Molecular epidemiology of infectious bronchitis coronavirus in southern African poultry from 2011 to 2020

by

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16406703

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Production Animal Studies

in the

FACULTY OF VETERINARY SCIENCE

at the

UNIVERSITY OF PRETORIA

Supervisor:

Prof. C. Abolnik

Date of submission

31-10-2023

Declaration

I, Christine Strydom, student number 16406703 hereby declare that this dissertation, "Molecular epidemiology of infectious bronchitis coronavirus in southern African poultry from 2011 to 2020" is submitted in accordance with the requirements for the Magister Scientiae degree at University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

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Christine Strydom

31 October 2023

Ethics approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

4 November 2021

Approval Certificate
New Application

AEC Reference No.: REC120-21
Title: Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011- 2020
Researcher: Mrs C Strydom
Student's Supervisor: Prof C Abolnik

Dear Mrs C Strydom,

The **New Application** as supported by documents received between 2021-08-24 and 2021-10-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-10-25.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Poultry	
Samples Allantoic Fluid	320 (Stored Historic/Retrospective)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-11-04.

3. Please remember to use your protocol number (REC120-21) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days and must be subsequently submitted electronically on the application system within 14 days.

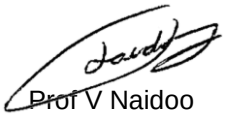
6. The committee also requests that you record major procedures undertaken during your study for own- archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely



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Lefapha la Diseanse tša Bongakadiruiwa

Dedication

I dedicate this research to my husband, Kobus Strydom, “op liefde en tee”

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- My two children, Kaleb, and Claire, I know I needed plenty breaks to work on this dissertation. I love you both endlessly.

Summary

Molecular epidemiology of infectious bronchitis coronavirus in southern African poultry from 2011 to 2020

By: Christine Strydom

Supervisor: Prof. Celia Abolnik

Department: Animal Production Studies

Degree: MSc (Veterinary Science)

Infectious bronchitis virus (IBV) (family *Gammacoronavirus)* is an ever-evolving avian pathogen that causes major economic losses within the poultry industry worldwide. Antigenic variations allow the virus to evade vaccine induced immunity and produce new variants at an alarming rate. Epidemiological studies of IBV are imperative to selection of vaccines. Field isolates (n=385) grown in SPF embryonated chicken eggs at Deltamune (Pty) Laboratory, that originated in commercial flocks were collected from Botswana, Eswatini, Namibia and South Africa's Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West, and Western Cape provinces from 2011-2020, were analysed in this study. A 745 bp region of the spike protein gene was amplified and sequenced, and phylogenetic analysis was performed. Twenty-four (6,2 %) samples contained mixed sequences from viral coinfections and were not evaluated further. In the remaining 364 isolates, 7 genetic lineages were identified. 184 (50,5%) viruses were identified as GI-19 (QX) strains and 78 (21,4%) as the GI-1 (Mass/H120) strain. 39 (10,7%) samples were identified as the GI-13 (4/91) lineage detected between 2011 and 2019, 29 (8,0%) as the GVI-1 (TC07-2) lineage detected from 2010-2020, 19 (5,2%) as the GI-23 (Var II) lineage from 2019-2020 with single isolates in 2010 and 2015, and 13 (3,6%) as the GI-11 (UFMG/G - Brazil) lineage detected from 2011-2020. Two (0.5%) viruses were closely related to GIV-1 (DE/072/92) lineages but were only isolated in 2011 and 2013. Three viruses from GI-1 and GI-13 may have been

recombinants, but further analysis is required to confirm this. Overall, this study reveals the co-circulation of diverse IBV field and vaccine-derived genotypes in southern African poultry flocks for the first time.

List of abbreviations

AI	Avian Influenza
APG	*Avibacterium paragallinarum*
BLAST	Basic Local Alignment Search Tool
CoV	Coronavirus
C-terminal	Free carboxyl group of a peptide
CPE	Cytopathic Effects
DNA	Deoxyribonucleic acid
E	Envelope protein
ELISA	Enzyme-linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic Acid
HI	Haemagglutination Inhibition
HVR	Hypervariable Region
IB	Infectious Bronchitis
IBV	Infectious Bronchitis Virus
IgG	Immunoglobulin G
ILT	Infectious Laryngotracheitis
ILTV	Infectious Laryngotracheitis Virus
IUPAC	International Union of Pure and Applied Chemistry
KZN	KwaZulu-Natal
M	Membrane protein
Mabs	Monoclonal antibodies
MASS	Massachusetts
MERS	Middle East Respiratory Syndrome
Min	Minutes

M-MLV	Molony Murine Leukaemia Virus
MTA	Material Transfer Agreement
N	Nucleocapsid protein
N-terminal	Free amine group of a peptide
NCBI	National Centre for Biotechnology Information
NDV	Newcastle Disease Virus
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cells
RdRP	RNA-dependant RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RO	Reverse osmosis
RT	Reverse-Transcriptase
rRT-qPCR	Real-time quantitative reverse-transcription PCR
S	Spike protein
S1	Subunit 1
S2	Subunit 2
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SAPA	South African Poultry Association
Sec	Seconds
TAE	Tris base, acetic acid and EDTA
TEM	Transmission Electron Microscope
UK	United Kingdom

USA	United States of America
US	United States
UTR	Untranslated Region
VI	Virus Isolation
VIM	Virus Isolation Medium
VN	Virus Neutralization
WGS	Whole Genome Sequencing
WOAH	World Organisation of Animal Health
ZA	South African

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Chapter 1

INTRODUCTION

Infectious bronchitis virus (IBV) is famous as the chicken (*Gallus gallus*) coronavirus. It is considered as one of the most significant diseases that has an economic impact on the poultry industry (Cavanagh, 2007). It can also be found on the World Organisation for Animal Health (WOAH) list under significant avian diseases and infections. The virus is prevalent in most every country where poultry farming is either established or on the rise (Jackwood et al., 2012) and affects both backyard as well as commercial poultry (Wickramasinghe, et al., 2014). While vaccines are readily available, the prevention of IBV is hindered by its great diversity in variants. Between serotypes there are little to non-cross-protection, which results in the administration of various different serotypes of vaccines in an attempt to protect against various potential field strains of IBV (Jackwood et al., 2012). In some cases, the use of a commercial vaccine can also result in poor immunity against local strains due to genetic variation (Bande, et al., 2015). It has also been proven that live vaccines can contribute to the emergence of new pathogenic variants through selection and mutation. Thus, the use of various strains of IBV simultaneously could potentially evolve to cause disease at a later stage (McKinley, et al, 2008).

Well known for its respiratory disease, the clinical symptoms of IBV can manifest in the form of coughing, sneezing, wheezing, watery eyes, and tracheal rales (Cavanagh, 2007, Jackwood et al., 2012). IBV is however not limited to the respiratory tract and can replicate within a variety of other organs. The level of pathogenicity observed depends on the type of variant the host has been infected with. These can manifest as nephritis and reproductive disorders (Cavanagh, 2007; Ignjatovic and Sapats, 2000). The virus can also infect the oviducts of hens, where it can cause permanent damage if infected at a very young age (before two weeks of age). This will result in poor laying abilities (Ignjatovic and Sapats, 2000). Thin walled, mishappened shells and loss of shell pigmentation are also indication of infection with IBV. In the kidneys, nephritis and urolithiasis could lead to mortality rates of about 25% in broiler chicks (Ignjatovic and Sapats, 2000). IBV can also reside within the intestinal tract and the

caecal tonsils and can then be excreted for weeks on end in those chickens that have recovered or that have been vaccinated (Cavanagh, 2007).

Direct contact between infected chickens is most likely the most common source of infection and transmission. This can be either through respiratory droplets and aerosols or through faeces (Cavanagh, 2007; Ignjatovic and Sapats, 2000). Indirect transmissions are also possible through contaminated utensils, boots, clothing and farm workers that do not follow good biosecurity practices(Ignjatovic and Sapats, 2000). Vertical transmission via eggs has previously been observed by McFerran et al., 1971 and demonstrated by Cook in 1971.

Virus isolation is one of the recommended methods for diagnostic confirmation of clinical cases, but not routinely performed due to time and cost constraints (Cook et al., 2012). It is however useful in circumstances where vaccine production is considered, or further tests are required such as whole genome sequencing (WGS) or for pathogenicity testing of the identified IBV variant (Bande et al., 2016; Legnardi et al., 2020). Cell cultures should not be used as the primary isolation method since it is most often necessary to adapt IBV strains in embryonated eggs before any cytopathic effects (CPE) can be observed in chick embryo kidney cells (Bande et al., 2016; Jackwood and De Wit, 2013). Real-time quantitative reverse-transcription PCR (rRT-qPCR) can also be used to confirm clinical cases, this however does not necessarily confirm the presence of live virus but gives an indication of virus presence at some recent point since viral RNA can remain for a period after infection has cleared (Legnardi et al., 2020). Indirect enzyme-linked immunosorbent assays (ELISAs) are mainly used to determine the immune status of a flock and whether seroconversion has taken place after vaccination (Ignjatovic and Sapats, 2000). It can also give insight as to whether a flock had been exposed to IBV infection a few weeks prior to testing serum or whether a field challenge had occurred due to large abnormal increase in titres. For proper interpretation, routine monitoring of flocks within a local area should be done and proper antibody baseline titres should be established (Legnardi et al., 2020).

Vaccines are currently one of the most significant tools used to control and prevent IBV infections from spreading within the poultry sector (Ignjatovic and Sapats, 2000).

Given the genetic diversity of IBV and the ability of RNA viruses to mutate at a rapid rate, it does however require careful consideration in the selection of the type of vaccine strain to be used. Biosecurity plays a vital role in preventing the transmission of IBV between sites and houses (Legnardi et al., 2020). At the rate at which new variants appear, it is, however, important to monitor antigenic drifts in the field since vaccines based on a specific serotype tend give little to no protection against IBV of a different genetic group. The regular emergence of IBV variants cause different disease situations as well as different vaccination programmes in different geographical locations (Jackwood et al., 2012).

The aim of this study was to investigate the genetic diversity and evolution of IBV in South African poultry and that of some neighbouring countries during the 2011-2020 period. With a better understanding of the disease's distribution and knowledge of the predominant IBV variants, targeted control measures can be implemented (such as choosing the correct vaccines) and thus ultimately reducing the impact of IBV on poultry populations within the sub-region.

Chapter 2

LITERATURE REVIEW

2.1 A brief history of IBV

In the early 1930s, Schalk and Hawn (1931) were the first to describe infectious bronchitis (IB) as a new respiratory disease in young chickens between the ages of 2 and 3 weeks in North Dakota, USA. Symptoms included listlessness and gasping (Cook, Jackwood and Jones, 2012; Fabricant, 1998) and the mortality rates ranged between 40-90%. In these early days, infections were often confused with infectious laryngotracheitis (ILT) (Fabricant, 1998). The confusion was later cleared up by Beach and Schalm (1936), who performed cross-immunity studies and proved that ILT was in fact distinguishable from IB, as well as from coryza (*Avibacterium paragallinarum*). Beaudette and Hudson (1937) were the first to isolate IBV in embryonated eggs using the chorioallantoic route. They noted that IBV was able to cause embryo mortalities. Furthermore, ILTV and Fowl Pox viruses caused lesions on the chorioallantoic membrane where IBV did not. They also observed that the longer IBV is passaged through embryonated eggs, the virus increased in pathogenicity towards embryos, but became less virulent towards chickens (Beaudette and Hudson, 1937).

Delaplane and Stuart (1939) described IBVs economic impact in 1939. A significant decrease in egg production was observed in older chickens. These claims were confirmed by similar observations made throughout the US in the years that followed (Fabricant, 1998).

Later, in the 1950's a new IBV strain was identified through viral neutralisation (Jungherr, et al., 1956). The researchers conducted a virus neutralisation study using four different strains, one of which was a field isolate from Connecticut, USA. Vaccination of birds with the Massachusetts strain could not provide cross-protection to infection with the Connecticut strain. Since then, many different serotypes and genotypes of IBV have been identified through viral neutralisation and molecular sequencing (Jackwood, 2012) and often, current vaccines that are being used commercially cannot provide cross protection against these new strains. There have also been several European, African, Australian, Asian, and South American variants

that have been identified as unique to the geographic region with no relation to the vaccine strains of the USA (Jackwood, 2012; Valastro, et al., 2016).

The first report of IBV in South Africa was in 1984 by Morley and Thompson. They reported that the most probable cause in their case of Swollen Head Syndrome (SHS) in chickens was likely to be IBV, since the virus was most consistently isolated in embryonated eggs from the affected flocks. They also proved through virus neutralization (VN) tests that this IBV was different strain to the most common genotypes known to be circulating within the USA and the Netherlands (Morley and Thompson, 1984).

Few studies have been published on the prevalence of IBV in South Africa. A study in the QwaQwa region (Thekisoe, et al., 2003) focused on the collection of serum samples in the area and testing for the prevalence of IBV-specific antibodies using ELISA's that could not identify the circulating variants. Knoetze and coworkers (2014) did a study on IBV variants present in the KwaZulu-Natal province, predominantly from 2011-2012, based on full spike (S1) gene sequencing. They found that the most prevalent genotypes circulating within the region were the Massachusetts (H120) and QX-like serotypes. Other genotypes were also present in this study, but no sequence data was obtainable even though there were clear bands after the RT-PCR. The reasons for this remain unknown and these isolates weren't studied any further (Knoetze et al., 2014).

Vaccination against IBV has been reported as early as the 1940's where protection was shown in laying birds by exposing them to attenuated IBV that had been passaged through embryonated eggs (Cook et al., 2012). The vaccine was administered to only a limited number of birds in the flock and allowed to spread further, naturally. Maternal antibodies allowed for initial protection of newly hatched chicks. The first IBV vaccine was developed in the 1950's using the Van Roekel M41 (Mass) strain. Later in the 1960's, the Netherlands produced what is known today as the most widely used vaccine worldwide i.e., the "H" strain of the Mass serotype, H120 and H52 was named after the farmer from where the isolate was acquired. H120 is a mild version of IBV, usually administered from day one of age, attenuated through 120 embryo passages,

while H52 is normally used as a second vaccine and was attenuated by only 52 passages (Cook et al., 2012).

By the 1970's new strains, also known as variants of IBV, were being described worldwide and it had become increasingly apparent that the Mass-type vaccines were unable to protect against these new variants (Cook et al., 2012). Some variants are indigenous to certain areas, while some are more globally distributed (De Wit, Cook and van der Heijden, 2011). A good example of a globally distributed strain is that of the GI-19 (QX-like) variants. This variant was believed to have originated in China and has since then spread across Europe, Asia and Africa in a short amount of time (De Wit, Cook and van der Heijden, 2011; Valastro et al., 2016). The GI-19 strain has also shown a tendency to cluster according to country (Franzo et al., 2017). The vast number of new variants of IBV being described was too large and it proved impossible to develop a new vaccine against each new strain. New variants were also disappearing at a high rate, so that by the time a new vaccine was evaluated, the strain was no longer relevant, and a new variant was a cause of concern. It is therefore imperative that new variants need to show sufficient importance to warrant the development of a new vaccine (Cook et al., 2012).

Currently, there is little information available on the viruses that circulated in the past and are currently circulating within South African poultry flocks. To select the best vaccines to help protect against circulating field viruses in South Africa, poultry veterinarians submit samples from clinical cases to diagnostic laboratories that offer specialised IBV diagnostic services. Service providers use Real-Time RT-PCR and DNA sequencing to obtain this information; however, the data is not publicly available. Once a variant is identified, the poultry veterinarian can select the most appropriate combination of registered vaccines to gain the best coverage and cross-protection (Bisschop, S. P. R. and Poolman P., personal communication, 2021). Live vaccines are typically given to protect broilers, layers and breeder pullets. Killed oil emulsion vaccines are usually administered to boost immune response of layers and breeders before egg production onset (De Wit and Cook, 2020).

2.2 Clinical signs and symptoms of IBV

Losses occur through mortalities, deficient performance through histopathological changes in the oviduct, decreased egg production and decreased quality of eggs (Ali, 2008). It is an acute, highly contagious viral disease that affects the domestic chicken (*Gallus Gallus domesticus*) and other poultry. Initially known for damaging the respiratory tract, different and emerging strains can also affect the reproductive system, cause nephritis in the renal system, and replicate in the epithelial cells of the intestinal tract (Abolnik, 2015; Albassam et al., 1986, Cavanagh 2007; Nieto et al., 2022; Winterfield and Albassam, 1984). IBV might also be linked to cockerel infertility (Cook et al., 2012). Other clinical symptoms include nasal and eye discharge, coughing, sneezing and depression. A loss of ciliated epithelial cells in the respiratory tract can be seen frequently, where histology is performed (Cook et al., 2012).

The clinical signs of reproductive damage can be linked to a decline in egg production and, early exposure of layer chicks to the virus can lead to what is known as “false layers.” The virus infects the oviduct and causes abnormalities such as cystic, non-patent ovaries, which in-turn leads to the inability to lay eggs externally (Broadfoot et al., 1956; Crinion and Hofstad 1972; Cook et al., 2012). Because of its contagious nature, the morbidity rate of IBV infection is usually at 100%. The mortality rate could be well below 50%, but opportunistic pathogens as *E. coli* cause secondary infections and further complications follow (Jackwood, 2012; King and Cavanagh, 1991; Wang et al., 1997).

2.3 Viral characteristics and structure

Coronaviruses are categorized into the order Nidovirales, family Coronaviridae, and are further divided into four genera: Alpha-, Beta, Gamma, and Deltacoronaviruses. Alphacoronaviruses have been known to infect cats, dogs, humans, and swine. *Betacoronaviruses* are better known to infect various mammalian species which include bats, humans, rodents, and ungulates (Abolnik, 2015; Woo et al., 2012).

The recent outbreak of SARS-CoV-2, as well as the previous outbreaks of SARS-CoV and Middle East Respiratory Syndrome (MERS CoV), are all classified under the *Betacoronavirus* genus (Pal et al., 2020 and Abolnik, 2015). *Gammacoronaviruses*

include strains that infect birds and whales while *Deltacoronaviruses* are known to infect birds, cats, and swine (Woo et al., 2012).

The name Coronavirus is famously derived from the Latin word “corona” is and has the meaning of “crown” or “wreath” (Estola, 1969). It is a characteristic feature of coronaviruses that they possess a "club-shaped" surface projection, which is formed by the Spike (S) protein and surrounds the virion. On an electron micrograph these projections form a solar crown or halo (Figure. 1).

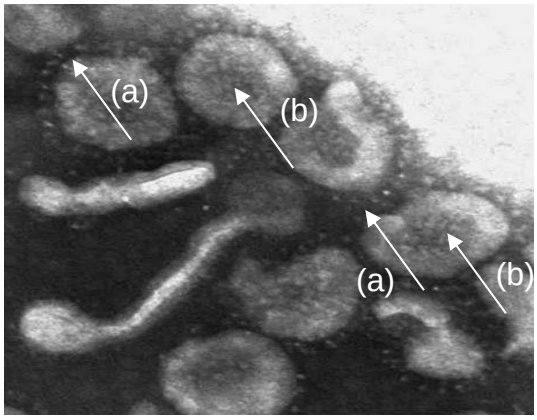

Figure 1. TEM micrograph of typical IBV-particles showing the characteristic depression and a “crown” of projections forming a corona (crown) at the periphery of each particle (source: Putterill, 2014). Arrows (a) indicate the spikes on the surface of the virus that forms the projection. Arrows (b) indicate characteristic depression of a virion.

The first ever reported coronavirus and only known CoV of domestic chickens, is Infectious Bronchitis Virus (IBV) (De Wit and Cook, 2020). It is one of the most studied coronaviruses and causes major economic burden to the poultry industry worldwide (Cavanagh, 1983; Hofstad, 1978).

IBV is classified within the *Gammacoronavirus* genus. The genome of IBV consists of a single-stranded, linear positive-sense RNA molecule that is 27.6 kb in length. The viral genome encodes various structural proteins such as, spike (S), envelope (E), membrane (M) and nucleocapsid (N). Additionally, it encompasses the RNA-dependant RNA polymerase (RdRp), polyproteins 1a and 1ab and several accessory or non-structural proteins (Jackwood, 2012; Lin and Chen, 2017). The whole genome

contains approximately 10 open reading frames (ORFs) (Leghari et al., 2016). Following a typical pattern, the IBV genome follows the sequence, 5' UTR-1a-1ab-S-3a-3b-E-M-5a-5b-N-3'UTR (Abolnik, 2015; Jackwood et al., 2012; Wibowo, et al. 2019).

