. These motifs were highly conserved within the same phylogenetic class, though some variations were observed.

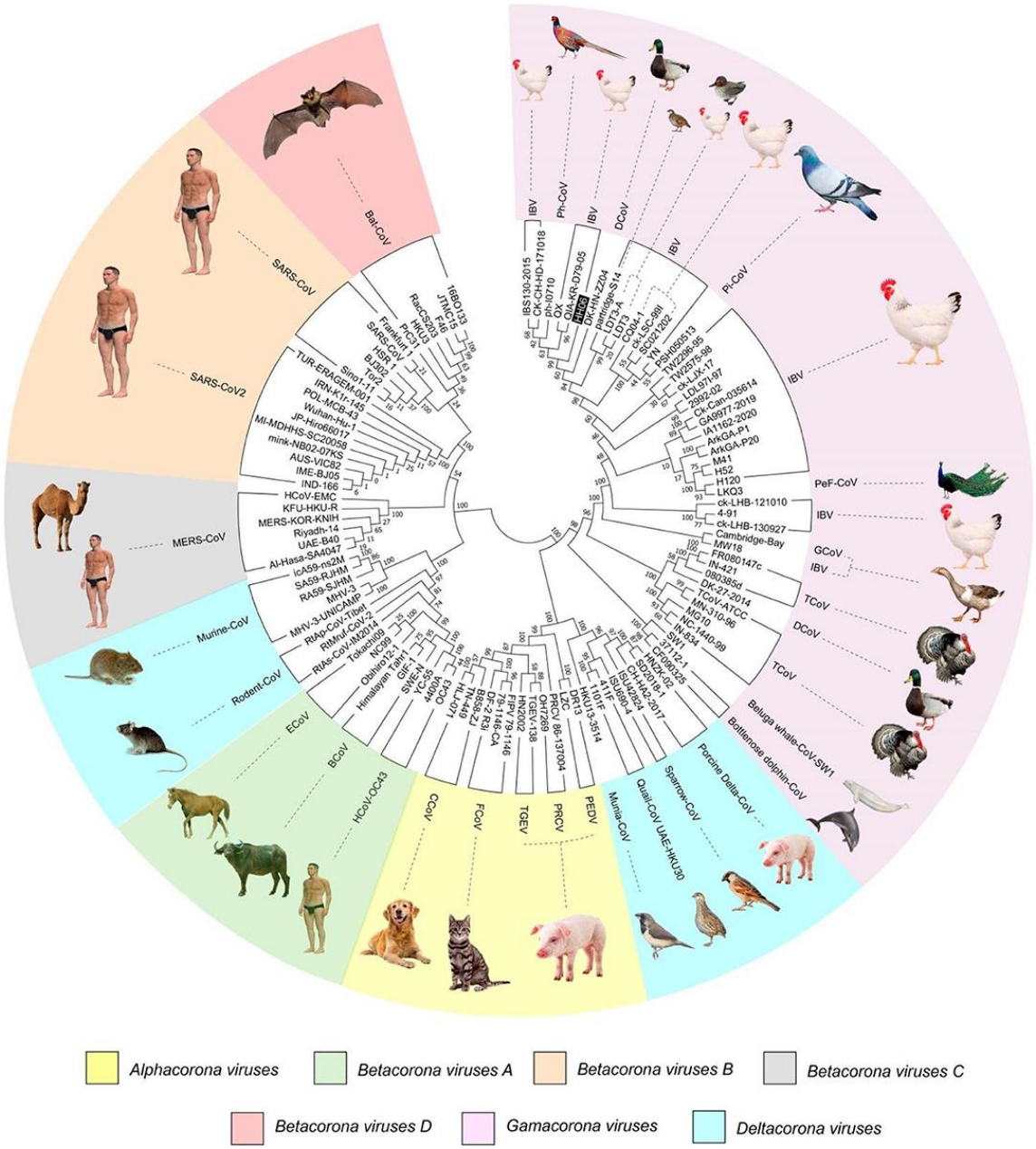

Figure 1. A phylogenetic tree was generated using the Spike (S) gene sequences from the four primary CoV genera (Alpha, Beta, Gamma, and Delta), encompassing the IBV HH06 isolate along with 110 reference CoV strains. The analysis was conducted with the Maximum-Likelihood method in MEGA7.0, with 1,000 bootstrap replicates to assess confidence levels. Different colors represent the hosts for each reference strain

