RESEARCH ARTICLE

Comparative Nucleotide Sequence Analysis of Glycoprotein B, C, and G of Infectious Laryngotracheitis Virus Isolated in Egypt During 2016-2018

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Abstract

Infectious Laryngotracheitis (ILT) is an economically important disease of poultry, causing a high mortality rate and /or reduced egg production. Tracheas from sever morbid and recently died unvaccinated household chicken were subjected to PCR assay to detect the presence of the virus through amplification of the glycoprotein B gene. All tested samples gave positive c_t ranging from 13.32 to 25.53. Virus isolation was performed by inoculation—the processed tracheal swaps onto the chorioallantoic membrane. Positive pock lesion has been developed within 7 days post-inoculation. The pock lesions were subjected to PCR assay for amplification of both the full-length glycoprotein B, C, and G for sequencing analysis. Positive amplicons migrating about 2600, 1250, and 890 bp were amplified corresponding to the *orf* of gpB, gpC and gpG genes. Sequence alignment and phylogenetic tree revealed that all sequenced isolates gave a high degree with wild type isolate of ILT and a high degree of genetic stability was clearly evident among strains isolated in different periods (2016-2018), indicating that these glycoproteins could be used as a vaccine candidate.

Keywords: Glycoprotein B, Glycoprotein C, Glycoprotein G, ILT, Surface glycoproteins.

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Introduction

Infectious laryngotracheitis virus (ILTV), is belonging to alpha herpesvirinae with a double-stranded DNA genome of approximately155 kb size (Guyand Garcia. 2008; Davison, 2010). ILTV strains of varying virulence have been isolated and characterized worldwide. Some strains were developed successfully into modified live vaccines but some of these vaccine strains could not be distinguished from virulent viruses by either immunological tests or PCR-based procedures (*Hanson and Bagust,* 1991). Additionally, some vaccines could revert to virulent viruses after bird-to-bird passage(Blacker *et al*., 2011 and Lee *et al*., 2012)

DNA vaccine coding for surface glycoprotein B, C, G, and D is promising in preventing the disease without any limitation. For example, Vaccination with a subunit vaccine made of a 205 kDa complex containing glycoprotein B (gpB) of ILTV gave 100% protection against clinical disease and also against viral replication (Chen *et al*., 2010, 2011).

The objective of the present study was to isolate ILT virus from unvaccinated household chicken and sequencing analysis of surface glycoprotein B, C and G genes as a primary step to investigate the validity of DNA vaccine coding for these glycoproteins.

Materials and Methods

From Dec 2016- March 2018, tracheas from unvaccinated, house hold (backyard) chicken suffering from the classical symptoms of ILT were collected from recently dead or severely morbid chickens after euthanization. Samples were kept in a sterile container and stored at -20 °C till processed within 24 h after collection.

Molecular detection of the ILTV by amplification of surface glycoprotein B gene

The presence of ILT virus in the tracheal swabs were confirmed by real-time PCR amplification of surface glycoprotein B gene (gpB). A set of primers that direct the glycoprotein B gene of ILT virus was designed using Lasergene molecular biology suite V15 (Table 1) and the designed primers were tested by

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Table 1: The primer sequence used in the current study.

Primer ID	** Seq 5′→3′*
Primers used for qPCR assay	
QgpB-f	CGACTAACATGACTGAAGGA
QgpB-r	ATGATACGTTGCTTTGGATGAG
Primers used for full-length amplification of gpB, gpC and gpG genes for sequencing	
gpB-f	ATGGCTAGCTTGAAAATGC
gpB-r	TTCGTCTTCGCTTTCTTCTGCC
gpC-f	ATGCAGCATCAGAGTACTGCGCT
gpC-r	TGTTGTCTTCCAGCACCAT
gpG-f	ATGAGCGGCTTCAGTAACATAGG
gpG-r	CTGCTGGAGCGTAGAGGGC

insilco amplification using fast PCR professional V6 software. Primer specificity was tested using the primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The scarped tracheas, along with the mucous plug, were subjected to DNA extraction using Trizol reagent (Thermo Scientific cat # 15596-026) according to the manufacturer's instructions.ThegpBgene was amplified from all the tested samples using Brilliant II SYBR® Green QPCR Master Mix (Agilent Cat# 600831) according to the manufacture instruction.