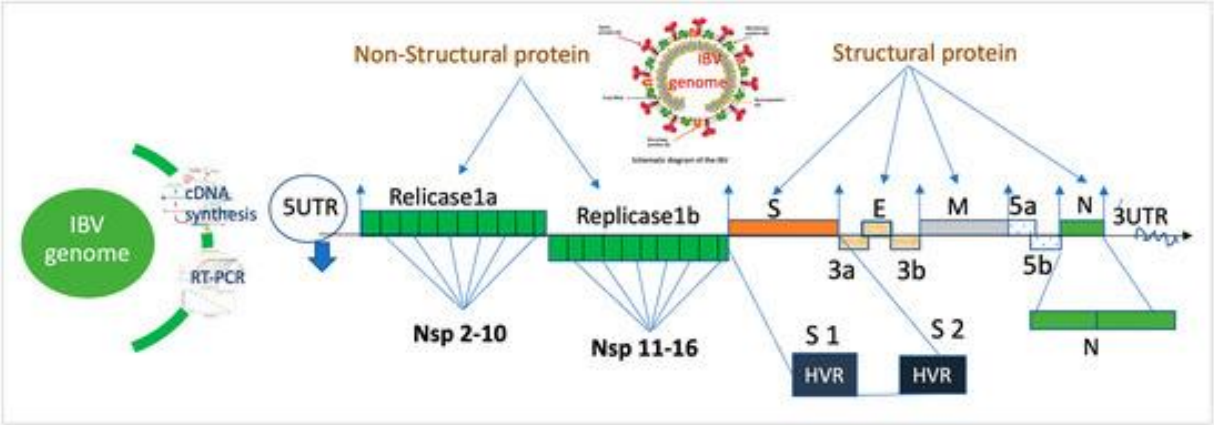

Figure 2. Morphology and genomic structure of IBV with different structural and non-structural viral genes (source: Bhuiyan, et al., 2023. Adapted from Bhuiyan et al.,2021).

The M protein is critical in all coronaviruses and is necessary for the incorporation of viral components into new virion. It gives the virus particle it is structural integrity through interactions with other structural proteins (Abro, 2013). The E protein is a small important structural protein that forms part of the virion envelope alongside the M protein and is essential for virus assembly. Any mutation to an envelope sequence could affect virus assembly within cells (Abro, 2013). The S protein is also known to interact with the transmembrane region of the M protein (Cavanagh, 2007). The N protein is a key component of the ribonucleoprotein (RNP) and is tightly bound to the RNA genome. It consists of 409 amino acids and is known to play an important part in various viral assembly processes. These include, viral assembly and packaging, core formations and conducting host cell processes (Abro, 2013). The S glycoprotein is the largest protein and is a polypeptide approximately 1162 amino acids in length. It is found on the surface of the viral membrane (Arbo, 2013.; Armesto et al., 2011; Ignjatovic and Sapats, 2005). It plays an important role in the infection of host cells by binding to target cell receptors and fusion of the virus membrane to the cell membrane (Armesto et al., 2011).

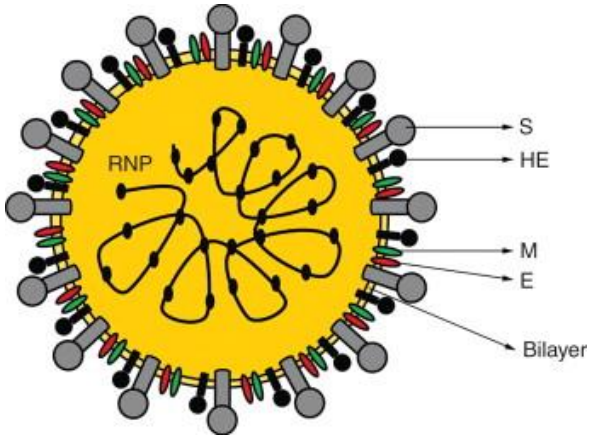

Figure 3. The schematic structure of the coronavirus displays a spherical virion envelope with a prominent crown of peplomers, comprising of S (spike) glycoprotein. Additionally, the virion includes HE (hemagglutinin), E (small envelope protein), and M (membrane glycoprotein). The genome is a positive-stranded RNA associated with the N (nucleocapsid phosphoprotein), forming the helical RNP (ribonucleoprotein) (source: Boncristiani et al., 2009).

2.4 The Spike protein

The S glycoprotein can form either a dimer or a trimer, and comprises four domains: a signal sequence, the ectodomain, the transmembrane region, and the cytoplasmic tail. (Armesto et al., 2011; Cavanagh, 2007). It is divided into two subunits namely, S1 and Subunit 2 (S2) that remain associated through noncovalent bonds (Callison, et al., 1999; Cavanagh, 2007; Cavanagh, et al., 1997). The S1 subunit is found on the N-terminus and forms the globular head of the S glycoprotein. It functions are the receptor-binding to host cells and the initiation of serotype specific, viral neutralizing, and haemagglutination inhibiting antibodies (Britton et al., 2012; Callison, et al., 1999; Ignjatovic and Galli, 1995). Glycans found on the surface of the S glycoprotein are known to play roles in the viral binding process, entry, protein folding and to help protect the spike protein from neutralizing antibodies (Wei et al., 2010; Zheng et al., 2018).

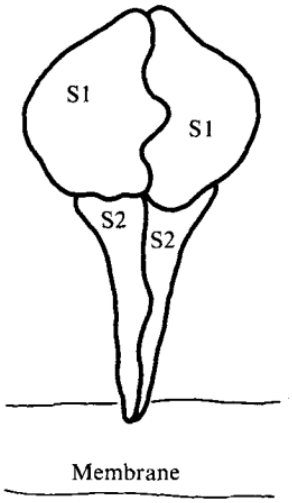

Figure 4. A speculative model for the S protein of IBV (source: Cavanagh, 1983).

The S1 subunit contains over 500 amino acids (Britton et al, 2012; Cavanagh, 2007) and forms the globular head of the S glycoprotein. The S1 region is one of the most studied gene components (Lee and Jackwood, 2001). The S1 subunit is not only extremely variable but also determines the different serotypes of IBV. For most IBVs, the difference in amino acids for serotypes can range anywhere between 20-25%, but can vary up to 50% (Cavanagh, 2003; Cavanagh, 2007; Cavanagh et al., 2005; Valestro, et al., 2016). In some cases, variant differences with as little as 2-3% in the amino acid sequence of the S1 subunit can present as a different serotype (Cavanagh, 2003; Wibowo et al., 2019; Zheng et al., 2018). A variation in amino acids of as little as 5% in the S1 sequence of IBV has also caused a lack of cross-protection in vaccines (Cavanagh, 2003). The variation that occurs throughout the whole genome is due to the limited proofreading abilities of the RdRp (Moreno et al., 2016). The S1 subunit can be divided into three different hypervariable regions namely: HVR 1, HVR 2 and HVR 3 (Moore et al, 1997; Valastro, et al., 2016). They are located at amino positions 38-67, 91-141, and 274-387, respectively (Abro, 2013). It is thought that neutralizing epitopes are mainly directed against the HVR regions (Moreno et al., 2016). The S2 subunit is found at the C-terminal and just over 600 amino acids (Britton et al, 2012). The S2 subunit's ectodomain has a fusion peptide-like region and two heptad repeat regions which through oligomerisation allows for membrane fusion to susceptible cells. It forms the stalk part of the S protein and anchors the S protein to the viral membrane. The S2 subunit is also the most conserved domain and different

serotypes have an amino acid sequence identity of ≥ 90% (Cavanagh, 2007; Wickramasinghe et al., 2014).

2.5 IBV classification

IBV has been classified using both functional and non-functional (phenotypic) tests (De Wit, 2000). Functional tests are those that focus on the biological functions (antibody-based methods) of a virus and can be divided into serotype, epitope types and protectotypes. Non-functional tests are those which focus more on the viral genome (nucleic acid-based methods) and is referred to as genotyping (De Wit, 2000). More recent reviews and studies focus on genotypes rather than the serotypes or protectotypes of IBV. The complexity, number of variants and costs of serotyping has led to phenotypic tests being used less frequently, though embryonated eggs are still being used for isolation and identification of the virus as well as vaccine production (Cook et al., 2012).

As mentioned previously, the first time it was proven that different variants exist was when Jungherr et al. (1956) observed that the Connecticut strain showed no cross-protection to a challenge of the Mass isolate. Even though a high number of variants can occur due to genetic mutations, errors, insertions, deletions, point mutations or RNA recombination only a few of these strains are likely become endemic to an area under the local conditions (Lee and Jackwood 2001 and Lin and Chen, 2017). Recombination within the S glycoprotein can give rise to novel serotypes or strains due to its role in host cell attachment and the presence of epitopes that can neutralize the virus (Jackwood and de Wit, 2013). Other strains either have no long-term effects or can disappear from where they first were identified, just to reappear in a different area but thrive there instead (Lin and Chen, 2017). The confirmation of more than a single IBV serotype co-circulation led to awareness that more effort needed to be afforded for control of the disease (Cook et al., 2012).

Recombination is common in some RNA viruses, including IBV, and plays a significant role in the virus' evolution (Cavanagh, 2007; Lee and Jackwood, 2000). The cause of recombination is thought to be that of template switching during viral replication when the host is infected with two different viral strains (Jarvis and Kirkegaard,1992; Lee and Jackwood, 2000). The recombination process involves exchange of genetic material between the two strains resulting in a new variant

carrying the genetic material from both parent strains (Jarvis and Kirkegaard,1992). The presence of compatible RNA segments in both parent strains and RNA secondary structure and interactions play a role in promoting RNA recombination (Jarvis and Kirkegaard,1992). A good example of RNA recombination would be that of the DE072 strain of IBV. The study, done by Lee and Jackwood (2000), revealed that the DE072 shared similar origins with that of the D1466 vaccine strain of the serotype D212 from the Netherlands, as well as similar sequences to the Mass41 strain of IBV, which was likely obtained through recombination.

Serotypes are classified using mainly viral neutralisation test through inoculation into either SPF embryonated eggs or tracheal tissue cultures. The serotype itself is based on the reaction of a specific IBV strain to antibodies raised against of different IBV strains. A binding neutralisation reaction of two different virus variants are considered as closely related, and thus grouped together as one serotype (Cowen and Hitchner, 1975). This method has been described previously as the “gold standard” for determining the different IBV serotypes despite the variances in methods used. Haemagglutination Inhibition has also been used to classify IBV into different serotypes as described by Alexander et al. (1983).

Epitope typing makes use of monoclonal antibodies (Mabs) and detects specific epitopes that are present within viral antigens through antigen-capturing enzyme-linked immunosorbent assay (ELISA) and immunofluorescent antibody techniques. Mabs specifically target the HVR’s of the S1 glycoprotein. False-negative results have been known to occur when conserved epitope regions are targeted. If an epitope is to show a mutation it does not necessarily change the serotype of the virus (Houta et al., 2020).

Protectotypes are those IBV’s classified according to an immune response of a chicken against a field challenge of IBV and offer direct insight into the efficacy of a vaccine (De Wit, 2000). They protect against a wider range of IBV’s than the strain contained within the vaccine. The number of protectotypes are unknown (De Wit, 2000). Small changes in amino acid sequence of S1 gene could lead to a new serotype of IBV, even if most of the viral genome remains the same. Vaccines containing a similar viral genome thus would be able to protect against the clinical disease of the said newfound serotype but to determine whether a strain is a

protectotype, cross-immunisation studies must be performed (De Wit, 2000). Combining two different vaccines, for example, the Mass strain at 1 day old and the 4/91 at 14 days of age, could also provide protection against different serotypes circulating within the region (Jordan, 2017).

Genotypes are the classification of IBV variants into different groups based on sequencing, detection of nucleic acids or the cleavage site determination of enzymes, targeting mainly from the spike glycoprotein (Houta et al., 2020) especially the S1 subunit due to its variability and antigenic properties. Genotypes and serotypes do not always correspond (Lee et al., 2003 and Gallardo et al., 2010). Genotyping to classify IBV variants can be done using both the complete S1 gene sequence or a smaller part of the sequence, usually limited to the three HVRs of the sequence (Cavanagh etal., 1988 and Moore, Jackwood and Hilt., 1997). Targeting only a small portion of the spike glycoprotein gene would not necessarily give a proper representation of which genetic group a particular IBV variant falls within. Longer sequence segments of the S1 region does however produce similar results to that of serotyping (Lee, et al., 2003 and Gallardo, et al., 2010, Valastro, et al., 2016). It is suggested that more than one HVR region is targeted. HVRs 1 and 2 is located between 112 and 423 nucleotide positions, and HVR 3 that is located between 820 and 1161 of the S1 gene, respectively (Valastro, et al., 2016). Similarly, there have been studies that show analysing a smaller portion of the S1 genome and comparing it to the results of the full genome could place an identified variant an entirely different lineage (Valastro, et al., 2016). The researchers demonstrated this by creating two additional Maximum-likelihood phylogenetic trees from their sub-sampled data set, targeting the three HVR regions (Valastro, et al., 2016). The contrast in results were striking, revealing that when comparing shortened sequences of HVR1 and 2 and that of the complete sequences of the S1 gene, the lineages GIII, V, and VI clustered within the GI lineage (Valastro, et al., 2016). Similarly using the HVR 3 region when compared to the data from full genome sequences, 8 of lineages (GI-5, -7, -10, -18, -22, -24, -25, and -27) were no longer classified as monophyletic (Valastro, et al., 2016).

2.6 IBV Vaccines

The spike protein has been proven to induce both cellular and humoral immunity within their poultry hosts. The circulation of IBV antibodies does not necessarily

provide protection against all IBV's since multiple serotypes can be present within a specific region (Ignjatovic and Sapats, 2000; Cavanagh. 2007). Antibodies generated are specific towards the vaccine strain that was administered and/or the field strains the chickens have been exposed to (Ignjatovic and Sapats, 2000; Cavanagh. 2007). The most effective preventative and control measure for IBV thus far has been vaccination (Ignjatovic and Sapats, 2000; Bhuiyan et al., 2021), but continuous appearance of new strains warrants the continuous development of new vaccines (Tizard, 2020). IBV is mainly controlled with a single or combination of live attenuated vaccines as well as inactivated booster vaccines (Vermeulen et al., 2023). Other vaccines available or being evaluated are viral vectors, DNA plasmids and recombinant vaccines (Bhuiyan et al., 2021).

Live attenuated vaccines are manufactured by means of the successive passage of field isolates in embryonated eggs. These isolates rapidly mutate which result in the ultimate attenuation of the virus. Live vaccines can range between mild to virulent depending on the degree of attenuation (Bhuiyan et al., 2021). Some virulent vaccines can cause tissue damage and other clinical symptoms such as airsacculitis under stressed and adverse environmental conditions. This could also pave the way for secondary infections especially in day-old chicks (Bhuiyan et al., 2021). The process becomes complicated since the virus has only a certain number of mutations that cause the loss of virulence and the retention of immunogenicity (Britton et al, 2012; Wickramasinghe, et al., 2014). It can take up to a year to produce an attenuated field strain. A balance needs to be found to keep the immunogenicity of the virus while altering the pathogenicity of the virus for commercial use. After this process however there is a possibility that the virus might regain its pathogenicity after being administered and revert to virulent virus, causing an undesirable outbreak on farms again (Britton et al, 2012). Despite the difficulties live attenuated vaccines are considered the gold standard to produce high immunity responses (Wickramasinghe, et al., 2014) and usually contain the Massachusetts strain alone or in combination with other strains.

Layers and broiler breeders usually receive inactivated vaccines right before the point of lay at 13 – 18 weeks (Bhuiyan et al., 2021). The serotype of inactivated vaccine administered depends on the geographical location. The most predominant

serotype present within the area would be the best option to provide cross-protection (Ignjatovic and Sapats, 2000). Inactivated vaccines are usually those vaccines produced from allantoic fluid containing IB virus, chemically inactivated with an agent that binds to and destroys the genomic RNA such as formalin, and adjuvanted by aluminium salt and formulated as an oil-emulsion (Bhuiyan et al., 2021; Ellis, 1999 and). They can be administered as either single or combination of antigens or as a multivalent vaccine that contains two or more serotypes of IBV (Bande et al, 2015; Bhuiyan et al., 2021). Inactivated vaccines stimulate only the humoral immune response but provide long lasting immunity without the side effects of live vaccines (Bande et al, 2015; Bhuiyan et al., 2021; Cook et al., 2012).

Recombinant vaccines have demonstrated the capacity to elicit immunity at levels like those achieved by live-attenuated vaccines. These vaccines are engineered to express specific immunogenic protein subunits without requiring the inclusion of a virulent organism (Bande et al, 2015). These vaccines have been investigated using Fowlpox virus, adenovirus, avian metapneumoviruses, as well as some other viruses (Tizard, 2020). Recombinant vaccines can be divided into different categories namely, viral vector-based, DNA plasmid, subunit, and peptide-based, and reverse genetic vaccines (Bhuiyan et al., 2021).

Viral vectored vaccines have resulted in variable immunity responses. Targeting of a single gene to induce both humeral and cellular immunity has been less successful than using those genes that encode multiple structures (Guo et al., 2010; Yang et al., 2009). Vector vaccines have proven rather successful in providing immunity against other poultry pathogens including Newcastle Disease Virus (NDV). Only partial protection has been proven so far by presenting the spike glycoprotein using organisms such as *Mycoplasma gallisepticum* and recombinant baculoviruses (Shil et al., 2011; Zhang et al., 2014). Fowlpox virus has been shown to stimulate an immune response with not only the expressing of the S1 protein but also that of the IFN-y gene to help protect against the side-effects of using Fowlpox virus as a vector and combatting stress (Shi et al., 2011; Tizard, 2020). In a different study Fowlpox virus expressing the S1 protein along with the IL-18 gene also produced favourable results, through enhancing immune responses (Chen et al., 2010). Recombinant

adenoviruses have also successfully expressed the S1 protein in their experimental hosts by a single dose (Johnson et al., 2003; Toro et al., 2014).

Plasmid DNA vaccines express a major antigen in the backbone of a specific virus, that is usually regulated by the host protein translators under a host promoter. They can express either a single or multivalent construct, using either single or multiple host promoters (Tizard, 2020). DNA vaccines injected *in ovo* at 18-19 days, while providing an immune response, did not provide sufficient protection as a single dose. When combined with the live vaccine administration at 14 days of age or injected intramuscularly twice, they could potentially play a role in eliciting a protective immune response against IBV (Kapczynski et al.,2003).

Subunit and peptide-based vaccines utilize a part of the antigen or antigenic characteristics presented by bacteria, yeast, or eukaryotic cell lines. Subunit vaccines are formulated like a purified virus, but only use part of the genome coding immunogenic epitopes or viral peptides (Tizard, 2020). One study reported that with the use of *Lactococcus lactis* expressing a multi-epitope gene (*EpiC*) of IBV along with the gene of the cell-wall anchoring domain of *Staphylococcus aureus* protein A, a significantly high humoral and mucosal immune response was possible (Cao et al., 2013). Reverse genetic vaccines are produced by altering the full genome cDNA copy of RNA viral genomes to reduce the pathogenicity of novel virus strains but still stimulate the immune response to produce antibodies against the specific antigen (Tizard, 2020).

A wide variety of vaccines are available in South Africa. Vaccine producing companies conduct local molecular epidemiological studies to determine which IBV variants are circulating in a particular region, including South Africa, but the information is only for company use and not publicly available. Table 1 below outlines the periods when certain IBV vaccines were registered in South Africa (Botha, C., Coetzer, M., Dawson, S., Malan, H., personal communication, 2022).

Table 1. Commercial IBV vaccines registered for use in South Africa.