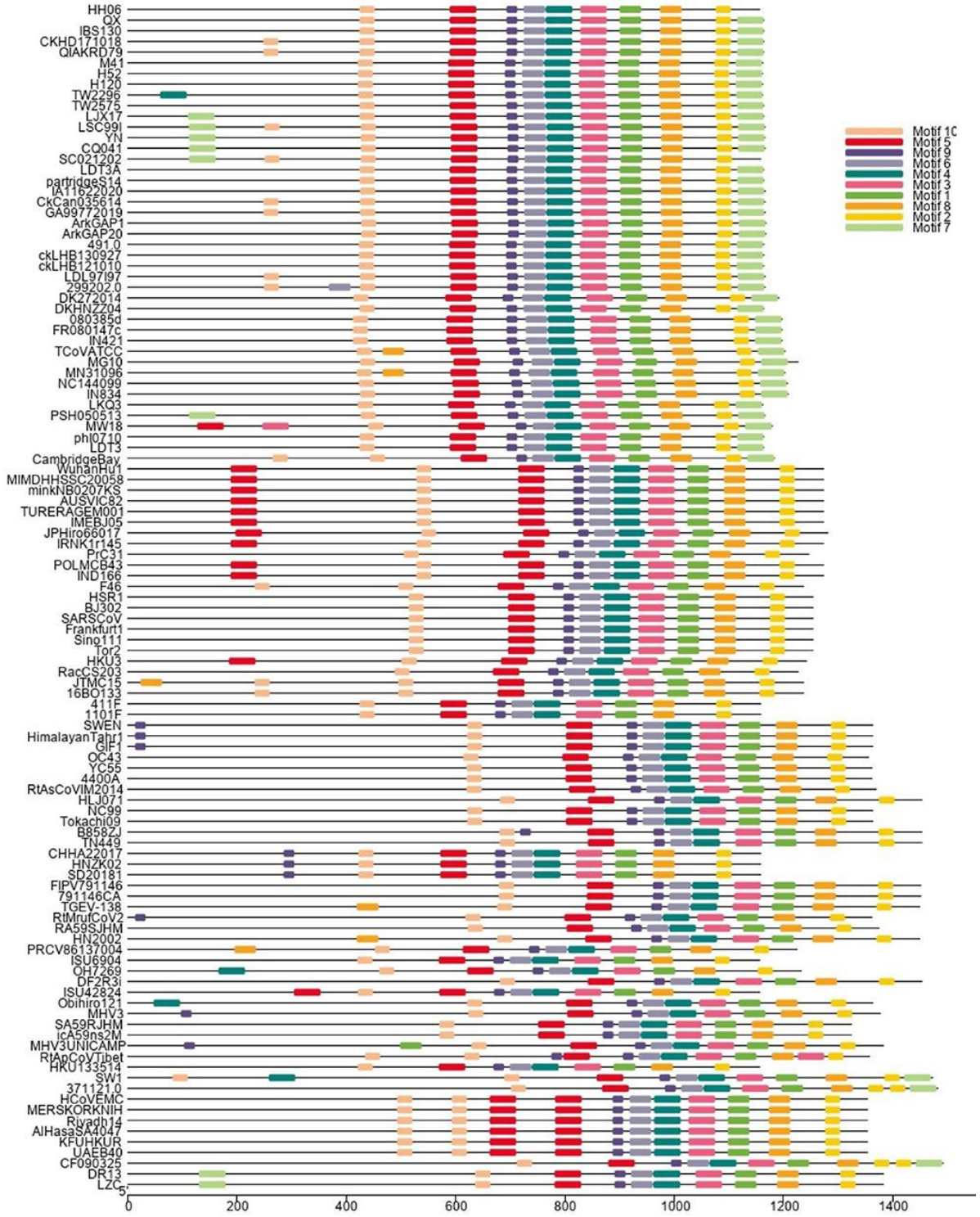

Figure 2. The Spike (S1 and S2) protein motifs are highlighted in different colors, reflecting their distribution across the genera and illustrating their evolutionary links