Isolation of ILT Virus on ECE and identification by amplification of the glycoprotein B gene

The tracheas (that were positive in PCR) each was scraped individually, and the bloody mucous were collected in 500µl of PBS pH 7.2. The suspensions were then centrifuged at 1500rpm for 5min at 4°C. The supernatants were collected and filtrated through 0.45 µØ syringe Millipore filter before inoculation in 9days old SPF chicken eggs (SPF-ECE) via the chorioallantoic membrane. Eggs were incubated at 37°C in a humid chamber for at least 7 days. The chorioallantoic membranes were then collected and examined for the pock lesions characteristics for the ILT. The membranes were stored at -20 °C till used.

The membranes that showed the pock lesions were subjected to DNA extraction using Trizol reagent and gpB gene was amplified as previously described above.

Virus purification by sucrose gradient ultracentrifugation

Membranes showed the characteristic pock lesions were homogenized by mechanical homogenizer (PRO 200homogenizer - Pro Scientific USA) using Sawtooth generator 10mm (dia.) x 115mm (L) at 4°C. The homogenates were then centrifuged at 5000rpm for 10min at 4°C. Cell debris and high molecular weight proteins were removed from the homogenized membranes by centrifugation at 1500rpm for 10min at 4 °C first. The virus suspension was laid onto sucrose cushion (3 mL 20% and 3 mL 50% sucrose prepared in TNE buffer [20 mM Tris-HCl (pH 7.), 100 mM NaCl and 2 mM EDTA]) and centrifuged for 2 h at 30000 rpm in Sorvall® Surespin™ 630 swinging bucket ultracentrifuge rotor using Sorvall WX 100 ultracentrifuge (Thermo Fisher Scientific, USA). The virus-containing layer was aspirated, and the virus particles were sedimented at 50000rpm for 3 hours at 4 °C using sorvall. The sediment virus was resuspended by gentle agitation in 1 mL of TNE (prepared with nuclease-free water) overnight at 4 °Cand stored at -80 °C till used in the PCR amplification and sequencing procedure.

Sequencing analysis of the surface glycoprotein B, C, and G genes

Viral DNA was extracted from the purified virus by Gene JET Genomic DNA Purification Kit (Thermo Scientific Cat. # K0721) according to the manufacturer's instructions and stored at -20°C till used. As per manufacturer's instructions, two µL of the eluted DNA was mixed with 5 µL of 10X high fidelity buffer (High Fidelity Hot Start Core Kit Jena Bioscience Cat # PCR 235S) and primer sets that amplify the full length surface glycoprotein B, C and G genes of ILT (Table 1).The PCR product was then electrophoresed on 1% agarose, stained with ethidium bromide and visualized under the U.V. transilluminator. A DNA ladder from 250bp- 10Kbp (GenRuler DNA ladder Thermo cat # SM0314) was used to determine the size of the amplicons.

Three strains isolate during Dec 2016 (designed as ILT- YA/16), Nov 2017(designed as ILT- YA/17) and Jan 2018 (designated as ILT-YA/18) were subjected for sequencing analysis of the surface glycoprotein B, C and G genes. The amplicons amplified by PCR were electrophoresed on a 1% low melting agarose and the size of the amplicons was determined using SynGene V4.01 (Synoptics – Cambridge- England). The amplicons were sliced off and purified using gel purification kit (Biobasic - Canada cat #.BSC02S1) according to the manufacturer's instructions. The nucleotide sequence of the genes of the isolated ILT strains was performed in (Macrogen USA). Sequencing reactions were performed in a M.J. Research PTC-225 Peltier Thermal Cycler using ABI PRISM 3730XL Analyzer Big DyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (F.S. enzyme Applied Biosystems). Nucleotide sequence analyses were conducted with the LaserGene sequence analysis software package (LaserGene,

version 10; DNAStar, Inc.). Alignments of the sequences were performed using the *Clustal W* module. Phylogenetic analysis was created using Maximum-likelihood (ML).