Vaccine/IBV Strain	Year of vaccine registration/first use	Manufacturer	Type
Nobilis IBMA5 (Mass-strain)	1996	MSD Animal Health	Live attenuated vaccine
Bioral H120 (H120-strain)	1998	Boehringer Ingelheim	Live attenuated vaccine
Gallimune 203 (Mass-strain)/407 (Mass-strain)	2001	Boehringer Ingelheim	Inactivated vaccines
Gallimune 503 (Mass-strain)	2004	Boehringer Ingelheim	Inactivated vaccine
TAbic H120 (H120-strain)	2005	Phibro	Live attenuated vaccine
TAbic VAR 223 (793/B-strain)	2013	Phibro	Live attenuated vaccine
Poulvac IB QX (QX-strain)	2013	Zoetis	Live attenuated vaccine
Nobilis IB 4-91 (4-91 (793/B)-strain)	2013	MSD Animal Health	Live attenuated vaccine
Volvac AC Plus + ND + IB + EDS KV (Mass-strain)	2013	Boehringer Ingelheim	Inactivated vaccines
Bioral H120 Neo (H120-strain)	2014	Boehringer Ingelheim	Live attenuated vaccine
TAbic IBVAR206 (Var-2-strain)	2015	Phibro	Live attenuated vaccine
Hatchpak IB H120 (H120-strain)	2015	Boehringer Ingelheim	Live attenuated vaccine
Nobilis IB Primo QX (QX-strain)	2016	MSD Animal health	Live attenuated vaccine
Poulvac IB Primer (793/B-strain)	2018	Zoetis	Live attenuated vaccine
CEVAC IBIRD (793/B-strain)	2018	Ceva	Live attenuated vaccine

2.7 Serotyping of IBV

The serotyping of IBV involves the identifying and categorising of different strains based on specific antigenic characteristics. Serotyping of IBV variants have been done using haemagglutination inhibition (HI) and virus neutralization (VN) tests.

The VN test in embryonated eggs was the first test used to determine the serotype of IBV circulating within a population, but repeated passaging could alter the virulence and the antigenic properties of the virus (Cook et al., 1976). VN using tracheal organ cultures were then demonstrated by Cook et al. (1976) who also devised a standardised method for VN tests to be performed. Two different methods of VN's exist: the alpha method uses a constant serum concentration that reacts with different dilutions of virus, whereas the beta method maintains a constant virus amount against various dilutions of serum (Hesselink, 1991 and De Wit, 2000).

Most but not all IBV strains have been proven to agglutinate Red Blood Cells (RBCs) after treatment with neuraminidase (Schultze et al, 1992). The neuraminidase treatment removes the α2,3-linked N-acetylneuraminic acid from the surface of an IB virion, which in turn gives IBV haemagglutination properties (Schultze et al, 1992). This allows for the use of HI tests for diagnosis of specific strains or the monitoring of vaccines. The procedure for HI testing on IBV variants was described first described by Alexander et al., 1983. HIs are used to quantify antibodies against that of IBV antigens and works on the principal that when IBV antigens are mixed with a specific anti-IBV antibody, they inhibit agglutination of RBC's. By measuring the highest dilution of antibodies that can inhibit haemagglutination, the HI titre is determined providing an indication of the level of immunity or exposure to a specific variant of IBV. HIs are considered less trustworthy than that of VN tests because strong and variable cross-reactions can occur (Houta et al., 2020)

Another serological test that is widely used but mainly only for the monitoring and determination of the immune status within a flock is ELISA (O'Connor et al., 2013). ELISA does, however, have the potential to be used for serotyping and experimental studies (with the use of S-specific antibodies) have proven somewhat successful (Karaca and Syed,1993).

Through development of a blocking ELISA and the use of Mabs the experiment proved to correlate with that of the VN test (Karaca and Syed,1993). However, similar cross-reactions, as with VN, were also observed. No commercial kits are available for this method.

2.8 Genotype grouping and classification

IBV genotyping research is predominantly focused on the surface S protein gene that is recognised for its antigenic variation (Cavanagh, 2007). IBV amino acids can differ with as little as 5% in the S1 protein, making it the ideal region for genetic variability determination through partial or complete S1 genome sequencing (Valastro, et al., 2016). The most variability amongst different IBV strains can be found in the S1 region due to recombination of the RNA, errors, and mutations (Nabavi et al., 2016). However, there have also been issues where the partial genotyping and amplification on different regions of the S1 gene has often led to the incorrect grouping between closely related strains (Valastro, et al., 2016). This makes full S sequencing the ideal method for classification of variants (Lee, et al., 2003).

Studies have been conducted that show the genotyping of the hypervariable regions containing both HVR I and HVR II could as successfully differentiate IBV types and recognise new variants as the whole genome sequence (Lee, et al., 2003; Gallardo, et al., 2010). Primers were developed to target as many variants as possible. This method may however not correlate with serotyping, since other regions in the S protein may also contribute to serotype differentiation (Lee, et al., 2003, Li etal., 2012). The classification of genotypes according to the system suggested by Valastro et al., (2016) is based on the S1 gene. 32 lineages based on geography were defined and in turn fall under 6 different genotype groups, namely, GI to GVI. 27 lineages cluster into the GI genotype whereas the remaining 5 genotypes comprise of a single lineage each (Valastro et al., 2016). Since then, there have been additional lineages added to the classification system resulting in 37 genetic lineages and 8 genotypes currently. The additional lineages originating in China were designated as GI-28, GI-29 (Chen, et al., 2017; Jiang et al., 2017; Ma, et al., 2019), GVIII-1 was described in Poland (Domanska-Blicharz, et al., 2020), GI-30 was discovered in Trinidad and Tobago

(Brown, et al., 2020) and tentatively the GI-31 strain was uncovered in the Ivory Coast (Bali, et al., 2022).

Table 2. Classification system of IBV, grouped by genotype (adapted from Valastro et al., 2016).

Lineage	Period of circulation	Prototype Strain/ Strain name	Country of origin	Collection date	GenBank accession number
GI-1	1937-2013	Beaudette	USA	1937	M95169
GI-2	1954-2006	Holte	USA	1954	GU393336
GI-3	1960-2006	Gray	USA	1960	L14069
GI-4	1962-1998	Holte	USA	1962	L18988
GI-5	1962-2012	N1/62	Australia	1962	U29522
GI-6	1962-2010	VicS	Australia	1962	U29519
GI-7	1964-2012	TP/64	Taiwan	1964	AY606320
GI-8	1965-1967	L165	USA	1965	JQ964061
GI-9	1973-2011	ARK99	USA	1973	M99482
GI-10	1970s-2000s	B	New Zealand	1970s	AF151954
GI-11	1975-2009	UFMG/G	Brazil	1975	JX182775
GI-12	1978-2006	D3896	The Netherlands	1978	X52084
GI-13	1983-2013	Moroccan-G/83	Morocco	1983	EU914938
GI-14	1984-2006	B1648	Belgium	1984	X87238
GI-15	1986-2008	B4	Korea	1986	FJ807932
GI-16	1986-2011	IZO 28/86	Italy	1986	KJ941019
GI-17	1988-1999	CA/Machado/88	USA	1988	AF419315
GI-18	1993-1999	JP8127	Japan	1993	AY296744
GI-19	1993-2012	58HeN-9311	China	1993	KC577395
GI-20	1996-1999	Qu_mv	Canada	1996	AF349621
GI-21	1997-2005	Spain/97/314	Spain	1997	DQ064806
GI-22	1997-2011	40GDGZ-971	China	1997	KC577382
GI-23	1998-2012	Variant 2	Israel	1998	AF093796
GI-24	1998-2013	V13	India	1998	KF757447
GI-25	2004-2013	CA/173704	USA	2004	EU925393
GI-26	2006-2007	NGA/B401/2006	Nigeria	2006	FN182243
GI-27	2008-2013	GA08	USA	2008	GU301925
GI-28	Not available	ck/CH/LGX/111119	China	2011	KX640829
GI-29	Not available	1011/14_China	China	2014	KY407556
GI-30	Not available	18RS1461-1	Trinidad and Tobago	2014	MN696789

Lineage	Period of circulation	Prototype Strain/ Strain name	Country of origin	Collection date	GenBank accession number
GI-31	Not available	D2334/11/2/13/ CI	Cote d'Ivoire (Ivory Coast)	2013	MZ325299
GII-1	1979-1984	D1466	The Netherlands	1979	M21971
GIII-1	1988-2008	N1/88	Australia	1988	U29450
GIV-1	1992-2003	DE/072/92	USA	1991	U77298
GV-1	2002-2008	N4/02	Australia	2002	DQ059618
GVI-1	2007-2012	TC07-2	China	2007	GQ265948
GVII-1	Not available	10636/26_Chin a	China	2016	MH924835
GVIII-1	Not available	PA/1220/98	Poland	1998	AY789942

2.9 Aims and objectives

The aim of the study was to identify the genotypes and determine the epidemiological distribution of the various infectious bronchitis coronavirus (IBV) variants within the southern African poultry and those of some neighboring countries from 2011 to 2020. IBV isolates were obtained from the Deltamune repository dating from 2011 to 2020. During my employment at Deltamune (2011 - 2020). I personally performed these virus isolations as a routine part of my duties.

Specific Objectives:

1. The extraction of the RNA from the stored IBV in allantoic fluid and ensure the quality and amount of RNA present was sufficient for analysis.
2. To use a conventional RT-PCR assay to amplify a 745 bp region of the hypervariable (HVR) region in the S1 protein.
3. To perform Sanger DNA sequencing on the purified RT-PCR amplicons and assemble a consensus sequence from forward and reverse sequencing reads.
4. To construct of phylogenetic trees with genotyping reference sequences, other international and local strains for the classification of southern African IBV strains.

Chapter 3

MATERIALS AND METHODS

3.1 Virus isolation

At Deltamune, IB viruses were isolated as per client requests. The specific route of inoculation depended on the virus in question. IBV is normally isolated using the allantoic sac route and samples were processed depending on sample type. Organ swabs were placed directly into a standard volume 9 ml of Virus Isolation Medium (VIM). VIM was prepared by adding 10 ml Gibco Antibiotic x Antimycotic solution (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), 2 ml of Gentamycin (Sigma-Aldrich, Saint Louis, U.S.), 2.5g of Bovine Serum Albumin (BSA) (Roche Diagnostics GmbH, Mannheim, Germany) and filled to 500ml of Gibco Phosphate Buffer Serum (PBS) (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The pH of the solution was adjusted to range between 7.0 and 7.4 and was filtered and sterilised before use. Trachea and caecal tonsils were scraped and the exudate and epithelial cells from the scrapings were added to VIM. Organs samples were excised and macerated before being added to VIM. The suspension was then vortexed and clarified by centrifugation at ± 4000 rpm for 10 min. Samples were then filtered through a 0.45µm filter to remove any bacteria that might affect the results. In rare circumstances, a smaller filter of 0.22µm had to be used to eliminate any smaller bacteria that were present.

Six 9-11-day-old Specific Pathogen Free (SPF) hens' eggs (Avi-farms Pty Ltd, South Africa) were used for allantoic sac route inoculations for each sample. A 1 mm hole was made in the egg with a sterilised, stainless-steel, stack point needle, where the allantoic sac meets the air cavity. Samples were inoculated by using a 25G x 25 mm needle and pointing the needle downwards, preferably away from the embryo. The holes were sealed using wood glue and the eggs were incubated for the next 7 days at 37°C ± 1°C. The stainless-steel containers at the bottom of the incubator were filled daily with Reverse Osmosis (RO) (demineralised) water to maintain humidity but the humidity was not monitored at a set value. Mortalities observed within the first 24hrs were considered non-specific. Eggs were candled daily, and any further mortalities were removed from the incubator and refrigerated until the last day of incubation. After the 7-day incubation period, the SPF eggs were placed in the refrigerator at 4°C ± 2°C

for at least 4 hours to kill the embryos before harvesting. Any embryo mortalities that occurred during the 7-day period (after the first day nonspecific deaths) were included in the harvesting process. Harvesting took place within a biohazard cabinet. The eggs were sprayed with 70 % ethanol before the shell was cracked open with sterilized metal forceps, at the top of the shell. A small amount of the shell was removed for entry. This is where the air sac is located of the egg and was the easiest access point for maximum harvesting of allantoic fluid with minimal invasion and contamination risk. The egg was then tilted slightly and, on the side, opposite of where the embryo was located a 15G needle was inserted and the allantoic fluid drawn up into a 10 ml syringe and dispensed to a 30ml McCartney bottle. Care was taken not to nick a vein to prevent any uptake of blood to the allantoic fluid as well. A minimum of 2 ml allantoic fluid was needed during harvesting for re-inoculation to a second or third passage. Depending on the embryo morphology the allantoic fluid was sent for RT-qPCR, at Deltamune' s diagnostic section, for confirmation of suspected viral presence or, the allantoic fluid was passaged further to a maximum of 3 passages before any confirmation tests were requested. If no morphology changes were observed in the embryos, Avian Influenza and IBV RT-qPCR was ordered to confirm the absence of any viruses. In some cases, there were limited morphological changes to embryos and confirmation with RT-qPCR was extremely important.

IBV was sometimes isolated from a different inoculation route such as chorioallantoic, yolk sac or intravenous routes. This would normally occur when other viruses were suspected but the morphological changes in the embryos were related to IBV instead. While all IBVs were confirmed using RT-qPCR for presence of IBV, the initial results before storage of the isolates were not available to report in this study.

3.2 Sample storage

Allantoic fluid confirmed positive for IBV using RT-qPCR were aliquoted in 1.0ml sterile cryotubes and placed at -80°C for long term storage. A material transfer agreement (MTA) was signed between Deltamune and the University of Pretoria that allowed for the transfer of the confirmed, IBV positive isolates for this study. They were marked with the laboratory number, the route of inoculation, the type of virus isolated, the passage number the virus was isolated on and the date of the last harvest.

The initial sample size was 447 isolates isolated at Deltamune over the period 2011-2020. The different geographical locations include Botswana, Eswatini, Namibia, and South Africa's Free State, Eastern Cape, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West, and Western Cape provinces (Table 3).

Table 3. Samples used in this study.

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
1	P4-30	147868	2010	Grand Parents	North West
2	P5-55	148576	2010	Broilers	North West
3	P5-39	149399	2010	Broilers	Western Cape
4	P4-7	152309	2010	Broilers	North West
5	P2-77	132309 H3	2010	Broilers	Gauteng
6	P4-76	151934 H6	2010	Broilers	North West
7	P5-51	151934 H6	2010	Broilers	North West
8	P4-15	167453	2011	Broilers	North West
9	P4-36	168218	2011	Broilers	Free State
10	P4-6	168355	2011	SPF	Gauteng
11	P4-17	174787	2011	Broilers	Western Cape
12	P5-37	175829	2011	Broilers	Mpumalanga
13	P3-50	175958	2011	Broilers	North West
14	P4-72	176633	2011	Broilers	Mpumalanga
15	P5-63	193307	2011	Broilers	North West
16	P5-36	152953 H9	2011	Broilers	North West
17	P3-84	154830 H6	2011	Broilers	Free State
18	P4-78	154830 H7	2011	Broilers	Free State
19	P3-61	169490 CT	2011	Broilers	North West
20	P3-68	172175 #2	2011	Broilers	North West
21	P3-92	172175 -7 #3	2011	Broilers	North West
22	P4-34	172324-2	2011	Broilers	Free State
23	P4-14	172324-4	2011	Broilers	Free State
24	P3-91	174443 TR	2011	Layers	Western Cape
25	P4-27	175744 KD H1	2011	Broilers	Free State
26	P4-12	175744 KD H1	2011	Broilers	Free State
27	P4-13	175744 KD H5	2011	Broilers	Free State
28	P4-28	175834 TR	2011	Broilers	North West
29	P1-54	193970-3	2011	broilers	Free State
30	P2-51	193970-4	2011	broilers	Free State
31	P1-8	193970-6	2011	broilers	Free State
32	P1-30	186039	2012	Broilers	Gauteng
33	P4-71	187596	2012	Broilers	North West
34	P4-84	187803	2012	Broilers	North West
35	P4-90	188172	2012	Broilers	North West
36	P2-21	188225	2012	Broilers	North West
37	P1-26	189122	2012	Broilers	North West
38	P2-62	189390	2012	Broilers	North West
39	P1-15	189628	2012	Broilers	North West
40	P2-38	189853	2012	Broilers	North West
41	P5-29	190866	2012	Broilers	Mpumalanga
42	P4-68	191145	2012	Broilers	Free State
43	P5-68	191930	2012	Broilers	North West
44	P5-11	192943	2012	Broilers	North West
45	P4-92	193013	2012	Layers	Gauteng
46	P4-82	193043	2012	Broilers	North West
47	P1-49	193138	2012	Broilers	North West
48	P5-24	193304	2012	Broilers	Free State
49	P1-28	193311	2012	Broilers	North West
50	P1-13	193366	2012	Layers	Mpumalanga

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
51	P4-83	193368	2012	Layers	Gauteng
52	P5-72	193602	2012	Broilers	Mpumalanga
53	P2-58	193774	2012	Broilers	Mpumalanga
54	P2-55	193921	2012	Layers	Western Cape
55	P5-69	194413	2012	Broilers	North West
56	P1-10	194822	2012	Broilers	North West
57	P5-28	195324	2012	Broilers	North West
58	P2-7	195696	2012	Broilers	Mpumalanga
59	P4-96	195854	2012	Broilers	North West
60	P5-66	196674	2012	Broilers	North West
61	P5-85	196723	2012	Broilers	North West
62	P1-9	197318	2012	Broilers	North West
63	P2-14	197346	2012	Broilers	Gauteng
64	P2-10	197346	2012	Broilers	Gauteng
65	P2-46	197826	2012	Unknown	Unknown
66	P1-78	198544	2012	Rearing	Gauteng
67	P5-10	198565	2012	Broilers	Free State
68	P2-39	198573	2012	Unknown	Unknown
69	P1-20	199262	2012	Broilers	Mpumalanga
70	P1-11	199341	2012	Broilers	North West
71	P4-95	200931	2012	Broilers	Free State
72	P5-20	200957	2012	Broilers	North West
73	P5-78	201106	2012	Broilers	North West
74	P5-76	201106	2012	Broilers	North West
75	P5-12	201208	2012	Broilers	Limpopo
76	P5-9	201216	2012	Broilers	North West
77	P5-1	201216	2012	Broilers	North West
78	P5-14	201216	2012	Broilers	North West
79	P3-88	201300	2012	Broilers	Botswana
80	P2-78	201351	2012	Broilers	North West
81	P2-5	201351	2012	Broilers	North West
82	P4-86	201591	2012	Broilers	North West
83	P1-52	201602	2012	Broilers	North West
84	P5-90	202348	2012	Broilers	North West
85	P2-96	203097	2012	Broilers	Gauteng
86	P5-22	203100	2012	Broilers	Gauteng
87	P5-70	203106	2012	Broilers	Gauteng
88	P3-32	203110	2012	Broilers	Gauteng
89	P1-81	206101	2012	Broilers	Mpumalanga
90	P5-15	233607	2012	Broilers	Free State
91	P5-48	187464 KN H2 AF	2012	Broilers	Western Cape
92	P5-35	187464 KN H3	2012	Broilers	Western Cape
93	P5-49	187464 TR H1 AF	2012	Broilers	Western Cape
94	P5-40	187464 TR H2 AF	2012	Broilers	Western Cape
95	P2-56	187464 TR H2 YS	2012	Broilers	Western Cape
96	P5-47	187464 TR H3 AF	2012	Broilers	Western Cape
97	P1-4	187521 H1	2012	SPF	Gauteng
98	P5-54	187521 H2	2012	SPF	Gauteng
99	P2-41	197270 H2	2012	Broilers	North West
100	P4-94	197270 H3	2012	Broilers	North West
101	P1-27	197666 H2	2012	Broilers	Gauteng
102	P1-7	197666 H4	2012	Broilers	Gauteng
103	P2-3	197677 H1	2012	Broilers	North West
104	P1-61	197677 H2	2012	Broilers	North West
105	P5-8	198007 H2	2012	Broilers	North West
106	P5-84	198007 H3	2012	Broilers	North West
107	P5-77	198007 H4	2012	Broilers	North West
108	P1-62	198442 CE	2012	Broilers	Free State
109	P2-9	198442 SF	2012	Broilers	Free State
110	P1-44	198442 TR	2012	Broilers	Free State
111	P2-28	198999 B9	2012	Broilers	North West
112	P5-3	200656 H3	2012	Broilers	North West