Recombinant network analysis

A network tree was constructed **(Figure 3A)** for 111 CoV strains across four genera (Alpha, Beta, Gamma, and Delta), representing viruses from humans, animals, and birds. CoVs from humans were clustered into distinct groups. Betacoronaviruses B (SARS-CoV-2 and SARS-CoV) and D (Bat-CoVs) shared a common cluster despite differing branching patterns, suggesting a bat origin. MERS-CoVs, originating from camels and humans within Betacoronaviruses C, formed a separate group but remained closely related to B and D Betacoronaviruses. HCoV-OC43, although a human virus, was more distantly related to other human CoVs such as SARS-CoV-2 and SARS-CoV, grouping instead with Bovine-CoVs. Feline- and Canine-CoVs shared a common evolutionary origin but

branched separately from SARS-CoVs. Avian-origin Gammacoronaviruses were distantly related to Betacoronaviruses, with species such as Beluga whale-CoV and Bottlenose dolphin-CoVs forming their own subgroup within Gammacoronaviruses. The HH06 isolate in this study was grouped with other Avian-CoVs, predominantly from chickens, but had close genetic ties to Duck-CoV and Pheasant-CoV. Interestingly, Turkey-CoVs formed a separate clade, diverging from other chicken-associated strains.

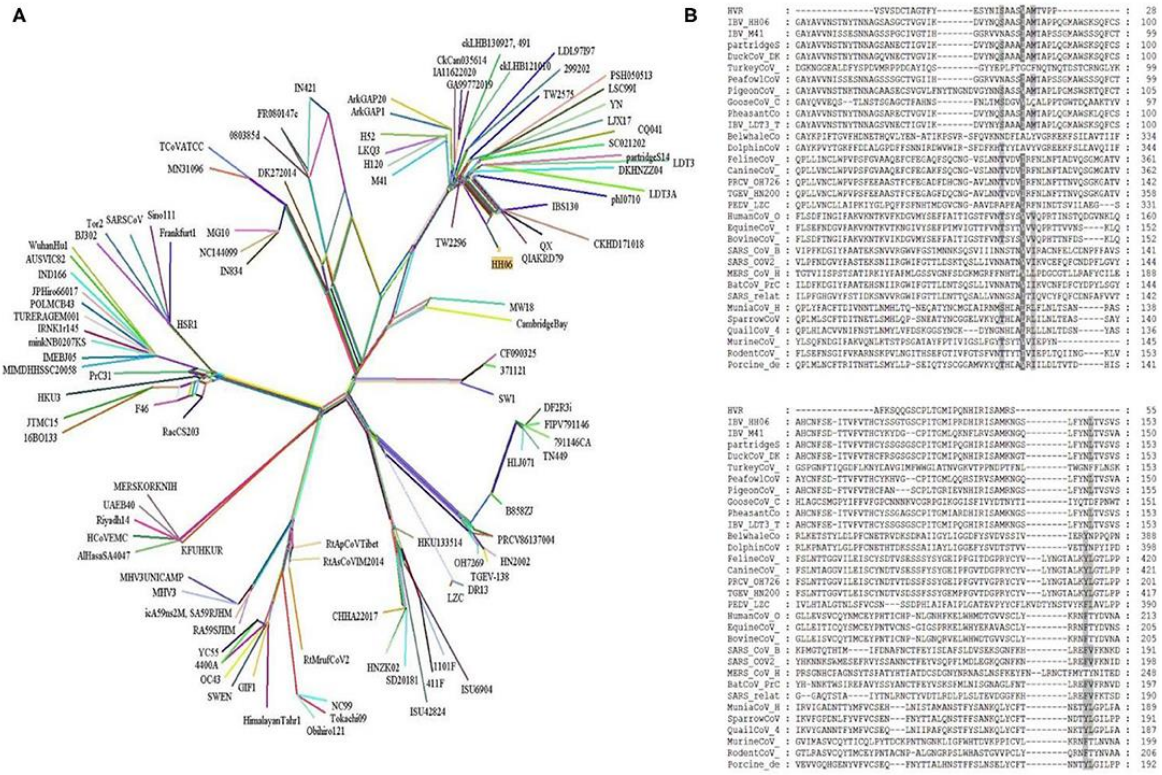