Results and Discussion

Virus isolation and characterization:

The clinical signs of ILT infection in chicken observed with the samples from which the virus was isolated ranging from extension of the neck and snoring due to moderate to severe dyspnea, lots of blood spotted mucous on the wall of the households. Some cases showed lacrimation, reddening of the conjunctiva, and swelling of the eyelids and paranasal sinuses.

PCR amplification of the surface glycoprotein B (gpB) gene of the ILT virus isolates from tracheal swabs gave a positive Ct ranging from 13.32- 25.53 and the dissociation curve revealed that all amplicons have Tm of 86.48 (Figs. 1 and 2)

Inoculation of the SPF embryonated chicken eggs revealed very dense necrotic foci (Pock lesions) at the site of

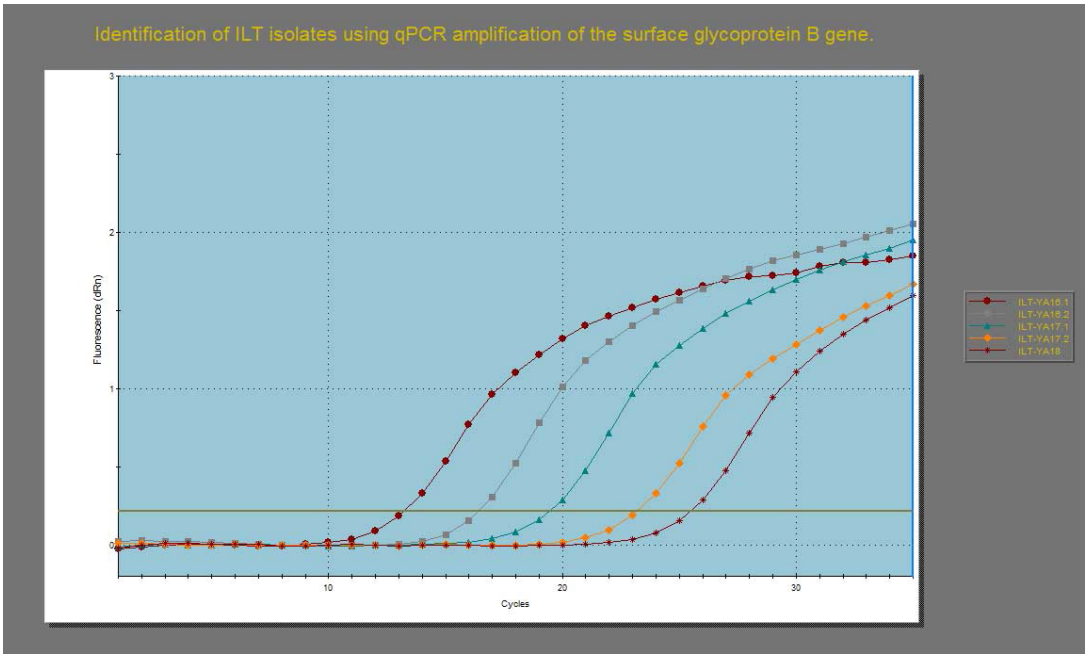

Fig. 1: Amplification of gpBgene from tracheal swaps from severely morbid and recently died chickens suffering from ILT signs. A positive *C*.T. were seen with all tested samples and it was ranging from 13.32 to 25.53