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
113	P5-80	200656 H4	2012	Broilers	North West
114	P5-82	200656 H4	2012	Broilers	North West
115	P5-7	200677 HA	2012	Broilers	North West
116	P5-87	200677 HB	2012	Broilers	North West
117	P5-2	200963 H5	2012	Broilers	North West
118	P5-59	200963 H7	2012	Broilers	North West
119	P5-86	200963 H7	2012	Broilers	North West
120	P2-91	205069 AF H6	2012	Broilers	Free State
121	P1-87	205069 YS H6	2012	Broilers	Free State
122	P1-86	205069 YS H6	2012	Broilers	Free State
123	P3-96	208097	2013	Broilers	Free State
124	P2-89	208136	2013	Layers	Gauteng
125	P2-92	208232	2013	Broilers	North West
126	P1-85	208890	2013	Broilers	Free State
127	P2-94	208981	2013	Broilers	Free State
128	P3-74	208982	2013	Broilers	Mpumalanga
129	P3-31	212412	2013	Grand Parents	Gauteng
130	P3-33	213416	2013	Broilers	Namibia
131	P3-34	215665	2013	Broilers	North West
132	P3-75	215735	2013	Broilers	Eswatini
133	P2-83	216935	2013	Unknown	Unknown
134	P2-95	217716	2013	Layers	Gauteng
135	P1-89	218586	2013	Broilers	Mpumalanga
136	P2-84	220197	2013	Broilers	Mpumalanga
137	P1-96	220680	2013	Broilers	Mpumalanga
138	P1-91	222046	2013	Layers	Gauteng
139	P1-84	222493	2013	Broilers	North West
140	P2-87	222625	2013	Broilers	North West
141	P1-92	222760	2013	Layers	Gauteng
142	P1-82	223677	2013	Layers	Eastern Cape
143	P2-44	224387	2013	Unknown	Unknown
144	P2-86	225510	2013	Unknown	Gauteng
145	P2-66	226220	2013	Broilers	North West
146	P3-11	226600	2013	Broilers	North West
147	P5-88	226789	2013	Unknown	KwaZulu Natal
148	P3-43	227152	2013	Broilers	KwaZulu Natal
149	P1-33	227659	2013	Broilers	North West
150	P1-31	227829	2013	Broilers	North West
151	P1-22	228016	2013	Broilers	North West
152	P2-37	229015	2013	Broilers	Free State
153	P4-60	229079	2013	Broilers	Free State
154	P4-70	229081	2013	Unknown	Gauteng
155	P5-64	229507	2013	Broilers	Free State
156	P2-82	230968	2013	Broilers	North West
157	P5-30	231139	2013	Broilers	Free State
158	P5-25	231217	2013	Broilers	Gauteng
159	P4-59	231808	2013	Broilers	North West
160	P1-37	231832	2013	Broilers	Mpumalanga
161	P4-91	232718	2013	Broilers	North West
162	P5-74	232719	2013	Broilers	Gauteng
163	P2-80	233942	2013	Broilers	Mpumalanga
164	P1-29	233979	2013	Broilers	North West
165	P2-52	234109	2013	Broilers	Free State
166	P2-71	234993	2013	Broilers	North West
167	P1-6	235198	2013	Broilers	Free State
168	P1-60	235447	2013	Broilers	North West
169	P1-66	236910	2013	Broilers	North West
170	P2-27	236912	2013	Broilers	North West
171	P1-40	238153	2013	Grand Parents	KwaZulu Natal
172	P2-24	240525	2013	Broilers	Free State
173	P1-57	242100	2013	Broilers	North West
174	P2-53	243570	2013	Breeders	North West

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175	P1-64	244155	2013	Broilers	Free State
176	P2-29	244663	2013	Broilers	Free State
177	P2-61	245067	2013	Broilers	Western Cape
178	P3-73	209783 CE	2013	Broilers	North West
179	P3-36	209783 TR	2013	Broilers	North West
180	P3-72	209842 H2	2013	Breeders	North West
181	P3-95	209842 H2	2013	Breeders	North West
182	P1-93	210882 H1	2013	Layers	Gauteng
183	P3-41	210882 H3	2013	Layers	Gauteng
184	P1-94	221316 - ORG	2013	Broilers	North West
185	P2-93	221316 - CE	2013	Broilers	North West
186	P2-85	221316 TR	2013	Broilers	North West
187	P1-88	222042 H1	2013	Rearing	North West
188	P2-90	222042 H2	2013	Rearing	North West
189	P1-83	225250 H1	2013	Broilers	Namibia
190	P2-88	225250 H5	2013	Broilers	Namibia
191	P1-95	225518 H10	2013	Broilers	North West
192	P5-31	225518 H11	2013	Broilers	North West
193	P5-89	225518 H12	2013	Broilers	North West
194	P3-60	226668 H2	2013	Unknown	Unknown
195	P3-40	226827 HD	2013	Unknown	Unknown
196	P3-77	226827 HE	2013	Unknown	Unknown
197	P3-86	226827 HF	2013	Unknown	Unknown
198	P4-80	227831 TR	2013	Broilers	North West
199	P2-72	227831 TR	2013	Broilers	North West
200	P5-16	229207 CE	2013	Broilers	Free State
201	P5-58	229207 TR	2013	Broilers	Free State
202	P5-18	229498 H1	2013	Broilers	North West
203	P4-73	229498 H2	2013	Broilers	North West
204	P2-57	232786 H4	2013	Unknown	Unknown
205	P2-63	232786 H4	2013	Unknown	Unknown
206	P1-50	233429 CE	2013	Broilers	North West
207	P3-30	233429 CE	2013	Broilers	North West
208	P1-24	234962 H13	2013	Broilers	North West
209	P1-2	234962 H7	2013	Broilers	North West
210	P2-49	235104 TR	2013	Layers	Western Cape
211	P2-15	236104 BR	2013	Broilers	North West
212	P1-51	236104 CE	2013	Broilers	North West
213	P3-14	246538	2014	Broilers	Western Cape
214	P1-5	249371	2014	Breeders	Gauteng
215	P2-12	253792	2014	Broilers	Free State
216	P1-14	253810	2014	Broilers	Free State
217	P1-69	253811	2014	Broilers	Free State
218	P2-25	256015	2014	Broilers	Limpopo
219	P2-6	259695	2014	Broilers	North West
220	P1-35	260228	2014	Broilers	Free State
221	P1-53	260229	2014	Broilers	Free State
222	P2-32	260600	2014	Vaccine production	Gauteng
223	P1-68	260884	2014	Broilers	Free State
224	P3-64	261158	2014	Broilers	Free State
225	P3-62	263752	2014	Broilers	Free State
226	P1-63	266932	2014	Broilers	North West
227	P2-20	266968	2014	Broilers	Free State
228	P1-45	267693	2014	Broilers	Mpumalanga
229	P3-16	267739	2014	Broilers	Free State
230	P3-48	267839	2014	Broilers	Western Cape
231	P3-6	268083	2014	Broilers	Gauteng
232	P3-9	268083	2014	Broilers	Gauteng
233	P3-70	268361	2014	Broilers	Gauteng
234	P3-23	269875	2014	Broilers	Free State
235	P3-46	270254	2014	Broilers	North West

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
236	P1-23	274387	2014	Broilers	Free State
237	P2-60	274626	2014	Broilers	Free State
238	P3-22	275478	2014	Broilers	Free State
239	P3-1	275644	2014	Broilers	Free State
240	P1-39	276515	2014	Broilers	Mpumalanga
241	P3-47	276628	2014	Broilers	Free State
242	P4-87	276753	2014	Broilers	Free State
243	P5-61	276811	2014	Layers	Gauteng
244	P5-62	276824	2014	Broilers	Free State
245	P4-81	277553	2014	Broilers	Free State
246	P5-23	277560	2014	Broilers	North West
247	P3-15	278583	2014	Broilers	Free State
248	P3-52	278628	2014	Broilers	Free State
249	P5-26	278832	2014	Broilers	Free State
250	P3-27	280023	2014	Broilers	Free State
251	P3-25	281216	2014	Broilers	Free State
252	P3-78	281572	2014	Broilers	Free State
253	P3-44	281574	2014	Broilers	Free State
254	P5-21	281975	2014	Broilers	Free State
255	P1-41	284195	2014	Broilers	Western Cape
256	P5-65	287871	2014	Broilers	North West
257	P5-83	288325	2014	Layers	Gauteng
258	P5-5	289015	2014	Broilers	North West
259	P2-11	295637	2014	Layers	Gauteng
260	P3-20	245842 H2	2014	Broilers	Free State
261	P2-35	245842 H3	2014	Broilers	Free State
262	P1-25	259214 S1	2014	Breeders	North West
263	P2-76	259214 S2	2014	Breeders	North West
264	P2-67	264213 H1	2014	Broilers	Gauteng
265	P2-79	264213 H1	2014	Broilers	Gauteng
266	P1-38	264213 H2	2014	Broilers	Gauteng
267	P3-59	269246 AF	2014	Broilers	KwaZulu Natal
268	P3-49	269246 BR	2014	Broilers	KwaZulu Natal
269	P3-79	269246 CT	2014	Broilers	KwaZulu Natal
270	P3-17	269246 KID	2014	Broilers	KwaZulu Natal
271	P3-53	271729 H1	2014	Broilers	Free State
272	P3-3	271729 H2	2014	Broilers	Free State
273	P4-35	274025 H	2014	Broilers	Free State
274	P2-45	274025 S	2014	Broilers	Free State
275	P3-57	276453 H1	2014	Layers	Gauteng
276	P3-10	276453 H2	2014	Layers	Gauteng
277	P2-13	282562 H15	2014	Broilers	Namibia
278	P1-21	282562 H4	2014	Broilers	Namibia
279	P5-60	282913 H2	2014	Layers	Gauteng
280	P5-13	282913 H3	2014	Layers	Gauteng
281	P5-4	288392 H2	2014	Broilers	Gauteng
282	P5-67	288392 H3	2014	Broilers	Gauteng
283	P5-6	289368 H10	2014	Broilers	North West
284	P1-79	289368 H10	2014	Broilers	North West
285	P5-75	289368 H7	2014	Broilers	North West
286	P2-54	Unknown	2014	Unknown	Western Cape
287	P3-67	309400	2015	Allantoic fluid	Western Cape
288	P3-8	314462	2015	Broilers	Gauteng
289	P3-12	316661	2015	Broilers	Western Cape
290	P3-45	317372	2015	Broilers	North West
291	P3-66	318879	2015	Broilers	North West
292	P3-80	319685	2015	Broilers	Free State
293	P3-18	320002	2015	Broilers	Free State
294	P3-28	320057	2015	Broilers	North West
295	P3-29	320213	2015	Broilers	Western Cape
296	P3-4	320709	2015	Broilers	North West
297	P3-21	320844	2015	Broilers	Free State

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
298	P3-58	321496	2015	Broilers	Western Cape
299	P2-18	321872	2015	Breeders	Gauteng
300	P4-38	321872	2015	Breeders	Gauteng
301	P3-55	322303	2015	Broilers	Gauteng
302	P3-5	322393	2015	Broilers	Free State
303	P2-43	322394	2015	Broilers	Free State
304	P3-54	322903	2015	Broilers	Free State
305	P3-19	323131	2015	Broilers	Free State
306	P1-73	325285	2015	Broilers	Gauteng
307	P2-22	327042	2015	Broilers	Free State
308	P1-67	327066	2015	Broilers	Free State
309	P1-59	329067	2015	Broilers	Free State
310	P2-8	329169	2015	Broilers	Free State
311	P1-18	330071	2015	Broilers	Free State
312	P2-69	332216	2015	Broilers	Free State
313	P1-65	332607	2015	Broilers	Limpopo
314	P1-70	332825	2015	Broilers	Free State
315	P3-24	335734	2015	Broilers	Free State
316	P2-70	335734	2015	Broilers	Free State
317	P2-33	335997	2015	Broilers	Namibia
318	P3-76	336625	2015	Broilers	Free State
319	P1-56	337288	2015	Broilers	Free State
320	P3-42	338905	2015	Broilers	Free State
321	P2-47	339318	2015	Broilers	Free State
322	P2-16	339989	2015	Broilers	Free State
323	P5-79	340340	2015	Broilers	Gauteng
324	P5-27	340461	2015	Broilers	Gauteng
325	P1-48	340744	2015	Broilers	Gauteng
326	P5-81	341521	2015	Broilers	Gauteng
327	P4-79	342217	2015	Broilers	North West
328	P5-17	344201	2015	Breeders	North West
329	P2-74	344383	2015	Broilers	Mpumalanga
330	P1-16	344385	2015	Broilers	Free State
331	P4-67	344385	2015	Broilers	Free State
332	P2-19	345298	2015	Broilers	Gauteng
333	P1-32	345568	2015	Broilers	Free State
334	P1-42	346464	2015	Broilers	Gauteng
335	P2-2	347675	2015	Broilers	Free State
336	P3-63	348514	2015	Broilers	North West
337	P2-64	348519	2015	Broilers	North West
338	P5-19	348520	2015	Broilers	North West
339	P4-75	348521	2015	Broilers	North West
340	P1-77	349150	2015	Broilers	Gauteng
341	P2-34	349476	2015	Broilers	North West
342	P1-71	349480	2015	Broilers	North West
343	P3-13	351011	2015	Broilers	Free State
344	P3-87	351288	2015	Broilers	Free State
345	P3-7	351505	2015	Broilers	Free State
346	P1-43	305670 H2	2015	Broilers	Gauteng
347	P1-1	305670 H3	2015	Broilers	Gauteng
348	P2-48	305670 H4	2015	Broilers	Gauteng
349	P1-46	309115 H3	2015	Broilers	Gauteng
350	P2-40	309115 H8	2015	Broilers	Gauteng
351	P2-42	313190 H1	2015	Broilers	North West
352	P3-65	313190 H3	2015	Broilers	North West
353	P1-90	327985 H12	2015	Broilers	North West
354	P2-75	327985 H18	2015	Broilers	North West
355	P3-35	336344 H1	2015	Broilers	Gauteng
356	P3-71	336344 H2	2015	Broilers	Gauteng
357	P4-9	336344 H7	2015	Broilers	Gauteng
358	P3-81	336344 H8	2015	Broilers	Gauteng
359	P1-72	337109 H5	2015	Broilers	Free State

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360	P1-12	337109 H7	2015	Broilers	Free State
361	P1-3	346613 H2	2015	Broilers	Free State
362	P2-36	346613 H4	2015	Broilers	Free State
363	P4-74	347477 H2	2015	Broilers	North West
364	P5-73	347477 H3	2015	Broilers	North West
365	P5-71	347626 H1	2015	Broilers	Gauteng
366	P4-69	347626 H2	2015	Broilers	Gauteng
367	P1-74	348513 AF	2015	Broilers	North West
368	P3-38	348513 CAM	2015	Broilers	North West
369	P1-58	348513 CAM	2015	Broilers	North West
370	P3-51	348517 H1	2015	Broilers	North West
371	P2-81	348517 H2	2015	Broilers	North West
372	P3-37	348517 H6	2015	Broilers	North West
373	P3-69	350676 C3	2015	Broilers	North West
374	P2-30	350676 C5	2015	Broilers	North West
375	P1-55	351290 H2	2015	Broilers	North West
376	P4-61	351290 H9	2015	Broilers	North West
377	P1-75	351290 H9	2015	Broilers	North West
378	P3-39	355673	2016	Broilers	North West
379	P2-4	363621	2016	Broilers	Gauteng
380	P4-2	372100	2016	Layers	Gauteng
381	P3-93	374409	2016	Broilers	Mpumalanga
382	P4-5	375003	2016	Broilers	Free State
383	P2-65	377455	2016	Broilers	Free State
384	P1-34	377465	2016	Broilers	Free State
385	P1-47	377467	2016	Broilers	Free State
386	P2-73	379353	2016	Broilers	Free State
387	P2-59	383258	2016	Broilers	Gauteng
388	P1-76	383258	2016	Broilers	Gauteng
389	P2-1	388559	2016	Broilers	Free State
390	P1-19	392670	2016	Broilers	Free State
391	P2-26	392673	2016	Broilers	Free State
392	P2-31	403218	2016	Broilers	Free State
393	P2-23	403218	2016	Broilers	Free State
394	P3-26	358886 AF H6	2016	Broilers	Gauteng
395	P3-2	358886 H4	2016	Broilers	Gauteng
396	P3-56	358886 YS H6	2016	Broilers	Gauteng
397	P2-50	359396 H4	2016	Broilers	Namibia
398	P1-80	359396 H5	2016	Broilers	Namibia
399	P1-36	409284	2017	Broilers	North West
400	P4-4	462411	2017	Broilers	Free State
401	P2-68	461064 H2	2017	Broilers	Free State
402	P2-17	461064 H4	2017	Broilers	Free State
403	P4-25	461064 H6	2017	Broilers	Free State
404	P4-1	526182	2018	Broilers	Gauteng
405	P3-82	526250	2018	Broilers	North West
406	P4-20	530520	2018	Broilers	North West
407	P1-17	531407	2018	Broilers	North West
408	P4-3	531796	2018	Broilers	Western Cape
409	P4-10	537307	2018	Broilers	Western Cape
410	P4-16	537309	2018	Broilers	Western Cape
411	P3-94	533511 H9	2018	Broilers	Free State
412	P3-90	533551 H6	2018	Layers	Free State
413	P4-11	538641 B4	2018	Broilers	Namibia
414	P4-8	538641 B5	2018	Broilers	Namibia
415	P4-19	537307	2019	Unknown	Unknown
416	P4-29	545467	2019	Broilers	Western Cape
417	P4-18	550819	2019	Broilers	Western Cape
418	P4-62	564876	2019	Broilers	Unknown
419	P5-43	568903	2019	Breeders	Mpumalanga
420	P5-34	579119	2019	Broilers	Eastern Cape
421	P5-41	586150	2019	Breeders	Gauteng

Sample number	Study reference number	Lab reference number	Year isolated	Chicken type	Location
422	P5-57	592655	2019	Broilers	KwaZulu Natal
423	P5-42	599490	2019	Broilers	North West
424	P5-50	601368	2019	Broilers	KwaZulu Natal
425	P4-89	603902	2019	Broilers	Mpumalanga
426	P4-85	605307	2019	Unknown	Unknown
427	P5-33	606426	2019	Unknown	Unknown
428	P5-56	608301	2019	Unknown	Unknown
429	P5-45	608305	2019	Unknown	Unknown
430	P3-85	578946 N2	2019	Layers	Gauteng
431	P3-83	578946 N5	2019	Layers	Gauteng
432	P4-23	578946 N6	2019	Layers	Gauteng
433	P4-93	599479 H3	2019	Broilers	North West
434	P5-32	599479 H4	2019	Broilers	North West
435	P4-88	617999	2020	Unknown	Unknown
436	P5-46	626739	2020	Unknown	Unknown
437	P5-52	634004	2020	Unknown	Unknown
438	P4-77	634038	2020	Unknown	Unknown
439	P5-44	635300	2020	Unknown	Unknown
440	P5-53	638883	2020	Unknown	Unknown
441	P4-31	656517	2020	Unknown	Unknown
442	P4-33	658187	2020	Unknown	Unknown
443	P4-32	659486	2020	Unknown	Unknown
444	P4-24	659489	2020	Unknown	Unknown
445	P3-89	662543	2020	Unknown	Unknown
446	P5-38	638634 IV	2020	Unknown	Unknown
447	P4-22	658421 H1	2020	Unknown	Unknown

3.3 RNA extraction

RNA was extracted using the automated 96-well extraction machine, the QIAcube HT (Qiagen, Hilden, Germany). The kit used for extraction was the IndiSpin QIAcube HT Pathogen kit and plasticware (Indical Biocscience, Leipzig, Sachsen, Germany). 200µl of each sample was allocated to a 96 deep well plate (called the “S-Block” on the protocol). An excel spreadsheet was created that had the sample information types in the respective well it was present. The spreadsheet was then saved as a .csv file, that enables the user to import the file into the machine protocol. The software “QIAcube HT” was then selected, and the protocol as saved on the quick start menu and by Qiagen as “cador Pathogen 96 QIAcube HT mit EtOH” was selected. The protocol was used as is saved on the computer and the instructions were followed as the program indicated. Filter tips were added when necessary and reagents were added to their respective wells in the volume indicated. The last reagent that needs to be allocated (VXL mixture) needs to be made immediately prior to starting the program, according to manufacturer’s instructions and the number of samples that are to be extracted. The protocol was then left to run on its own except for checking if any errors occur during the process. At the end of the run, a report was generated to note any errors that might have occurred during the extraction process. The 96-well plate in the end consisted of

50µl of RNA/DNA for each sample that was stored either short term in the refrigerator at 4°C ± 2°C before being processed the following day or for a longer term at -20°C before continuing to the next step. Each sample was named according to the plate they were run on and the sample number they represented on the 96 well plate e.g., P1-1. The last run included a negative control (PBS) as QC to ensure no contamination occurred during the extraction process. This sample was processed up to the gel visualisation step to ensure negativity, throughout the entire process.

3.4 Primer sequences and RT-PCR

Primer sequences as published in Gallardo et al., 2010 (S17F 5'-TGA AAA CTG AAC AAA AGA CCG ACT TAG-3' and S18R 5'-GGA TAG AAG CCA TCT GAA AAA TTG C-3') were ordered from Integrated DNA Technologies (IDT) (Coralville, Iowa, United States) via Whitehead Scientific. These primers target roughly the first 720 nucleotides of the S1 gene in the IBV genome and encompass HVRs. Primers were reconstituted according to manufacturer's instructions with nuclease free water (Merck Life Science (Pty) Ltd) (Darmstadt, Germany) and further diluted to a concentration of 10 pmol. Aliquots of the primers were made and stored at -20°C. The Master mix reaction contained volumes as set out in Table 4.