Figure 3. A network tree for 111 CoVs was produced from the protein sequence alignments of the spike protein using SplitsTree 4.14.5. (A) The tree displays multiple reticulate patterns, indicative of recombination events among various CoV strains. The HH06 isolate is highlighted in yellow, and major reference sequences like SARS-CoV-2, SARS-CoV, and MERS-CoVs are included. (B) The alignment of S protein sequences from four genera was conducted using MEGA7, and the GeneDoc program was used to shade amino acids based on similarity (dark shading for identical amino acids, gray for similar ones)

Comparative sequence alignment

The S gene sequence alignment across the four CoV genera revealed specific mutations in the hypervariable regions (HVRs) of the spike protein. Notably, the IBV HH06 isolate showed several mutational sites, particularly in the HVRs, which were consistent with the sequence alignments of selected 31 CoVs from across the four genera of Coronaviridae **(Figure 3B)**.

Selection pressure analysis by amino acid position

The SELECTION server (http://selecton.tau.ac.il/) using the Mechanistic Empirical Combination (MEC) model was employed to analyze selection pressure at specific codons in the S protein. Positive selection was detected at multiple codons, with 36.63% of amino acids under positive selection, while the remaining amino acids underwent purifying selection **(Figure 4A)**, suggesting evolutionary pressure primarily driving the gene's maintenance and stability.