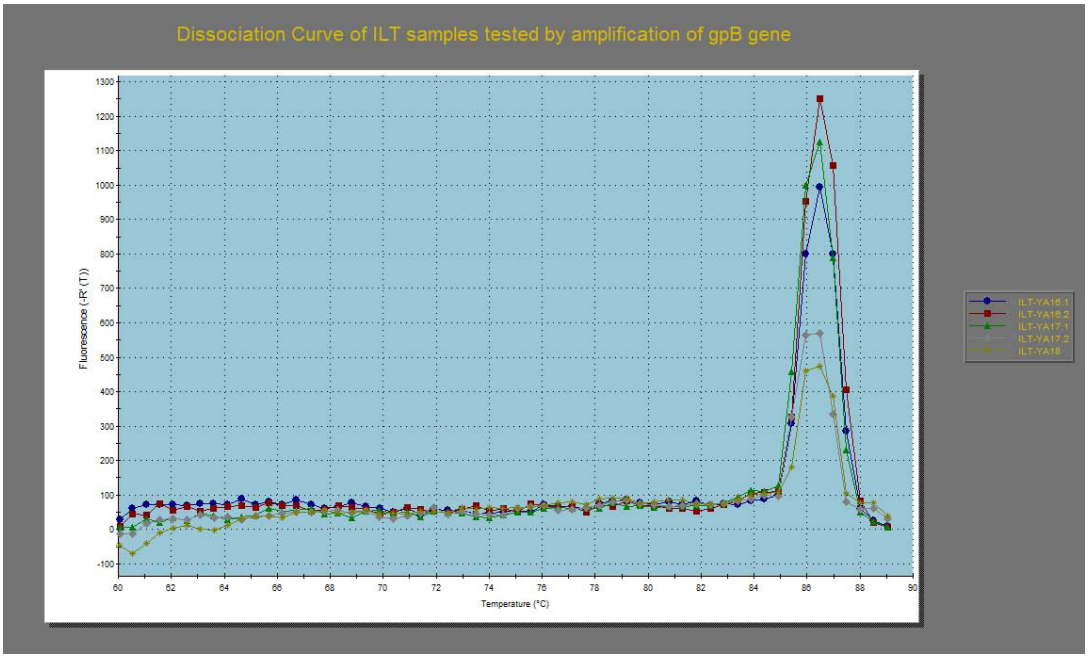

Fig. 2: The dissociation curve of the amplified gpB gene from tracheal swaps from severely morbid and recently died chickens suffering from ILT signs. The Tm for all amplified products. Were 86.48

inoculation and spreading over the membranes, although no deaths of the embryos were observed even after 7 days post-inoculation (Fig. 3).

Embryonated chicken eggs 9 days old, was used for isolation of the ILT virus from suspected tissue homogenate. Inoculation of the Chorioallantoic membrane with the specimen showed characteristic pock lesions 5-7 days post-inoculation. Indeed there were no differences between the intensity of the pock lesions produced by the three isolated viruses during this study

All the membranes with pock lesions gave a positive amplicon migrating at about 2600, 1200 and 800 bp corresponding to the surface glycoprotein B, C and G genes respectively

Sequencing analysis of the surface glycoproteins B, C, and G of ILT virus

The nucleotide sequences of the gpB, gpC and gpG genes **(table 2)** were aligned using the clastal*W* algorithm (Fig. 7, 9, 11). High identity percentage (over 98%) was found between