Table 4. Master mix reaction per sample.

Reagent	Volume
Nuclease free water	5 µl
Phusion Flash (Thermo Fisher Scientific, Waltham, Massachusetts)	10 µl
mmLV RT enzyme (Thermo Fisher Scientific, Waltham, Massachusetts)	0.3 µl
RNAse Inhibitor (Thermo Fisher Scientific, Waltham, Massachusetts)	0.2 µl
Primers (S17F and S18R)	1 µl
RNA template	3.5 µl
Total reaction volume	20 µl

The PCR was performed using a LifeECO (Bioer) thermal cycler under the following conditions: Incubation for RT-Step at 60 °C for 10 min, denaturation at 98 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 50 °C for 10 sec, and polymerization at 72 °C for 2 min with a final elongation step of 5 min at 72 °C.

3.5 Gel visualization, extraction, and cleanup

A 1% agarose gel was prepared using 1.5g of molecular grade agarose powder, 150 ml of TAE buffer (Omega Bio-tek, Norcross, Georgia, United States) and 15 µl of a 10% Ethidium bromide solution (Merck KGaA, Darmstadt, Germany). The mixture was microwaved until all particles were dissolved and cooled to a temperature of approximately 60°C before pouring into the gel mould with comb. The gel was left to set before use. As a control and for visualization purposes, 10µl of a 100bp ladder (New England BioLabs, Ipswich, Massachusetts, United States) was added to the first well of the gel and 3.3 µl of loading dye (New England BioLabs, Ipswich, Massachusetts, United States) was added to each sample before adding 12µl of sample to separate wells within the gel. The gel was left to run at a voltage of 230V, 100A for 30-60 minutes and visualised under a UV light. cDNA fragments of the correct size (745 bp) were then excised using a clean scalpel blade and placed into sterile 1.5ml microcentrifuge tubes for gel extraction.

To purify the excised fragments the QIAquick Gel extraction/QIAquick PCR and gel cleanup kit (Qiagen, Hilden, Germany) was used. 100µl of Buffer QG was added to each gel slice and heated to 50°C for 10 minutes on a heating block until dissolved. The colour of each sample needed to be yellow in colour to continue (a pH indicator). A 1:1 volume of isopropanol was then added to the tube and mixed. The samples were then aliquoted to a QIAquick spin column and centrifuged for 1 min at 10 000 rpm. The flow through was discarded. 500 µl of Buffer QG was added to the spin column, centrifuged again for 1 min and the flow through discarded. 750 µl of washbuffer was then added (Buffer PE) to the tube and centrifuged for 1 min. The column was then placed into a sterile 1.5 ml microcentrifuge tube and 50µl of elution buffer was placed in the centre of the column and centrifuged for 1 min. The column was discarded, and the purified DNA was then used further in conventional DNA sequencing.

3.6 DNA sequencing

The purified 745 bp amplification products were sent to Inqaba Biotechnical Industries (Pty) Ltd in Pretoria for Sanger DNA sequencing. DNA sequences were visualized in Chromas Lite software (Version 2.6.6) (Technelysium Pty Ltd), inspected, and edited where necessary (visual base calling). Overlapping bases (point mutations without clear consensus) were designated according to the International Union of Pure and Applied Chemistry (IUPAC) nucleotide codes (Table 5) (https://www.cottongen.org/help/nomenclature/IUPAC_nt).

Table 5. IUPAC nucleotide code

Nucleotide	Common display	Meaning	Mnemonic
A	A	A	Adenine
C	C	C	Cytosine
G	G	G	Guanine
T (or U)	T	T (or U)	Thymine (or Uracil)
R	A/G	A or G	Purine
Y	C/T	C or T (or U)	Pyrimidines
K	G/T	G or T (or U)	Bases which are ketones
M	A/C	A or C	Bases with amino groups
S	C/G	C or G	Strong interaction
W	A/T	A or T (or U)	Weak interaction
B	C/G/T	Not A (i.e., C, G, T or U)	B comes after A
D	A/G/T	Not C (i.e., A, G, T or U)	D comes after C
H	A/C/T	Not G (i.e., A, C, T or U)	H comes after G
V	A/C/G	Neither T nor U (i.e., A, C or G)	V comes after U
N	A/C/G/T	A or C or G or T (or U)	Nucleic acid
. or -	-	Gap of indeterminate length	

The sequences were saved as the reverse or forward sequences based on the position they were allocated on the plate and the plate number in sequence of extraction (Table 3). The forward sequence was opened into the BioEdit software (Version 7.2.5) (Hall, 1999) and the reverse sequence was imported into the same file. The reverse sequence was selected and changed to the reverse complement. Both sequences were then aligned using the pairwise alignment tool and by allowing the ends to slide. The consensus sequence was deduced from the forward and reverse sequences. Any differences between the forward and reverse sequences were double checked in the original Chromas Lite software version of the sequence before finalizing the consensus sequence for further analysis.

3.7. Multiple sequence alignments and phylogenetic analysis

Assembled consensus sequences were imported into multiple sequence alignments in BioEdit software (Version 7.2.5) (Hall, 1999). The sequences were aligned using the online platform MAFFT (Version 7) (Katoh, et al., 2019 and Kuraku, et al., 2013) which is a multiple alignment program for amino acid and nucleotide sequences. An initial phylogenetic tree was generated using the online platform IQ-TREE (Version 2.2.2) (Trifinopoulos, et al., 2016) with 1000 bootstrap replicates, with reference sequences retrieved from the GenBank nucleotide sequence database (Bali, et al., 2022, and Houta et al., 2021) by Basic Local Alignment Search Tool (BLAST) homology searches. (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastnandPAGE_TYPE=BlastSearchandLINK_LOC=blasthome). The initial phylogenetic tree allowed for grouping of samples according to lineage. Reference sequences used are listed in Table 6.

Table 6. Reference sequences.

Lineage	GenBank accession number
GI-1	FJ888351, AY561711, M95169
GI-2	GU393336, DQ070840
GI-3	L14069, L14070
GI-4	L18988, AY251816
GI-5	DQ490215, U29522
GI-6	U29519, DQ515802
GI-7	AY606320, DQ646405
GI-8	M99484
GI-9	AF006624, M99482, DQ912831
GI-10	AF15159, AF15160, AF15154
GI-11	GU393339, JX182783
GI-12	X52084, X15832
GI-13	Z83975, EU914938
GI-14	X87238, FN182277
GI-15	FJ807932, FJ807944
GI-16	AF286302, KJ941019
GI-17	AF510656, AF027509, AF419315
GI-18	AY296744, KC577391
GI-19	AF193423, KC577395, AY189157
GI-20	AF349620, AF349621
GI-21	AJ457137, DQ064808
GI-22	GQ265940, DQ167147, KC577382
GI-23	KY805846, AF093796
GI-24	KF757447, KF809796
GI-25	KM660636, KP085595, EU925393
GI-26	MZ325299_D2334, FN182268, FN182270
GI-27	GU301925, KM660634
GI-28	JX291989, KX640829
GI-29	KY407556, KY407558
GII-1	M21971, M21968
GIII-1	U29450, U29521
GIV-1	U77298, AF274436
GV-1	FJ235194, JX018208, DQ059618
GVI-1	KF007209, GQ265948
GVII-1	MH924835, KM365468
GVIII	AY789942

Duplicate samples or samples of similar origin aligned in the BioEdit Software to calculate the pairwise identity and when closely related a single sequence or a consensus sequence was selected as a representative. If they were not closely related

both sequences were added separately. The sequences were then verified again in the BioEdit software, to ensure that open reading frame of the partial gene was intact. A new phylogenetic tree was then reconstructed, again using the MAFFT alignment program and the IQ-TREE online platform for phylogenetic tree assembly. In the IQ-TREE data base, the MAFFT alignment file was selected to be uploaded. The sequence type was left at “Auto-detect” and the partition type at “Edge-linked”. The substitution model was left on “Auto” with rate categories at 4 and state frequency at “Empirical”. In the Bootstrap analysis category “Ultrafast” was selected with 1000 bootstrap replicates for the number of alignments allocated. The maximum iterations were also left at 1000 and the minimum correlation coefficient at 0.99. The search parameters were left at 0.5 for the perturbation strength and 100 for the IQ-TREE stopping rule. When all parameters and settings were correct the job was submitted.

The Phylogenetic trees were visualized in the Figtree (Version 1.4.4) saved as .svg files and edited in Inkscape (Version 1.2) (Inkscape Developers, 2022). Single sequences were subjected to the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastnandPAGE_TYPE=BlastSearchandLINK_LOC=blasthome) online server and the first 100 similar field sequences were additionally added to the individual lineage alignments and phylogenetic trees. The closest related outgroups of each lineage identified, were used as root for each lineage’s individual tree.

3.8 Genbank submission

Representative sequences were selected after the phylogenetic analysis for deposition into the Genbank sequence database. Isolate descriptions and Genbank accession numbers are listed in Annexure 1.

Chapter 4
RESULTS AND DISCUSSION

4.1 Virus isolation results and storage of isolates

After harvesting, embryo morphology was noted. When IBV was suspected observations included curling, stunting, growth retardation, feather club-down (Figure 7). 447 isolates with characteristic IBV embryo morphology were confirmed by RRT-PCR

Figure 5. The morphological characteristics of a normal embryo (source: Deltamune, 2023).

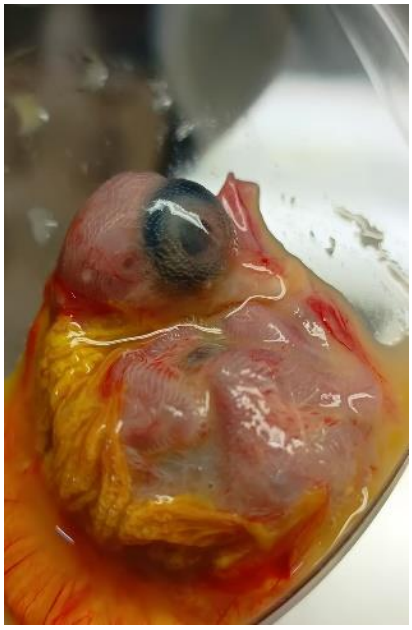

Figure 6. An embryo displaying morphological characteristics of IBV – curling, stunting growth retardation, and feather club-down (source: Deltamune, 2023).

For IBV, the allantoic fluid was harvested most often as this was the main route inoculated to specifically isolate respiratory viruses such as Avian Influenza (AI), Newcastle Disease Virus (NDV) and IBV. If any significant morphology relating to IBV was observed during a different route of inoculation, the allantoic fluid as well as the preferred organ was harvested for confirmatory tests. IBV did not always produce any morphological characteristics. This depended on the pathogenicity of the strain and its ability to adapt in eggs. When any morphological changes were observed, or after the third passage in eggs if no changes in morphology was observed, the allantoic fluid (or any other routes' organs when necessary) was sent to the diagnostic molecular department of Deltamune where IBV and AI RT-qPCRs were performed. Other morphological characteristics are observed for diseases such as AI and NDV, but AI also can go unnoticed in eggs if a low pathogenic strain is present. The PCR's either confirmed suspected IBV cases, identified unsuspected cases or confirmed a negative result. After three passages the cases with no results were closed. In some cases, IBV was sent for sequencing at Molecular Diagnostic Services (Pty) Ltd (MDS) for strain confirmation.

4.2 RT - PCR results

Table 7. DNA concentrations and RRT - PCR Results

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
1	P4-30		147868	Yes		16,2	
2	P5-55		148576	Yes		14,7	
3	P5-39		149399	Yes		12	
4	P4-7		152309	Yes		8,1	
5	P4-15		167453	Yes		12,7	
6	P4-36	P8-27	168218	No	Yes		10,8
7	P4-6		168355	Yes		8,3	
8	P4-17		174787	Yes		10,2	
9	P5-37		175829	Yes		13,4	
10	P3-50		175958	Yes		16,6	
11	P4-72		176633	Yes		12,6	
12	P1-30		186039	Yes		20,7	
13	P4-71		187596	Yes		30,1	
14	P4-84	P8-32	187803	Yes	Yes	13,5	15,3
15	P4-90		188172	Yes		13,9	
16	P2-21		188225	Yes		8,3	
17	P1-26		189122	Yes		10,7	
18	P2-62		189390	Yes		16,4	
19	P1-15		189628	Yes		10,2	
20	P2-38		189853	Yes		13,1	
21	P5-29		190866	Yes		15	
22	P4-68		191145	Yes		11,9	
23	P5-68		191930	Yes		17	
24	P5-11		192943	Yes		26,1	
25	P4-92		193013	Yes		22,6	
26	P4-82		193043	Yes		8,7	
27	P1-49		193138	Yes		14,9	
28	P5-24		193304	Yes		21,2	
29	P5-63		193307	Yes		17,3	
30	P1-28		193311	Yes		12,9	
31	P1-13		193366	Yes		12,8	
32	P4-83	P8-31	193368	Yes	Yes	6,5	12,3
33	P5-72		193602	Yes		8,4	
34	P2-58		193774	Yes		16,2	
35	P2-55	P9-29	193921	Yes	Yes	22,6	4,9
36	P5-69		194413	Yes		15,8	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
37	P1-10		194822	Yes		13,6	
38	P5-28		195324	Yes		14,4	
39	P2-7		195696	Yes		8,1	
40	P4-96		195854	Yes		16,2	
41	P5-66		196674	Yes		14,5	
42	P5-85	P8-52	196723	No	No		
43	P1-9	P9-2	197318	No	Yes		8,5
44	P2-14		197346	Yes		6,5	
45	P2-10	P9-13	197346	Yes	Yes	0,4	8,1
46	P2-46	P9-20	197826	Yes	Yes	7,5	4,1
47	P1-78		198544	Yes		9,4	
48	P5-10	P8-34	198565	Yes	Yes	41,4	9,5
49	P2-39		198573	Yes		13,1	
50	P1-20		199262	Yes		5,8	
51	P1-11		199341	Yes		13,1	
52	P4-95		200931	Yes		15	
53	P5-20		200957	Yes		19,1	
54	P5-78	P8-50	201106	Yes	Yes	8,5	8,2
55	P5-76	P8-49	201106	Yes	Yes	15,7	29,9
56	P5-12	P8-35	201208	Yes	No	4,5	
57	P5-9		201216	Yes		13,6	
58	P5-1		201216	Yes		26,1	
59	P5-14	P8-37	201216	Yes	Yes	12,8	5,7
60	P3-88	P8-19	201300	Yes	Yes	6	8,8
61	P2-78	P9-43	201351	Yes	Yes	13,7	5,1
62	P2-5	P8-4	201351	Yes	No	1,2	
63	P4-86		201591	Yes		6,2	
64	P1-52		201602	Yes		8,3	
65	P5-90		202348	Yes		13,7	
66	P2-96	P9-52	203097	Yes	Yes	17,4	11
67	P5-22		203100	Yes		10,5	
68	P5-70		203106	Yes		13,7	
69	P3-32	P9-69	203110	Yes	Yes	31,3	7
70	P1-81		206101	Yes		9,8	
71	P3-96	P8-22	208097	Yes	Yes	14,6	3,7
72	P2-89	P8-8	208136	Yes	Yes	16,2	4,5
73	P2-92	P8-9	208232	Yes	No	17,8	
74	P1-85		208890	Yes		16,2	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
75	P2-94		208981	Yes		16,5	
76	P3-74	P9-83	208982	Yes	Yes	17,8	14,1
77	P3-31		212412	Yes		20,4	
78	P3-33	P9-70	213416	Yes	Yes	19,2	11,5
79	P3-34		215665	Yes		20,2	
80	P3-75	P8-13	215735	Yes	Yes	10,6	9,2
81	P2-83	P9-48	216935	Yes	Yes	8,2	9,5
82	P2-95		217716	Yes		27,3	
83	P1-89		218586	Yes		13,8	
84	P2-84	P9-49	220197	Yes	Yes	13,4	12,4
85	P1-96	P9-9	220680	Yes	Yes	0,4	21,8
86	P1-91		222046	Yes		20,6	
87	P1-84	P9-6	222493	No	Yes		7,8
88	P2-87	P8-7	222625	Yes	Yes	13,1	4,7
89	P1-92	P9-7	222760	No	Yes		9,1
90	P1-82		223677	Yes		2,8	
91	P2-44	P9-18	224387	Yes	Yes	9	11,1
92	P2-86	P9-51	225510	Yes	Yes	13,9	13,1
93	P2-66	P9-34	226220	No	No		
94	P3-11		226600	Yes		25,8	
95	P5-88		226789	Yes		5,8	
96	P3-43		227152	Yes		12,9	
97	P1-33		227659	Yes		9,6	
98	P1-31		227829	Yes		22,2	
99	P1-22		228016	Yes		17,9	
100	P2-37		229015	Yes		13,5	
101	P4-60		229079	Yes		13,8	
102	P4-70	P8-29	229081	No	Yes		13,5
103	P5-64		229507	Yes		33,4	
104	P2-82	P9-47	230968	Yes	Yes	25,3	14,8
105	P5-30	P8-39	231139	No	Yes		10,2
106	P5-25		231217	Yes		22	
107	P4-59		231808	Yes		15,9	
108	P1-37		231832	Yes		14,7	
109	P4-91		232718	Yes		13	
110	P5-74		232719	Yes		34,8	
111	P5-15	P8-38	233607	No	No		
112	P2-80	P9-45	233942	Yes	Yes	27,1	21,3

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
113	P1-29		233979	Yes		12,4	
114	P2-52	P9-26	234109	Yes	Yes	19,1	5,5
115	P2-71	P9-38	234993	Yes	Yes	25,1	8
116	P1-6		235198	Yes		5,8	
117	P1-60		235447	No			
118	P1-66		236910	Yes		7,8	
119	P2-27		236912	Yes		21,4	
120	P1-40		238153	Yes		25,1	
121	P2-24		240525	Yes		18,1	
122	P1-57		242100	Yes		11,4	
123	P2-53	P9-27	243570	Yes	Yes	12,3	7,3
124	P1-64		244155	Yes		9,3	
125	P2-29		244663	Yes		12,8	
126	P2-61		245067	Yes		21,8	
127	P3-14	P9-63	246538	Yes	Yes	23,7	11,8
128	P1-5		249371	Yes		6,7	
129	P2-12		253792	Yes		3,1	
130	P1-14		253810	Yes		11,7	
131	P1-69		253811	Yes		10,6	
132	P2-25		256015	Yes		18	
133	P2-6	P8-5	259695	No	Yes		7,5
134	P1-35		260228	Yes		13,1	
135	P1-53		260229	Yes		14,1	
136	P2-32		260600	Yes		10,5	
137	P1-68	P9-4	260884	No	Yes		11,9
138	P3-64		261158	Yes		27,6	
139	P3-62		263752	Yes		25	
140	P1-63		266932	Yes		16	
141	P2-20		266968	Yes		17,2	
142	P1-45		267693	Yes		27,1	
143	P3-16		267739	Yes		23,2	
144	P3-48	P9-74	267839	Yes	No	7,9	
145	P3-6	P9-58	268083	Yes	Yes	18,2	13,5
146	P3-9	P9-60	268083	Yes	Yes	25,1	9,7
147	P3-70		268361	Yes		15,1	
148	P3-23	P9-67	269875	Yes	Yes	40,7	10,1
149	P3-46		270254	Yes		8,6	
150	P1-23		274387	Yes		22,3	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
151	P2-60	P9-33	274626	Yes	Yes	21,9	5,9
152	P3-22	P9-66	275478	Yes	Yes	21,2	9,1
153	P3-1	P9-53	275644	Yes	Yes	26,8	13,5
154	P1-39		276515	Yes		12,8	
155	P3-47		276628	Yes		20,6	
156	P4-87		276753	Yes		13,1	
157	P5-61		276811	Yes		20,8	
158	P5-62		276824	Yes		16,9	
159	P4-81		277553	Yes		6,3	
160	P5-23		277560	Yes		18,5	
161	P3-15	P9-64	278583	Yes	Yes	17,4	10,5
162	P3-52		278628	Yes		25,7	
163	P5-26		278832	Yes		21,1	
164	P3-27		280023	Yes		30	
165	P3-25		281216	Yes		28,1	
166	P3-78	P8-16	281572	Yes	No	17,9	
167	P3-44		281574	Yes		21,6	
168	P5-21		281975	Yes		21,2	
169	P1-41		284195	Yes		17,2	
170	P5-65		287871	Yes		17,7	
171	P5-83		288325	Yes		28,5	
172	P5-5		289015	Yes		14,5	
173	P2-11		295637	Yes		5,3	
174	P3-67	P9-78	309400	Yes	Yes	16,2	8,2
175	P3-8		314462	Yes		31,4	
176	P3-12	P9-62	316661	Yes	Yes	17,4	14
177	P3-45	P9-73	317372	Yes	Yes	12,3	13,8
178	P3-66		318879	Yes		18,5	
179	P3-80	P8-18	319685	Yes	Yes	17,7	12,3
180	P3-18	P9-65	320002	Yes	Yes	14,8	10,5
181	P3-28		320057	Yes		26,9	
182	P3-29		320213	Yes		35,6	
183	P3-4	P9-56	320709	Yes	Yes	16,4	13,2
184	P3-21		320844	Yes		20,2	
185	P3-58		321496	Yes		27	
186	P2-18		321872	Yes		13,9	
187	P4-38	P8-28	321872	Yes	Yes	23,4	10
188	P3-55	P9-75	322303	No	No		