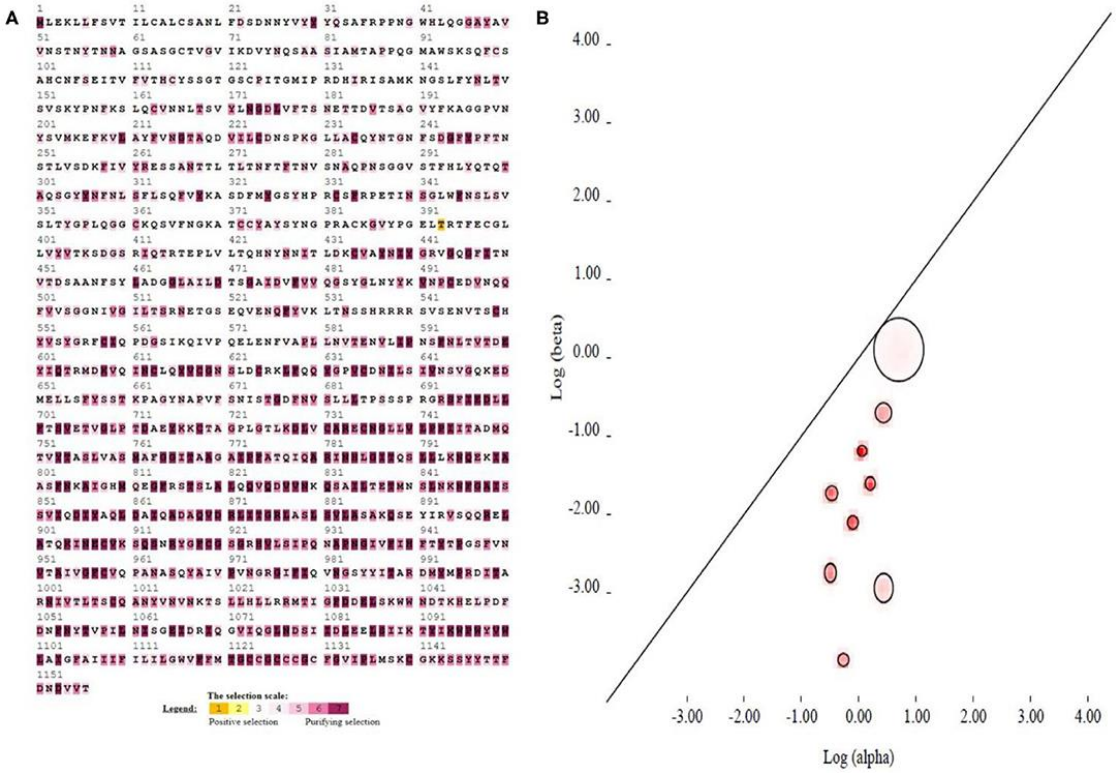

Figure 4. (A) The MEC model in the Selection Server was used to evaluate the selection pressure on spike protein sequences of 111 CoVs. Codons under positive selection are marked in brown and yellow, neutral codons are in white and gray, and purifying selection codons are indicated in purple.
(B) The evolutionary fingerprint plot, based on 1,000 distribution samples, estimates the distribution of synonymous and non-synonymous rates. The plot, on a log-log scale, shows ellipses representing variance in each rate estimate, with colored pixels indicating density for a given rate. The diagonal line reflects neutral evolution (ω = 1), with points above it indicating positive selection (ω > 1) and below it indicating negative selection (ω < 1)

Evolutionary fingerprinting

Evolutionary fingerprinting of the spike gene was assessed using a codon-based evolutionary model, focusing on synonymous and non-synonymous substitution rates with a genetic algorithm. The ω ratio (ω = β/α) was calculated, reflecting site-to-site distribution of substitution rates, using likelihood log and Akaike information criterion (AIC) to determine neutral evolution across the spike gene. The likelihood log value was −129131.88712, and the AIC was 258361.77424 for the nine rate classes, with parameters indicating the neutral evolutionary pattern. The ω values across nine CoV categories were 0.004, 0.022, 0.117, 0.275, 0.077, 0.151, 0.262, 0.312, and 0.543, respectively. The graph indicated that most sites were neutral, with a few showing positive selection above the diagonal in **Figure 4B**.

Positive and purifying selection

Out of 2,625 codon positions in the spike gene sequences, covering the 1st, 2nd, 3rd codon positions, and non-coding regions, only 18 positions (codons 26-866) showed signs of positive selection, indicated by dN-dS > 1. Synonymous and non-synonymous mutation counts were tabulated, and synonymous/non-synonymous substitution sites were calculated. The MEME analysis revealed 46 sites under episodic diversifying selection, all with p-values <0.01 **(Table 1)**. The model estimates synonymous (α) and non-synonymous (β) substitution rates, identifying sites where β > α, indicating significant diversifying selection. These sites, under positive selection (β+ > α), provided strong evidence of evolutionary pressure on the S gene. The Simes' method reduced false discovery rates under a strict null model **(Table 1)**.

Table 1. Mixed-Effect Model Evolution (MEME) – Episodic Diversifying Selection of the S Gene

Codon	α	β^+	Pr[$\beta = \beta^+$]	β^-	Pr[$\beta = \beta^-$]	*q*-value	*p*-value
23	0.763537	6.94636	0.208058	0.0876926	0.791942	0.306802	0.00599763