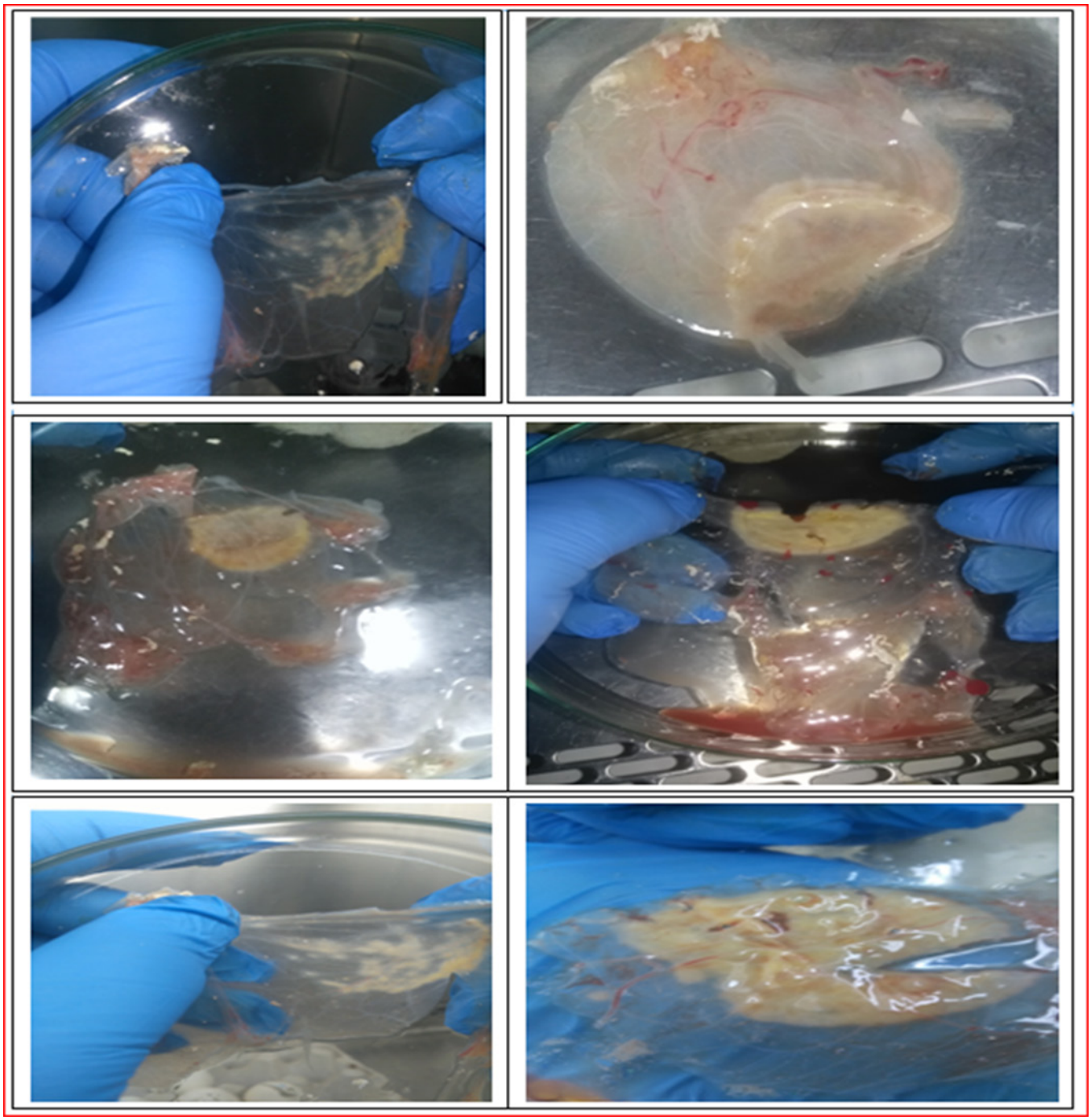

Fig. 3: Chorio-allantoic membrane showing the pock-like lesions 7 days post-inoculation of the ILTV isolates.

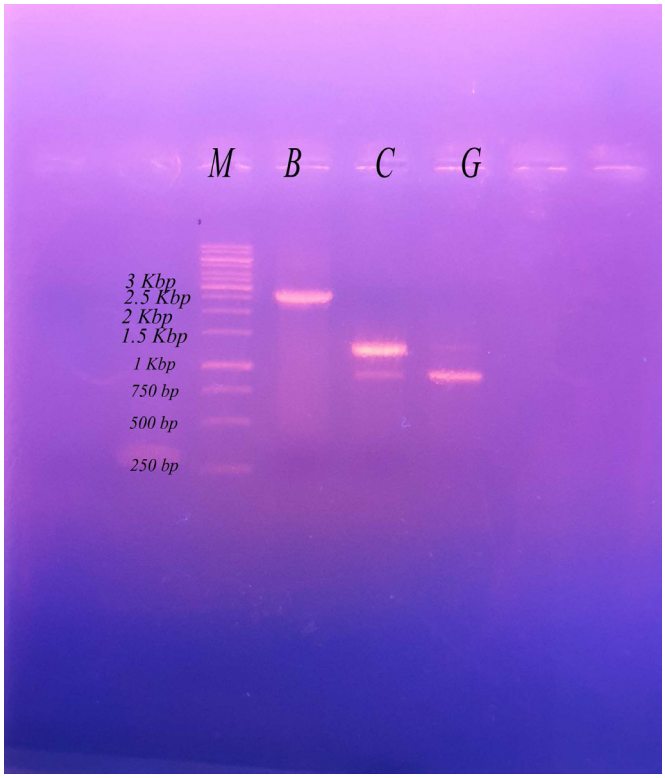

Fig. 4: Amplification of the full-length surface glycoprotein B, C, and G genes from the purified virus by ultracentrifugation. M is the DNA ladder

the three sequenced isolates (ILT.YA/2016, ILT.YA/2017 and ILT.YA/2018) regarding the three genes.