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
189	P3-5	P9-57	322393	Yes	Yes	15,1	12,4
190	P2-43		322394	Yes		21	
191	P3-54		322903	Yes		22	
192	P3-19		323131	Yes		18,8	
193	P1-73		325285	Yes		14	
194	P2-22		327042	Yes		15,9	
195	P1-67		327066	Yes		12,4	
196	P1-59		329067	Yes		7,3	
197	P2-8	P8-6	329169	Yes	Yes	8,2	4
198	P1-18		330071	Yes		13,2	
199	P2-69	P9-36	332216	No	No		
200	P1-65		332607	Yes		6,1	
201	P1-70		332825	Yes		9,2	
202	P3-24		335734	Yes		29,9	
203	P2-70	P9-37	335734	Yes	No	9,2	
204	P2-33		335997	Yes		19,8	
205	P3-76	P8-14	336625	Yes	Yes	21,1	8,9
206	P1-56		337288	Yes		14	
207	P3-42	P9-72	338905	No	No		
208	P2-47	P9-21	339318	Yes	Yes	19,6	12,1
209	P2-16		339989	Yes		23,1	
210	P5-79		340340	Yes		25,1	
211	P5-27		340461	Yes		13,4	
212	P1-48		340744	Yes		12,9	
213	P5-81		341521	Yes		34,8	
214	P4-79		342217	Yes		16,5	
215	P5-17		344201	Yes		16,8	
216	P2-74		344383	No			
217	P1-16		344385	Yes		21,3	
218	P4-67		344385	Yes		7,1	
219	P2-19		345298	Yes		17,4	
220	P1-32		345568	Yes		28,3	
221	P1-42		346464	Yes		13,9	
222	P2-2		347675	Yes		8,8	
223	P3-63		348514	Yes		30,9	
224	P2-64		348519	Yes		28,7	
225	P5-19		348520	Yes		22,5	
226	P4-75		348521	Yes		15,4	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
227	P1-77		349150	Yes		7,3	
228	P2-34		349476	Yes		10,7	
229	P1-71		349480	Yes		13,1	
230	P3-13		351011	Yes		11,3	
231	P3-87		351288	Yes		22,9	
232	P3-7	P9-59	351505	No	Yes		11,1
233	P3-39		355673	Yes		29,3	
234	P2-4	P9-11	363621	Yes	Yes	11,6	10,1
235	P4-2		372100	Yes		6	
236	P3-93		374409	Yes		25,8	
237	P4-5		375003	Yes		12,9	
238	P2-65		377455	Yes		21,3	
239	P1-34		377465	Yes		11,4	
240	P1-47		377467	Yes		21,2	
241	P2-73	P9-39	379353	Yes	Yes	10,5	6,3
242	P2-59	P9-32	383258	Yes	Yes	14,8	13,5
243	P1-76	P9-5	383258	No	Yes		14,2
244	P2-1		388559	Yes		11,4	
245	P1-19		392670	Yes		23,3	
246	P2-26		392673	Yes		13,9	
247	P2-31		403218	Yes		13,4	
248	P2-23		403218	Yes		18,7	
249	P1-36		409284	Yes		15,2	
250	P4-4		462411	Yes		15,3	
251	P4-1		526182	Yes		9,9	
252	P3-82		526250	Yes		19,3	
253	P4-20		530520	Yes		20,2	
254	P1-17		531407	Yes		22,7	
255	P4-3		531796	Yes		20,8	
256	P4-10		537307	Yes		10,7	
257	P4-19	P8-23	537307	No	Yes		6,3
258	P4-16		537309	Yes		15,5	
259	P4-29		545467	Yes		12,3	
260	P4-18		550819	Yes		11,3	
261	P4-62		564876	Yes		7,9	
262	P5-43		568903	Yes		8,6	
263	P5-34		579119	Yes		16,9	
264	P5-41		586150	Yes		5,1	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
265	P5-57		592655	Yes		11,8	
266	P5-42		599490	Yes		14,4	
267	P5-50	P8-43	601368	No	Yes		14
268	P4-89		603902	Yes		9,8	
269	P4-85	P8-33	605307	Yes	Yes	18,9	11,1
270	P5-33	P8-40	606426	No	Yes		14
271	P5-56		608301	Yes		16,2	
272	P5-45		608305	Yes			
273	P4-88		617999	Yes		14,2	
274	P5-46	P8-42	626739	No	Yes		12,9
275	P5-52		634004	Yes		13,4	
276	P4-77		634038	Yes		12,5	
277	P5-44		635300	Yes		25,1	
278	P5-53		638883	Yes		11,1	
279	P4-31		656517	Yes		8,9	
280	P4-33		658187	Yes		17,9	
281	P4-32		659486	Yes		10,7	
282	P4-24	P8-25	659489	Yes	Yes	14,7	12,9
283	P3-89		662543	Yes		26,2	
284	P2-77	P9-42	132309 H3	Yes	Yes	21,4	5,7
285	P4-76		151934 H6	Yes		12	
286	P5-51		151934 H6	Yes		14	
287	P5-36		152953 H9	Yes		29,1	
288	P3-84		154830 H6	Yes		24,5	
289	P4-78		154830 H7	Yes		16,4	
290	P3-61	P9-77	169490 CT	Yes	Yes	4,3	8,4
291	P3-68		172175 #2	Yes		17,8	
292	P3-92		172175 -7 #3	Yes		17,8	
293	P4-34		172324-2	Yes		19,4	
294	P4-14		172324-4	Yes		6,8	
295	P3-91		174443 TR	Yes		21,9	
296	P4-27		175744 KD H1	Yes		24,9	
297	P4-12		175744 KD H1	Yes		8,3	
298	P4-13		175744 KD H5	Yes		10,6	
299	P4-28		175834 TR	Yes		11,2	
300	P5-48		187464 KN H2 AF	Yes		16,1	
301	P5-35		187464 KN H3	Yes		15,7	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
302	P5-49		187464 TR H1 AF	Yes		18,6	
303	P5-40		187464 TR H2 AF	Yes		18,7	
304	P2-56	P9-30	187464 TR H2 YS	No	No		
305	P5-47		187464 TR H3 AF	Yes		35,1	
306	P1-4		187521 H1	Yes		6,3	
307	P5-54	P8-44	187521 H2	Yes	Yes	13,4	14,8
308	P1-54		193970-3	Yes		19	
309	P2-51	P9-25	193970-4	Yes	Yes	15,9	7,6
310	P1-8		193970-6	Yes		8,7	
311	P2-41	P9-17	197270 H2	Yes	Yes	5,8	7,8
312	P4-94		197270 H3	Yes		14,8	
313	P1-27		197666 H2	Yes		17,6	
314	P1-7		197666 H4	Yes		11,2	
315	P2-3	P9-10	197677 H1	Yes	Yes	9,3	9,4
316	P1-61	P9-3	197677 H2	Yes	Yes	16,1	9,6
317	P5-8		198007 H2	Yes		22,9	
318	P5-84		198007 H3	Yes		17	
319	P5-77		198007 H4	Yes		2,9	
320	P1-62		198442 CE	Yes		3,4	
321	P2-9	P9-12	198442 SF	No	No		
322	P1-44		198442 TR	Yes		18,1	
323	P2-28	P9-15	198999 B9	Yes	Yes	21	10,8
324	P5-3		200656 H3	Yes		12,8	
325	P5-80		200656 H4	Yes		6,2	
326	P5-82	P8-51	200656 H4	No	Yes		18,3
327	P5-7		200677 HA	Yes		11,8	
328	P5-87		200677 HB	Yes		5,2	
329	P5-2		200963 H5	Yes		14,4	
330	P5-59	P8-46	200963 H7	Yes	Yes	4,3	9,9
331	P5-86		200963 H7	Yes		4,4	
332	P2-91		205069 AF H6	Yes			
333	P1-87		205069 YS H6	Yes		15,4	
334	P1-86		205069 YS H6	No			
335	P3-73	P9-82	209783 CE	Yes	Yes	8,8	7,4
336	P3-36	P9-71	209783 TR	No	No		
337	P3-72	P9-81	209842 H2	Yes	Yes	25,3	17

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
338	P3-95	P8-21	209842 H2	Yes	No	24,7	
339	P1-93		210882 H1	Yes		18,4	
340	P3-41	P8-12	210882 H3	Yes	Yes	30,1	12,6
341	P1-94	P9-8	221316 - ORG	No	Yes		13,1
342	P2-93		221316 - CE	Yes		19,4	
343	P2-85	P9-50	221316 TR	Yes	Yes	19,7	13,4
344	P1-88		222042 H1	Yes		17,5	
345	P2-90		222042 H2	Yes		13,1	
346	P1-83		225250 H1	Yes		15,2	
347	P2-88		225250 H5	Yes		15,2	
348	P1-95		225518 H10	Yes		6,2	
349	P5-31		225518 H11	Yes		19,3	
350	P5-89		225518 H12	Yes		25,5	
351	P3-60		226668 H2	Yes		17,4	
352	P3-40		226827 HD	Yes		21,3	
353	P3-77	P8-15	226827 HE	Yes	Yes	10	5,2
354	P3-86		226827 HF	Yes		23,8	
355	P4-80	P8-30	227831 TR	No	Yes		5,2
356	P2-72		227831 TR	Yes		11,1	
357	P5-16		229207 CE	Yes		6,5	
358	P5-58	P8-45	229207 TR	Yes	Yes	15,8	9,4
359	P5-18		229498 H1	Yes		16,3	
360	P4-73		229498 H2	Yes		10,5	
361	P2-57	P9-31	232786 H4	Yes	Yes	13,1	4,7
362	P2-63		232786 H4	Yes		18,6	
363	P1-50	P8-2	233429 CE	No	No		
364	P3-30	P9-68	233429 CE	No	No		
365	P1-24		234962 H13	Yes		8,8	
366	P1-2		234962 H7	Yes		9,4	
367	P2-49	P9-23	235104 TR	Yes	Yes	12,4	12,5
368	P2-15		236104 BR	Yes		23,5	
369	P1-51		236104 CE	Yes		19,6	
370	P3-20		245842 H2	Yes		39,9	
371	P2-35		245842 H3	Yes		16,3	
372	P1-25		259214 S1	Yes		16,8	
373	P2-76	P9-41	259214 S2	Yes	Yes	17,7	6,8
374	P2-67		264213 H1	Yes		15,3	
375	P2-79	P9-44	264213 H1	Yes	Yes	13,9	8,5

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
376	P1-38		264213 H2	Yes		25,8	
377	P3-59	P9-76	269246 AF	No	Yes		11,4
378	P3-49		269246 BR	Yes		22	
379	P3-79	P8-17	269246 CT	Yes	No	18,4	
380	P3-17		269246 KID	Yes		37	
381	P3-53		271729 H1	Yes		45	
382	P3-3	P9-55	271729 H2	No	Yes		12,1
383	P4-35	P9-19	274025 H	Yes	Yes	33,3	10,1
384	P2-45		274025 S	Yes		9,8	
385	P3-57		276453 H1	Yes		27,1	
386	P3-10	P9-61	276453 H2	Yes	Yes	16,3	10,1
387	P2-13		282562 H15	Yes		26,7	
388	P1-21		282562 H4	Yes		22,1	
389	P5-60	P8-47	282913 H2	No	Yes		17,8
390	P5-13	P8-36	282913 H3	No	Yes		12,1
391	P5-4		288392 H2	Yes		18,6	
392	P5-67		288392 H3	Yes		13	
393	P5-6		289368 H10	Yes		18,1	
394	P1-79		289368 H10	Yes		13,9	
395	P5-75		289368 H7	Yes		14,5	
396	P1-43		305670 H2	Yes		13,7	
397	P1-1	P9-1	305670 H3	No	Yes		13,4
398	P2-48	P9-22	305670 H4	Yes	Yes	15,7	13,7
399	P1-46		309115 H3	Yes		20,3	
400	P2-40	P9-16	309115 H8	Yes	Yes	17,8	10,9
401	P2-42		313190 H1	Yes		20,4	
402	P3-65		313190 H3	Yes		36,8	
403	P1-90		327985 H12	Yes		17,8	
404	P2-75	P9-40	327985 H18	No	Yes		6,8
405	P3-35		336344 H1	Yes		27,9	
406	P3-71	P9-80	336344 H2	Yes	Yes	16,9	13,2
407	P4-9		336344 H7	Yes		24,8	
408	P3-81		336344 H8	Yes		22,6	
409	P1-72		337109 H5	Yes		11,2	
410	P1-12		337109 H7	Yes		4,8	
411	P1-3		346613 H2	Yes		14,6	
412	P2-36		346613 H4	Yes		10,6	
413	P4-74		347477 H2	Yes		10,3	

Sample number	Study reference number	Repeat reference number	Lab reference number	Band present	Repeat band present	DNA Conc. (ng/µl)	Repeat Conc. (ng/µl)
414	P5-73		347477 H3	Yes		14,4	
415	P5-71	P8-48	347626 H1	No	Yes		14,6
416	P4-69		347626 H2	Yes		12	
417	P1-74		348513 AF	Yes		8,8	
418	P3-38	P8-11	348513 CAM	No	Yes		3,8
419	P1-58	P8-3	348513 CAM	No	No		
420	P3-51		348517 H1	Yes			
421	P2-81	P9-46	348517 H2	Yes	Yes	15,8	19,7
422	P3-37	P8-10	348517 H6	Yes	No	33,4	
423	P3-69	P9-79	350676 C3	Yes	Yes	24,2	10,9
424	P2-30		350676 C5	Yes		8,5	
425	P1-55		351290 H2	Yes		21,6	
426	P4-61		351290 H9	Yes		45,3	
427	P1-75		351290 H9	Yes		10,9	
428	P3-26		358886 AF H6	Yes		37,4	
429	P3-2	P9-54	358886 H4	Yes	Yes	15,9	12,5
430	P3-56		358886 YS H6	Yes		28,1	
431	P2-50	P9-24	359396 H4	Yes	Yes	5,5	10,4
432	P1-80		359396 H5	Yes		17,2	
433	P2-68	P9-35	461064 H2	No	Yes		5,7
434	P2-17	P9-14	461064 H4	Yes	Yes	13,2	10,3
435	P4-25	P8-26	461064 H6	Yes	No	16,9	
436	P3-94	P8-20	533511 H9	Yes	Yes	22,6	8,1
437	P3-90		533551 H6	Yes		25,2	
438	P4-11		538641 B4	Yes		11,6	
439	P4-8		538641 B5	Yes		21,2	
440	P3-85		578946 N2	Yes		21,6	
441	P3-83		578946 N5	Yes		3,2	
442	P4-23		578946 N6	Yes		17,6	
443	P4-93		599479 H3	Yes		16,6	
444	P5-32		599479 H4	Yes		12,7	
445	P5-38	P8-41	638634 IV	No	Yes		16,9
446	P4-22	P8-24	658421 H1	Yes	Yes	18,5	12,3
447	P2-54	P9-28	Unknown	Yes	Yes	29,4	7,1

432 samples produced a detectable band of approximately 745 base pairs (bp). Results for some of the agarose gel electrophoretic separation of RT-PCR products are presented in Figures 7 and 8.

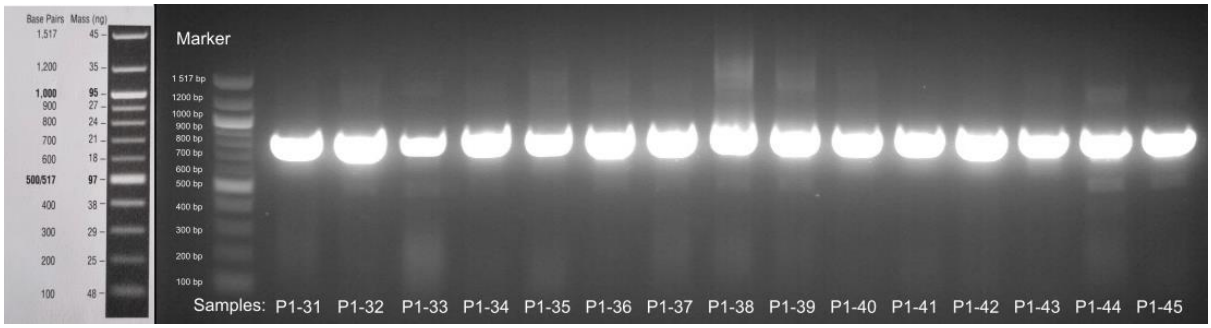

Figure 7. 1% Agarose gel of partial S1 gene bands of 745 bp.

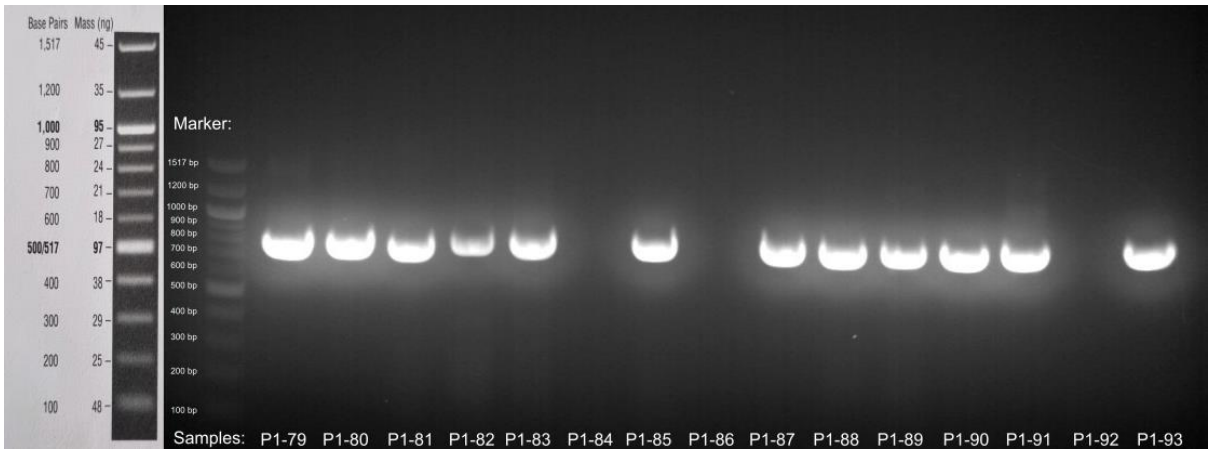

Figure 8. 1% Agarose gel of partial S1 gene bands 745 bp.

112 samples produced a band on the repeat PCR. In total, 385 purified samples were suitable for Sanger DNA sequencing.

4.3 Sequencing results and analysis

An example of a sequence where editing was done using the IUPAC nucleotide codes is displayed in Figure 9.