32	0.0303846	16.0443	0.154643	0.0303846	0.845357	0.0217625	0.000109085
183	0	1385.75	0.191579	0	0.808421	0.0121202	4.86022e-05
292	2.25206	1017.51	0.0166862	0.196196	0.983314	0.0488714	0.000440945
298	1.42265	100.502	0.0601715	0.174859	0.939828	0.0158631	7.95141e-06
317	1.04444	18.7376	0.112989	0.137501	0.887011	0.0301017	0.00021124
325	1.13163	20.307	0.07924	0.232676	0.92076	0.249942	0.00463552
342	1.57608	79.7256	0.04149	0.159258	0.95851	0.24093	0.00362301
353	1.29453	28.1257	0.0750881	0.352534	0.924912	0.345888	0.0069351
386	0.753244	8.28076	0.199246	0.0399839	0.800754	0.0997	0.00114942
425	0.410353	11.616	0.176644	0.02881	0.823356	0.169125	0.00245846
433	0.593223	78.4249	0.06212	0.231261	0.93788	0.0254667	0.000153183
439	0.479128	72.4365	0.0355088	0.113517	0.964491	0.103465	0.00124469
552	0.99667	19.789	0.0949856	0.123464	0.905014	0.254245	0.00458789
665	0.438912	16.7519	0.0830372	0.068045	0.916963	0.0431592	0.000346139
800	0.697221	28.2982	0.103285	0.0904626	0.896715	0.0120883	5.45339e-05
801	1.19665	59.5819	0.0549798	0.104413	0.94502	0.0141066	3.53549e-05
812	0.435179	209.337	0.0172323	0.0407753	0.982768	0.0174943	2.63072e-05
945	0.311495	1385.76	0.0682191	0.311495	0.931781	0.114277	0.00160388
1010	0.666632	6.50562	0.205446	0.0706994	0.794554	0.0121043	4.24713e-05
1036	1.07674	11.1189	0.192209	0.149688	0.807791	0.395497	0.00872274
1063	1.34172	29.3489	0.227819	0.051046	0.772181	0.0722627	0.000724438
1065	0.23116	4.16857	0.371122	0.0690992	0.628878	0.372842	0.00766241
1070	1.27829	156.206	0.0497822	0.088108	0.950218	0.118014	0.00159718
1089	0.461102	39.1503	0.0444409	0.0974815	0.955559	0.0170905	3.42666e-05
1171	0.702561	13.1505	0.0805655	0	0.919435	0.387482	0.00835174
1176	1.23305	1292.18	0.00752452	0.10632	0.992475	0.0247109	0.000136251
1241	0.43565	7.65695	0.176822	0.142802	0.823178	0.100245	0.00110546
1282	2.62966	41.2155	0.0646616	0.0713705	0.935338	0.0550796	0.000524567
1285	0.384711	15.0998	0.110134	0.103926	0.889866	0.0126936	3.81763e-05
1381	0.443969	54.664	0.0439479	0.102546	0.956052	0.0262251	2.62908e-05
1446	1.01357	22.6903	0.0619309	0.0717032	0.938069	0.233577	0.00362951
1519	0.586696	186.959	0.0294032	0.0355755	0.970597	0.0474378	0.000404232
1563	0.141133	192.671	0.0414764	0.0203677	0.958524	0.0251713	0.000164024
1606	0.906054	9.24085	0.0971675	0.0786118	0.902833	0.36508	0.00768589
1658	1.94819	210.228	0.0147941	0.170368	0.985206	0.419478	0.00967217
1694	0.331159	432.483	0.0138985	0.0278589	0.986102	0.0998924	0.00125178
1727	0.453456	22.5383	0.0674039	0.155761	0.932596	0.241765	0.00387794
1819	0.480708	49.0222	0.0129895	0.031874	0.987011	0.255805	0.00435959
1840	0.515394	250.885	0.0191948	0.0643477	0.980805	0.0334543	0.000251536
1866	0.776281	12.702	0.112381	0	0.887619	0.289249	0.0055095
1939	0.232632	33.5588	0.0218743	0	0.978126	0.388338	0.00875951
1942	0.500544	25.6181	0.0370913	0	0.962909	0.117311	0.00152886
1972	0.115119	4.66122	0.105195	0.0412817	0.894805	0.239631	0.00396383
1973	0.310806	21.6742	0.03523	0.0140975	0.96477	0.0762024	0.000802131
1989	0	1.51907	0.153316	0	0.846684	0.251072	0.00440476

Discussion

Genetic methodologies are essential for understanding the evolutionary relationships between pathogens, including viruses, and the diseases they cause. In this study, we compared the phylogenetic relationship of the IBV HH06 strain to available genome and protein data using network analysis, multiple sequence alignments, selection pressure, and evolutionary fingerprinting. The COVID-19 pandemic highlighted the zoonotic potential of coronaviruses.

Genetic recombination network analysis provides valuable insights into the complex evolutionary relationships of viruses [46]. The network analysis showed that the IBV HH06 strain clustered with Duck-CoV and Pheasant-CoV, highlighting an evolutionary link. Previous studies have reported similar findings, suggesting the susceptibility of both host species to IBV [24, 47, 48].