Many nucleotide substitutions have been found between the three isolates for gpB, gpC, and gpG, yet no frameshift has been detected. Nucleotide blast revealed that there were a great percentage of identity between three isolates in the current study and the wild field type isolates in the gene bank database (identity % ranges from 98% -100%), which emphasized that those three strains are wild type virus strain, and not revertants of the modified live vaccine. Phylogenetic tree construction based on the nucleotide sequence; however, revealed the three isolated strains in the current study were closely related concerning the three glycoproteins B, C, and G.

Surface glycoproteins of ILTV have the main role in viral entry, replication, and release. Also, it involved in modulation of the immune response. Glycoprotein G facilitates virus entry through apical surface of polarized epithelial cells (Devlin *et al*., 2006); it also acts as viral chemokine binding protein (Bryant *et al*., 2003and Coppo *et al*., 2018). Initial attachment involves interaction between Glycoprotein C and heparan sulfate; likewise, glycoprotein B can also bind heparan sulfate(Pavlova *et al.,* 2010). Thus DNA vaccine coding for these genes would be a good alternative for the live attenuated vaccine.

Conclusion

During this study, we have amplified the full-length *orf* of the Egyptian locally isolated ILTV glycoprotein B, C, and G genes spanning the three years (2016-2018). Sequencing analysis (alignment) and phylogenetic tree revealed that those three genes have genetic stability and a high degree of identity with the wild type virus. Now, as per the important role of those three genes in viral entry, replication, and shedding, it makes them a good candidate for DNA vaccine production to overcome the drawback of the live attenuated vaccines.