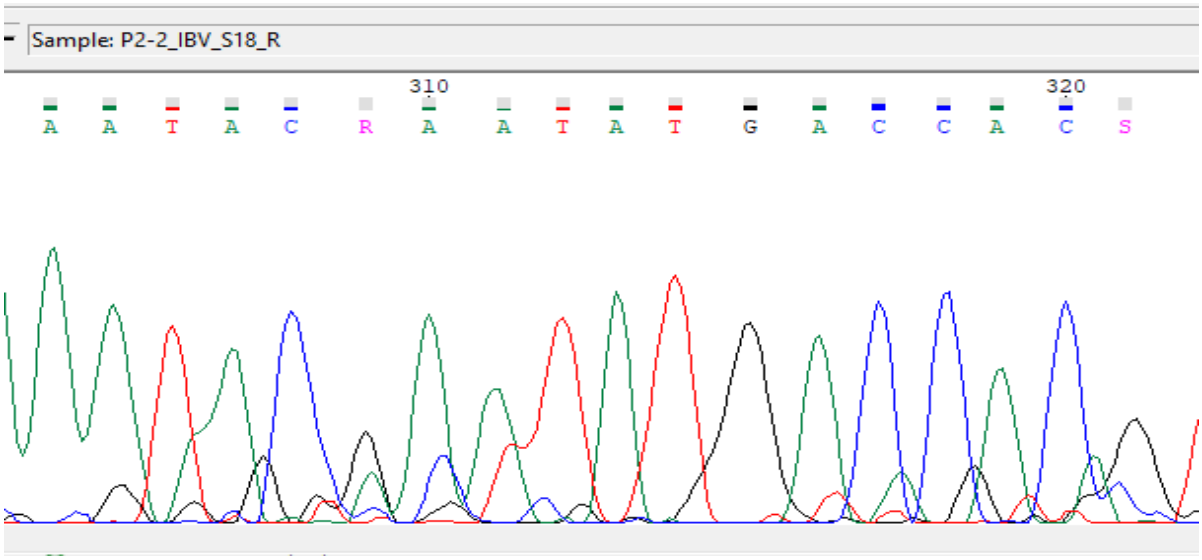

Figure 9. Sample number P2-2 showed as an example of where the letter “R” and “S” would have been changed to a “G” according to the IUPAC nucleotide guidelines.

11 samples had either duplicate sequences available or originated from the same site or house where a single or consensus sequence was chosen as representative. Twenty-four (6.2%) sequences (Table 7) showed a possible mixed sequence that could be caused by infections from viral coinfections. These sequences were removed from any further data analysis. Figure 10 shows an example of such a mixed sequence.

Table 8. Mixed sequences excluded from the study.

Study reference code	Lab reference number	Analysis
P1-20	199262	Mixed
P5-20	200957	Mixed
P2-96	203097	Mixed
P5-22	203100	Mixed
P1-91	222046	Mixed
P2-86	225510	Mixed
P1-66	236910	Mixed
P3-47	276628	Mixed
P3-8	314462	Mixed
P1-18	330071	Mixed
P1-65	332607	Mixed
P2-16	339989	Mixed
P1-16	344385	Mixed
P2-64	348519	Mixed
P1-71	349480	Mixed
P3-7	351505	Mixed
P5-45	608305	Mixed
P1-61	197677 H2	Mixed
P5-86	200963 H7	Mixed
P5-58	229207 TR	Mixed
P1-79	289368 H10	Mixed
P1-1	305670 H3	Mixed
P2-75	327985 H18	Mixed
P1-75	351290 H9	Mixed

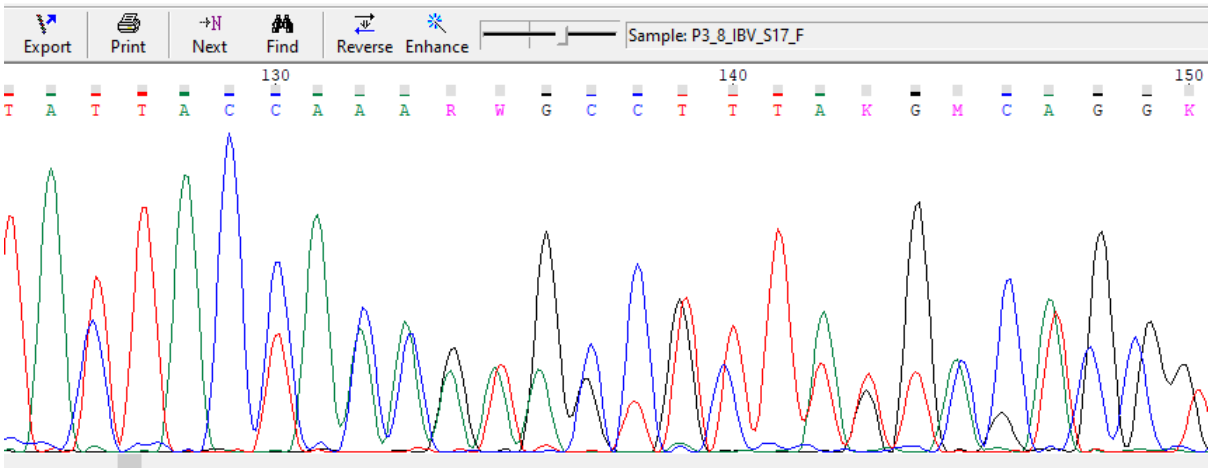

Figure 10. Sample P3-8 displayed as an example of overlapping sequences that indicate a possible mixed sequence.

After editing the sequences were saved in FASTA format and further analyzed in BioEdit software (Version 7.2.5) (Hall, 1999) by comparing forward and reverse sequences with each other. The finalized products were all grouped together, and an initial phylogenetic tree was created for guidance. The initial tree gave an indication of where isolates would classify (Figure 11). An additional 100 field reference samples identified by BLAST homology searches were added, and individual phylogenetic trees for specific genotypes were created.

Seven genotypes were identified in the southern African IBV isolates, namely, GI-1, GI-11, GI-13, GI-19, GI-23, GIV-1, and GVI-1 (Figures 13-19, respectively). The individual phylogenetic trees were prepared for each genotype and are presented from pg. 74 onwards.

184 (50,5%) viruses were identified as GI-19 (QX) strains and 78 (21,4%) as the GI-1 (Mass/H120) strain. 39 samples grouped within the GI-13 (4/91) (10,7%) lineage, 29 in the GVI-1 (TC07-2) (8,0%) lineage, 19 in the GI-23 (Var II) (5,2%) lineage, and 13 in the GI-11 (UFMG/G - Brazil) (3,6%) lineage. Two unique viruses appeared more closely related to GIV-1 (DE/072/92) (0.5%) lineages (Figure 12).

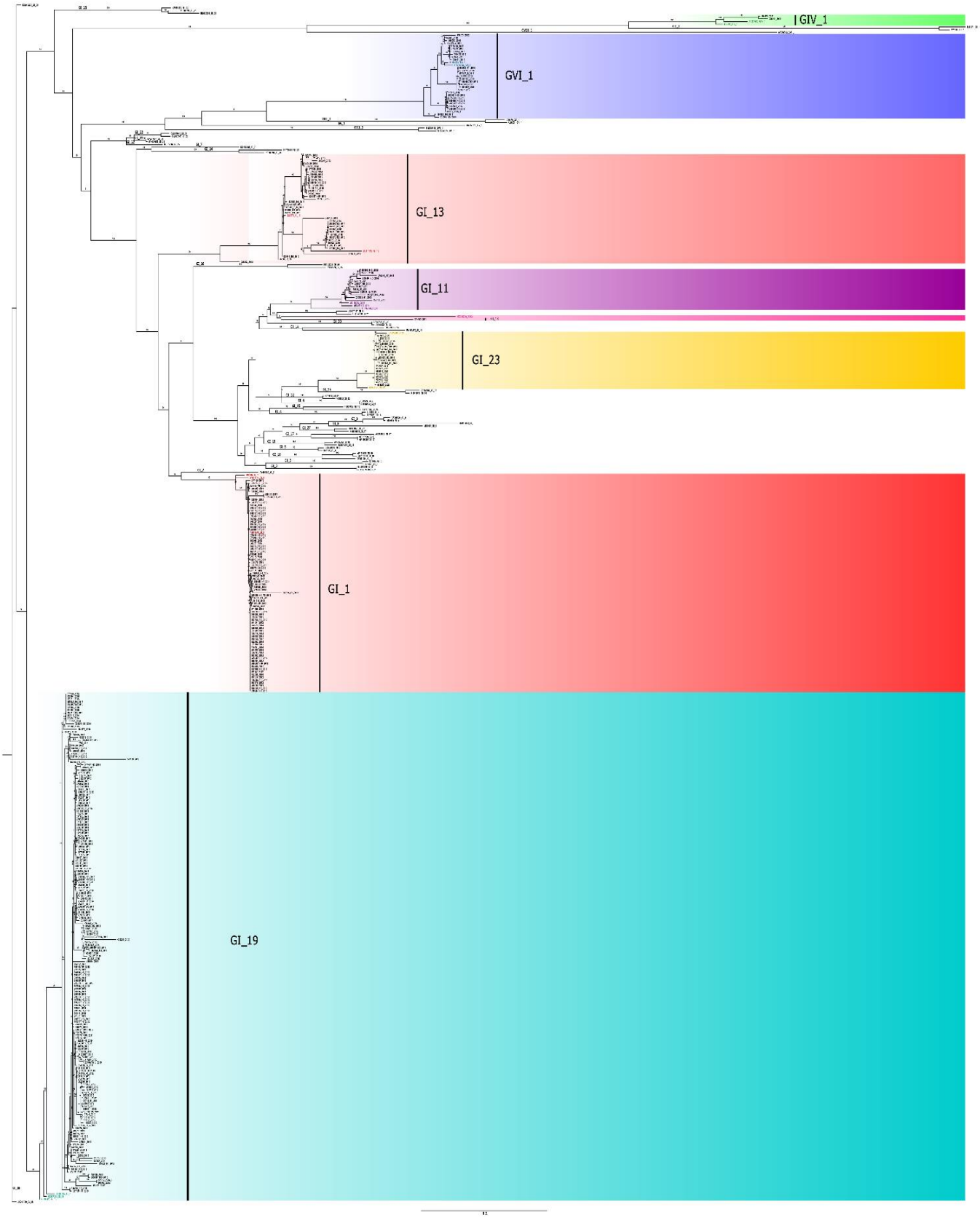

Figure 11. Maximum-likelihood phylogenetic tree based on a partial sequence of the S1 gene that includes all reference and isolate sequences.

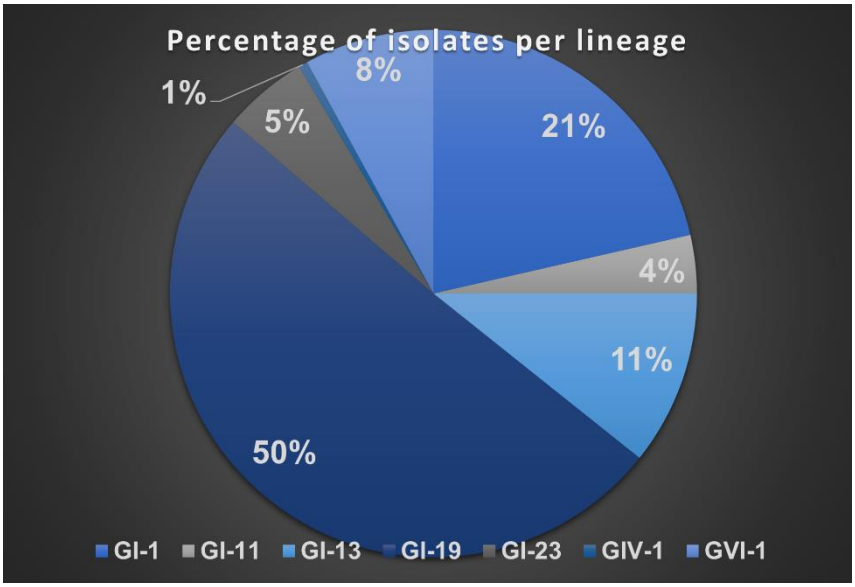

Figure 12. The percentage of IBV isolates identified per lineage during the study period of 2011-2020.

48 Samples had previous sequencing results were available from previous result reports (Molecular Diagnostic Services but only 70% correspond with the outcome of this study (Table 8.)

Table 9. Samples with previous sequencing data available versus current study data

Sample Number	Study Reference Code	Lab Reference Number	Lineage as presented in this study	Previous sequencing results
1	P1-28	193311	GI-19	GI-19
2	P1-10	194822	GI-19	GI-19
3	P5-20	200957	Could not be determined	4-91 Path
4	P3-32	203110	GI-19	GI-19
5	P2-84	220197	GI-1	Mixed: GVI-1, GI-19
6	P1-84	222493	GI-19	GI-19
7	P1-92	222760	GI-11	GI-19
8	P2-52	234109	GI-19	GI-19
9	P2-71	234993	GI-1	GI-1
10	P1-5	249371	GI-19	GI-11
11	P3-16	267739	GI-19	GI-1
12	P3-6	268083	GVI-1	GI-19
13	P3-9	268083	Could not be determined	GI-11
14	P3-22	275478	GI-19	GI-1
15	P3-15	278583	GI-19	GI-19
16	P5-26	278832	GI-19	Mixed unable to type
17	P5-83	288325	GI_11	GI-19

Sample Number	Study Reference Code	Lab Reference Number	Lineage as presented in this study	Previous sequencing results
18	P3-8	314462	Could not be determined	GI-1
19	P3-12	316661	GI-1	GI-19
20	P3-80	319685	GI-19	GI-19
21	P3-28	320057	GI-13	GI-19
22	P3-4	320709	GI-13	GI-19
23	P3-58	321496	GI-1	GI-19
24	P2-18	321872	GVI-1	GI-19
25	P4-38	321872	GI-19	GI-19
26	P3-19	323131	GI-19	GI-11
27	P1-59	329067	GI-1	GI-1
28	P2-8	329169	GI-19	GI-1
29	P1-70	332825	GI-19	GI-19
30	P2-70	335734	Could not be determined	GI-1
31	P2-33	335997	GI-13	GI-1
32	P1-56	337288	GI-13	GI-19
33	P2-16	339989	Could not be determined	GI-1
34	P5-81	341521	GI-13	GI-19
35	P4-79	342217	GI-19	GI-19
36	P1-16	344385	Could not be determined	GI-1
37	P4-67	344385	GI-19	GI-19
38	P1-42	346464	GI-13	GI-19
39	P3-63	348514	GI-19	GI-19
40	P2-64	348519	Could not be determined	GI-19
41	P4-75	348521	GI-1	Mixed: GI-1, GI-13
42	P3-87	351288	GI-13	GI-19
43	P2-4	363621	GI-13	GI-19
44	P2-73	379353	GI-13	GI-19
45	P4-31	656517	GI-23	GI-19
46	P3-36	209783 TR	Could not be determined	GI-1
47	P2-75	327985 H18	Could not be determined	GI-19
48	P3-83	578946 N5	GI-23	GI-19

4.3.1 Lineage GI-1 (Mass/H120-type strains)

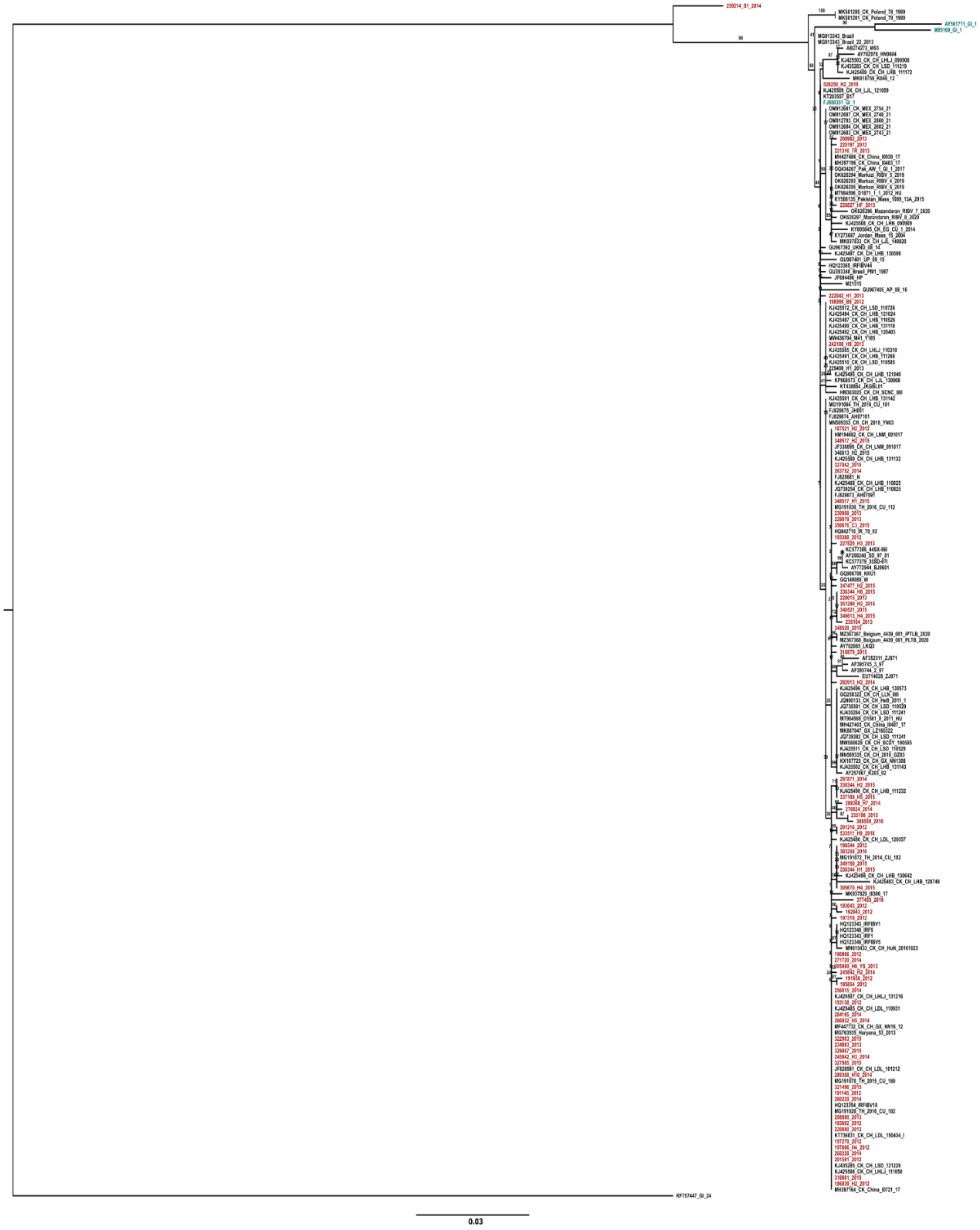

Figure 13(a). Maximum-likelihood phylogenetic tree of lineage GI-1 IBV. Study isolates are highlighted in red, genome classification reference samples are in light blue, and field viruses retrieved from Genbank are in black.

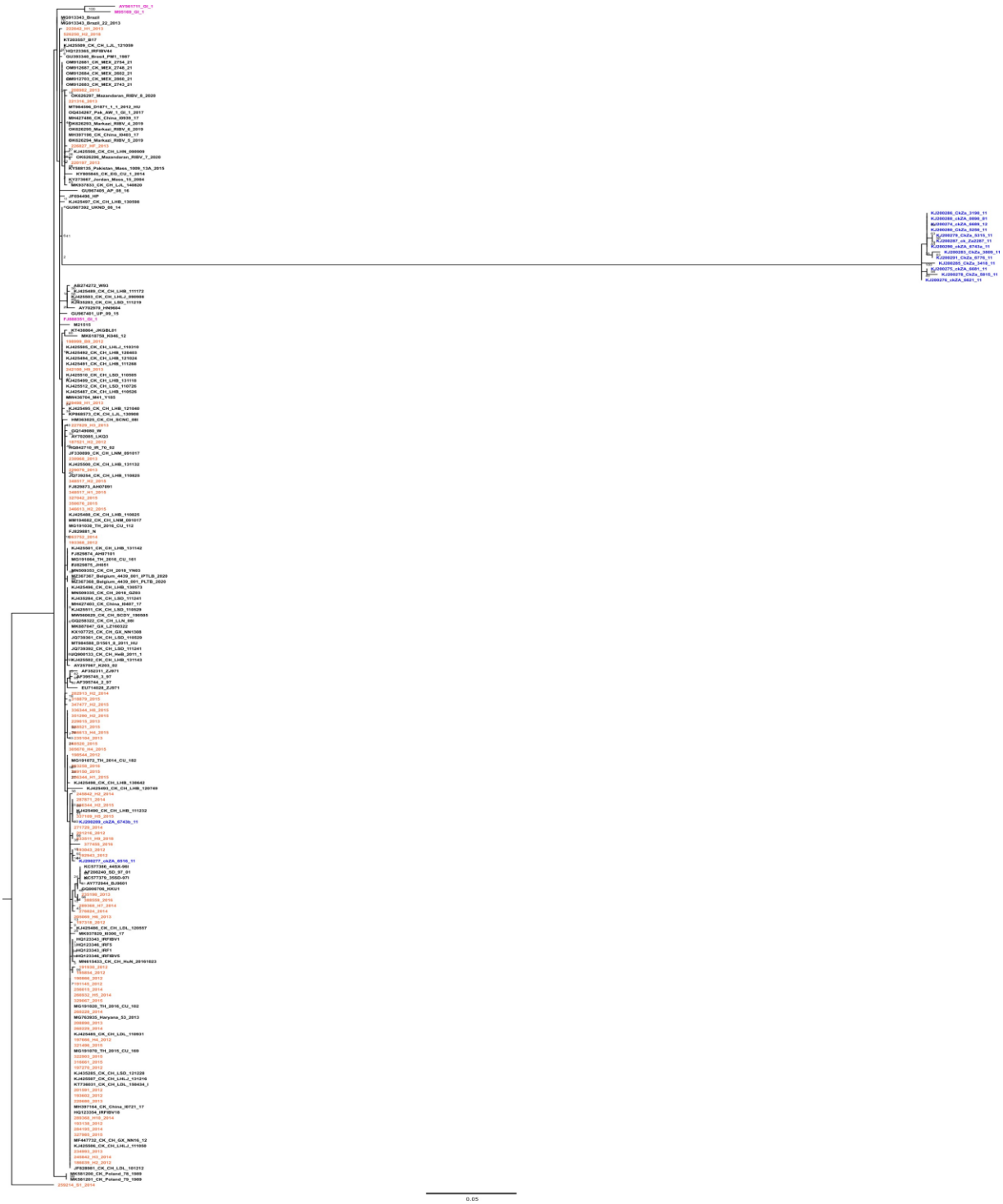

Figure 13(b). Maximum-likelihood phylogenetic tree of lineage GI-1 that includes previously identified ZA H120-like strains from Knoetze et al., 2014 (KJ200286, KJ200283, KJ200280, KJ200279, KJ200278, KJ200291, KJ200275, KJ200276, KJ200277, KJ200290, KJ200289, KJ200274, KJ200288, KJ200287, KJ200285) that were retrieved from Genbank. Study isolates are in orange font, ZA strains are in blue font, genome classification reference samples are in magenta, field viruses retrieved from Genbank are in black.