Sequence homology alignment revealed numerous mutations in the hypervariable region (HVR) of the spike protein, supporting the current classification of CoVs based on the structural protein, particularly the spike gene [49]. The spike protein is responsible for viral entry into host cells by interacting with surface receptors [50]. It exists as two subunits, S1 and S2, with S1 mediating attachment to host receptors and S2 facilitating fusion,

forming the stalk of the spike molecule. The C-terminal region of S2 is highly conserved and contains a furin cleavage site (S2'), crucial for the virus's ability to invade host cells [51]. Mutations in the S protein play a key role in viral adaptation and organ tropism, potentially altering the targeted host organ [52]. We identified 10 motifs in the S gene, which were confirmed through Pfam database annotations, supporting their roles in viral attachment and infection.

Evolutionary dynamics

Approximately 36.63% of the amino acids in the spike (S) protein underwent positive selection, suggesting that negative selection influenced all gene pairs. The process of stabilizing selection can occur when harmful mutations are removed from the gene pool. To evaluate the evolutionary adaptability of IBV strains, particularly regarding the S gene, Gaussian-approximate variance analysis was employed, indicating that most of the evolution was neutral, with minimal evidence for positive evolution in the S gene. To understand the dynamics of the molecular sequence evolution, we used the MEME approach to calculate the synonymous (α) and non-synonymous (β) substitution rates, revealing 46 sites under episodic diversifying selection. Our phylogenetic analysis demonstrated a broad evolutionary framework in which MERS-CoVs of Beta-CoV group C showed a close relationship with SARS-CoVs-2 and SARS-CoVs from Beta-CoV group B. Similarly, Bat-CoVs were also tightly linked to SARS-CoVs, supporting the theory that SARS-CoV-2 originated from bats or pangolins [29]. This suggests that the adaptation and recombination of SARS-CoV-2 might have occurred in an intermediate host, possibly involving contact with bats or pangolins. CoV mutations and adaptations have been key drivers of the co-evolution between CoVs and their hosts [9]. Recombination and mutation are both fundamental in the formation of new CoV strains, as various studies have shown [53]. Recombination events between strains, as well as changes in the antigenic profile of the resulting viruses, are crucial for predicting future CoV evolution and guiding control efforts [54, 55]. Evolutionary conservation plays a vital role in pinpointing essential amino acid positions that help maintain the function of structural proteins. The sites identified as under purifying or positive selection during selection pressure analysis provide valuable insights into the role and evolution of the spike protein gene.

Selection pressure analysis

Purifying selection was evident throughout the selection pressure analysis in this study. Li *et al.* [56] highlighted purifying selection in various host species, coupled with frequent recombination events among coronaviruses, pointing to a shared evolutionary pathway that could lead to the emergence of new human coronaviruses. Similarly, our network tree analysis showed that Betacoronaviruses B (SARS-CoV-2 and SARS-CoV) and Betacoronaviruses D (Bat-CoVs) shared clades, although they branched differently, indicating a potential bat origin. Such intra-species transmission events in birds and mammals likely result from the widespread presence of CoVs [57]. The analysis of Gamma-CoVs revealed interesting patterns. The Beluga whale- and Bottlenose dolphin-CoVs clustered closely with avian CoVs, and avian-CoVs like sparrow, munia, and quail grouped with Porcine-CoVs, hinting at potential cross-species transmission. These findings suggest that variations in the S gene allowed CoVs to adapt to a wide variety of host species. Analysis of IBV HH06, isolated in this study, showed that it belonged to the QX-type G-19 IBV but had close associations with Duck-CoV and Pheasant-CoV. Abro *et al.* [58] also observed inter-species transmission between avian and mammalian CoVs, which aligns with our results.

Conclusion

The genetic analysis of the S glycoprotein reveals that SARS-CoV-2, SARS-CoV, Bat-CoV, and MERS-CoV share a close genetic relationship. IBV has the potential to infect various bird species beyond its primary host, chicken, including ducks, teal, partridges, turkeys, and pheasants. The close clustering of Duck-CoV and Pheasant-CoV with IBV HH06 and other QX-type viruses indicates the need for further studies on the transmission dynamics of these CoVs. The selection pressure analysis showed that purifying selection predominates in the evolutionary process. The recombination and evolution of CoVs continue to give rise to novel strains with significant intra-species transmission and zoonotic potential. Therefore, enhanced global control measures are necessary to mitigate the continued emergence of CoVs.

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