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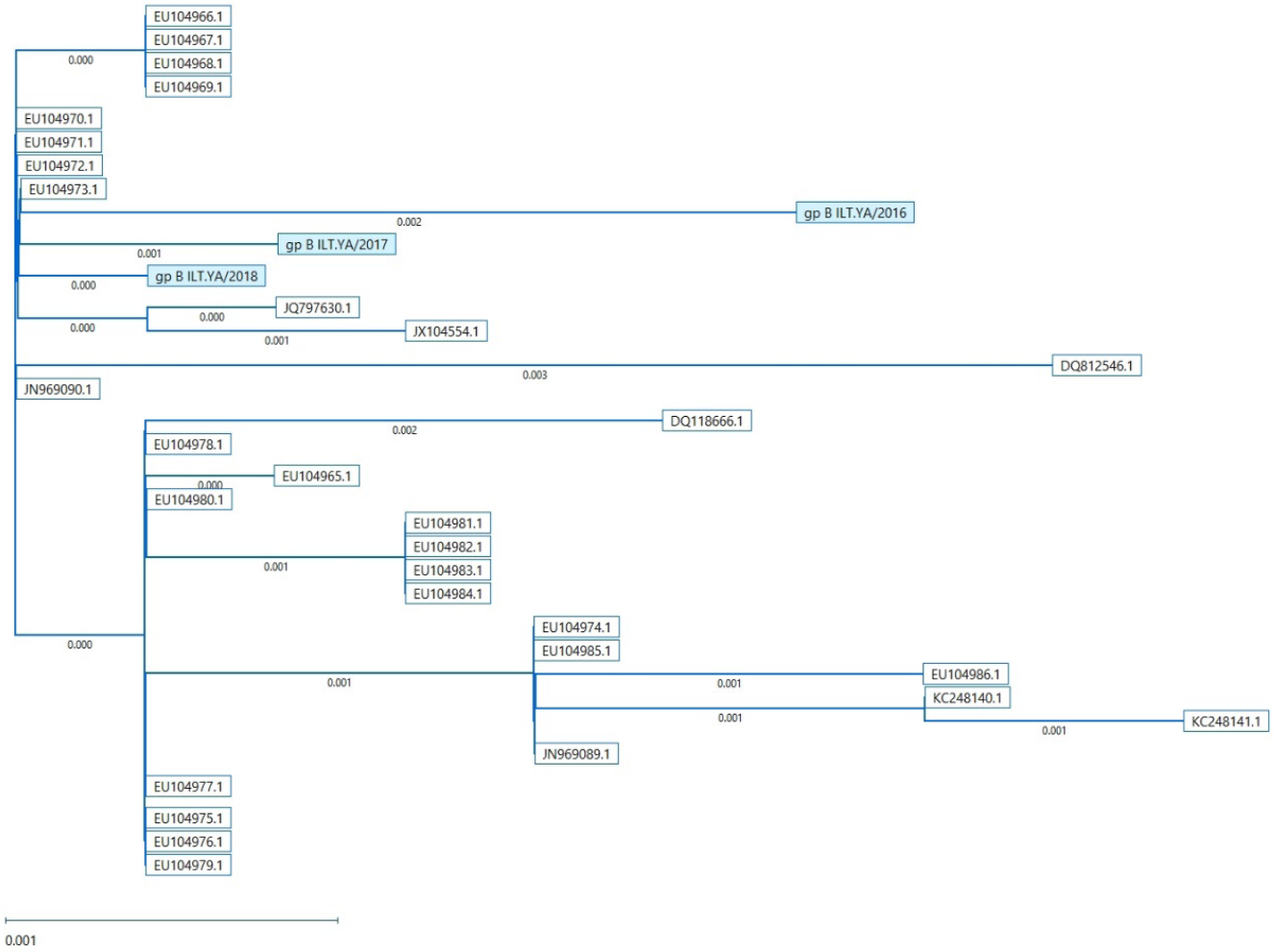
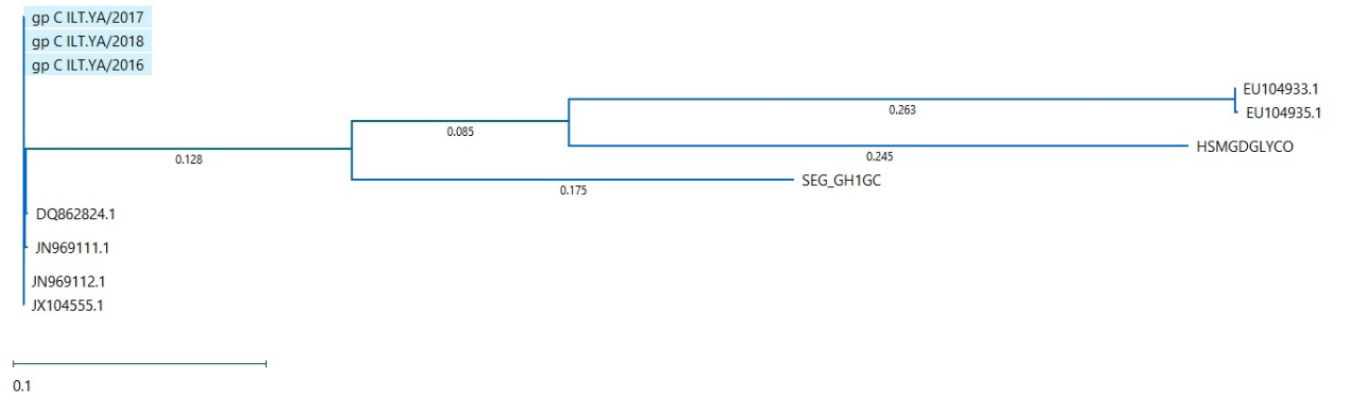
Appendix

The aligned nucleotide sequence of assembled glycoprotein B of the three ILT isolates (ILT.YA/16, ILT.YA/17

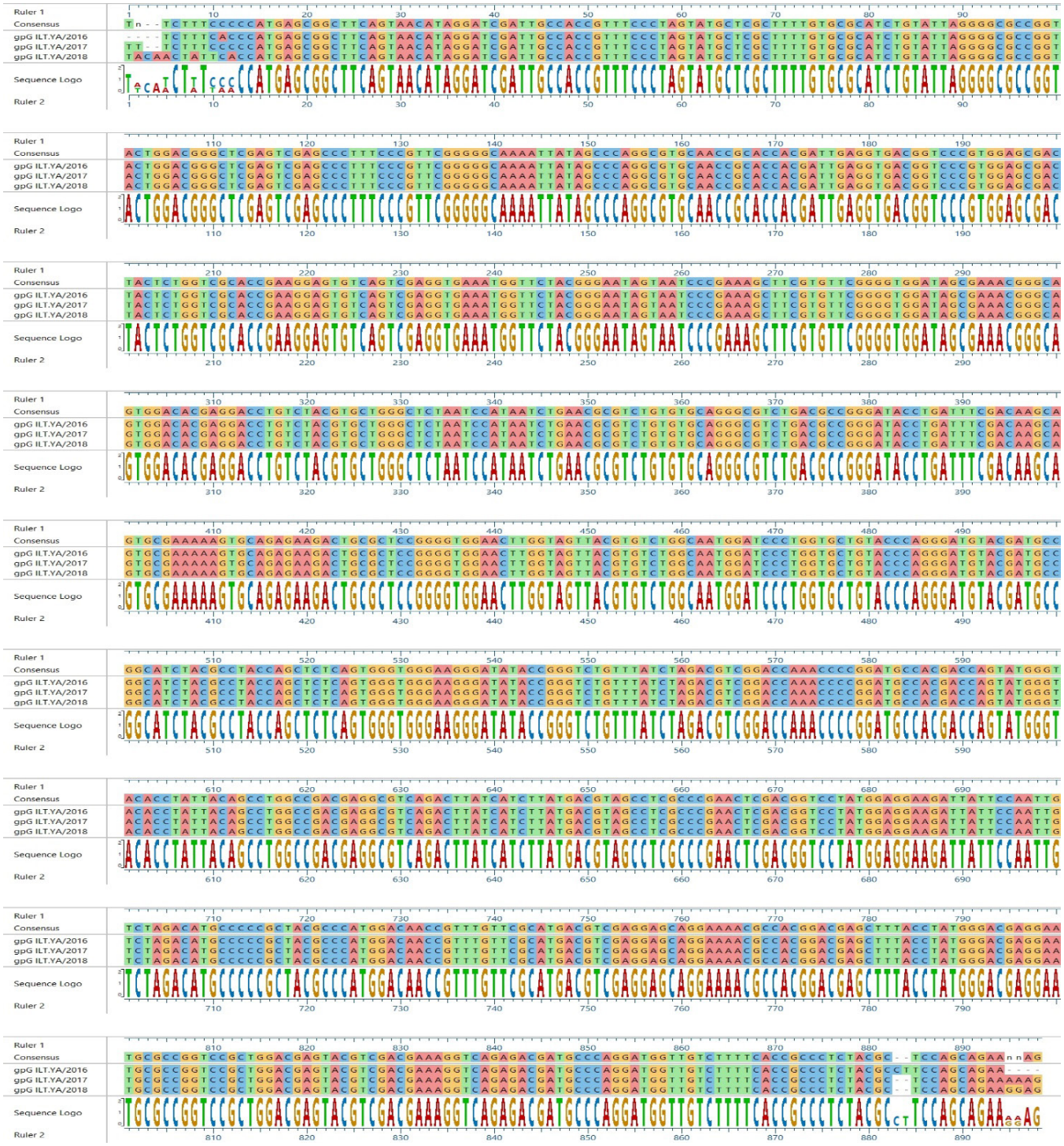


Phylogenetic tree constructed based on the three nucleotide sequence of ILTV glycoprotein B (blue rectangles) and the sequences obtained by blast search of the homologues sequences with identity over 90%

The aligned nucleotide sequence of assembled glycoprotein C of the three ILT isolates (ILT.YA/16, ILT.YA/17 and ILT.YA/18)



Phylogenetic tree constructed based on the three nucleotide sequence of ILTV glycoprotein C (blue rectangles).



The aligned nucleotide sequence of assembled glycoprotein G of the three ILT isolates (ILT.YA/16, ILT.YA/17 and ILT.YA/18)

Phylogenetic tree constructed based on the three nucleotide sequence of ILTV glycoprotein G (blue rectangles)