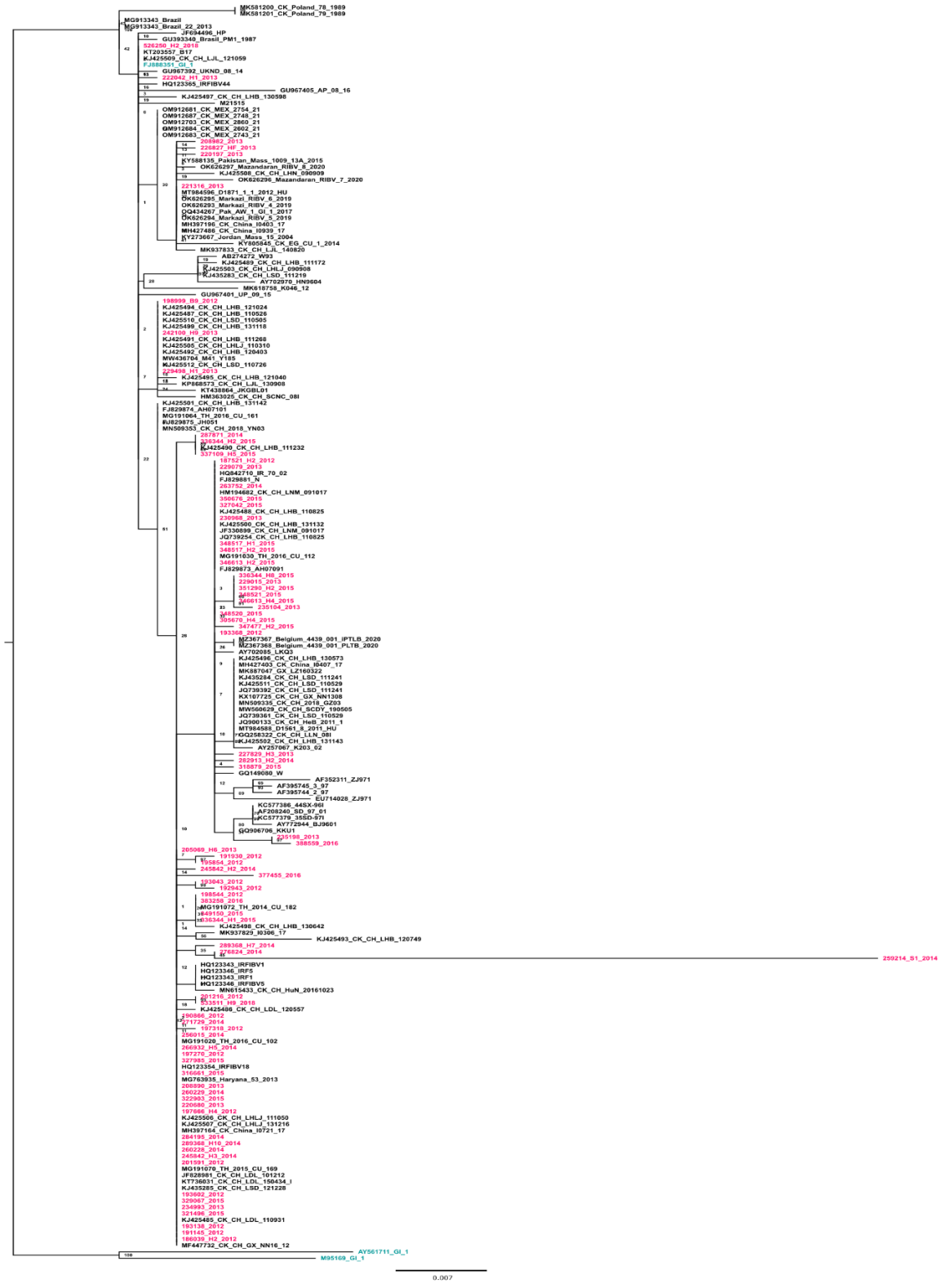

Figure 13(c). Maximum-likelihood phylogenetic tree of lineage GI-1 rooted with a more closely related reference sequence (AY561711 and M95169) for a better visualization. Study isolates are highlighted in pink, genome classification reference samples are in light blue, field viruses retrieved from Genbank are in black.

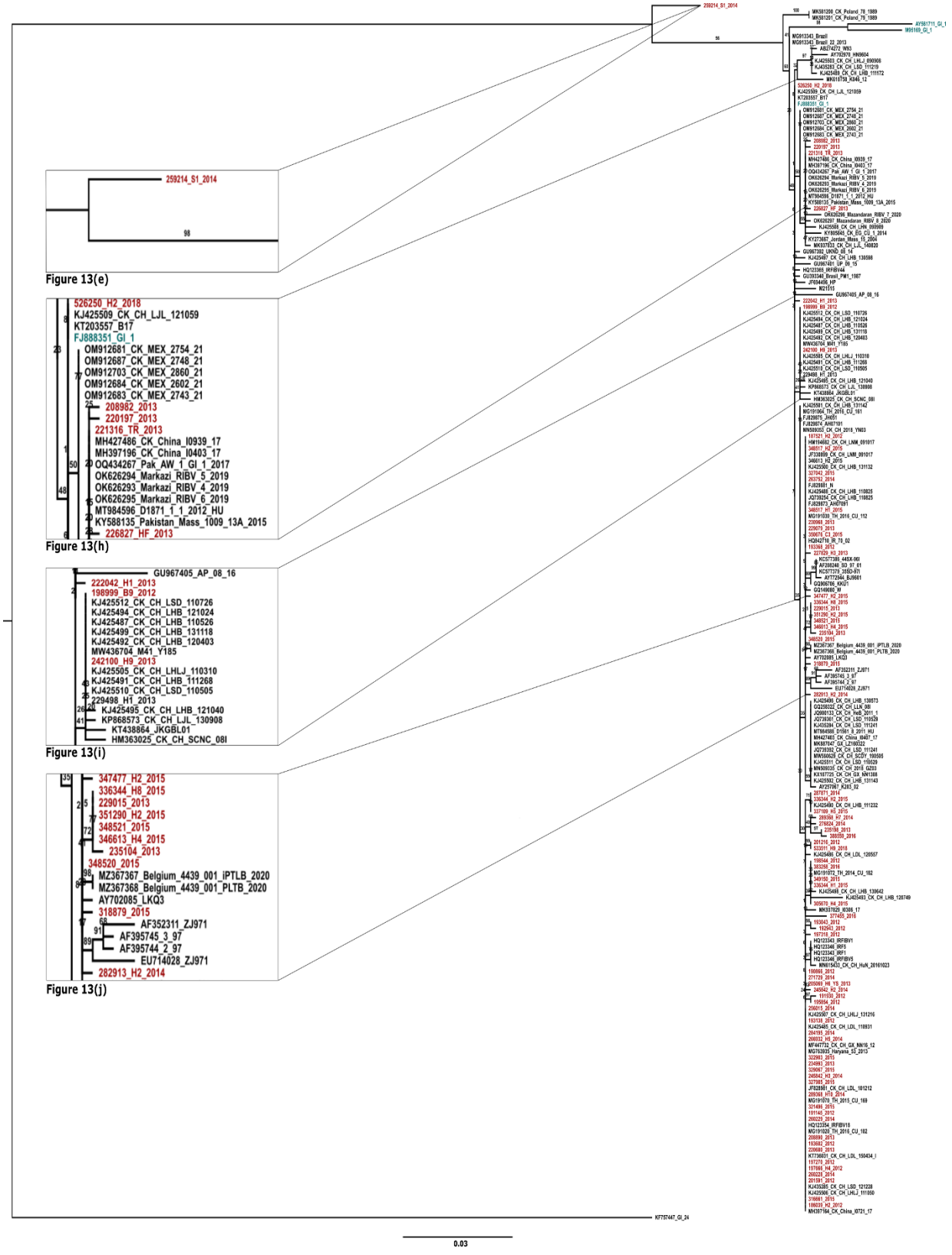

Figure 13(d). Maximum-likelihood phylogenetic tree of lineage GI-1 highlighting sub-set-based trees in Figs 13 (e to j) that follow for a better visualization and discussion. Study isolates are in red font, genome classification reference samples are in light blue font, field viruses retrieved from Genbank are in black font.

Figure 13(e). Maximum-likelihood phylogenetic tree of the GI-1 lineage re-rooted for an enlarged view of isolate 259214_S1_2014(shaded in purple). Genome classification reference samples are in purple font and field viruses retrieved from Genbank are in black font.

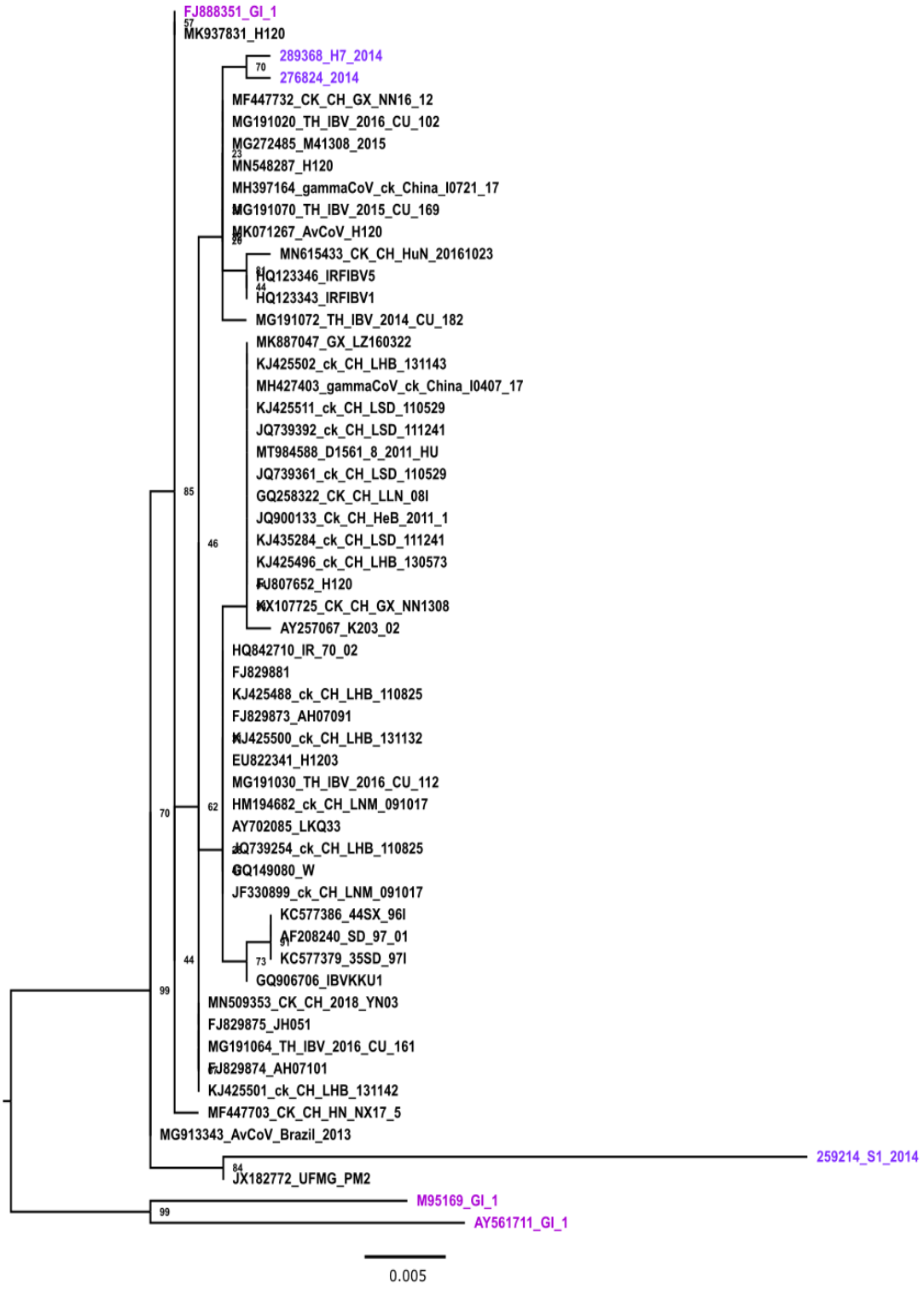

Figure 13(f). Maximum-likelihood phylogenetic tree of the GI-1 lineage, re-rooted for an enlarged view of isolates 259214_S1_2014, 289368_H7_2014 and 276824_2014. Study isolates are highlighted in purple, genome classification reference samples are highlighted in magenta and field viruses retrieved from Genbank are in black.

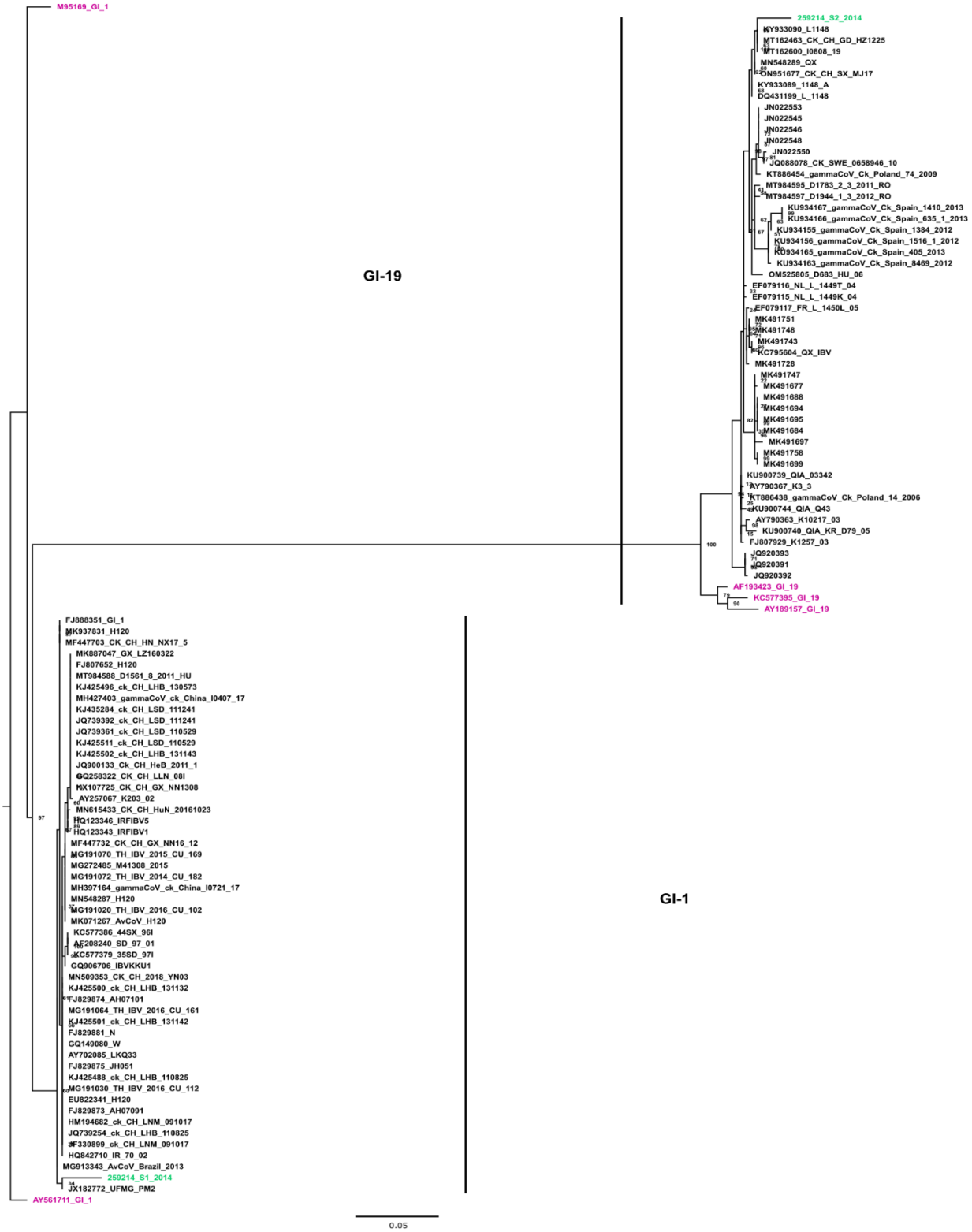

Figure 13(g). Maximum-likelihood phylogenetic tree re-rooted for an enlarged view of isolate 259214_S1_2014 and 259214_S2_2014 in the GI-1 and GI-19 lineage. Study isolates are highlighted in light blue, genome classification reference samples are highlighted in magenta and field viruses retrieved from Genbank are in black.

The reference sample with accession number FJ888351, is a complete genome of an attenuated H120 strain collected in 1960 that originated from the Netherlands. Although H120 vaccines had been in put in use since at least 1998 in South Africa (Table 1.), there is proof that the Mass strains have been circulating within the region since 1981 (Knoetze et al., 2014). Most samples isolated from the GI-1 lineage, appeared to be more closely related to the FJ888351 reference strain rather than that of AY561711 (strain M41, USA, 1956) and M95169 (strain Beaudette, USA, 1984).

During BLAST homology searches it was noticed that none of the previous South African sequences studied and submitted to Genbank (Knoetze et al., 2014) formed part of the first 100 most closely related sequences to add to the phylogenetic tree analysis. Figure 13(b) shows the relationship of these sequences to the isolates used in this study. Fifteen of the sequences submitted to Genbank were identified as Mass type isolates (Knoetze et al., 2014). Interestingly, thirteen of these isolates (KJ200286, KJ200283, KJ200280, KJ200279, KJ200278, KJ200291, KJ200275, KJ200276, KJ200290, KJ200274, KJ200288, KJ200287, KJ200285) form a separate cluster and excluded any of the other isolates found in the current study. This included the isolate from 1981 (KJ200288), which was initially classified as an unknown variant by Cook et al., 1999. Two other ZA (KJ200277, KJ200289) strains appeared to be more closely related to isolates used found this study.

Isolate 259214_S1_2014 can be differentiated from all other isolates of GI-1 lineage viruses on the phylogenetic tree. Initially it appeared to be closely related to isolates 289368_H7_2014 and 276824_2014 (Figure 13(c)) the bootstrap values however are a mere 45%. Further analysis suggests it may be a unique sequence (Figure 13(f)). It also appears to be more closely related to a sample isolated from Brazil in 1987 (JX182772) (Figure 13(e) and 13(f)) with bootstrap values at 80%. There is limited information available on the Brazilian strain, but the abstract explains two different types of IBV circulating in the South east area of Brazil namely, one group containing only Brazilian isolates and a second Massachusetts-like group (Santos et al., 2012). Full genome sequencing of this isolate could reveal a more extensive look into its ancestry and show the most likely lineages that combined to form this sequence. However unique this sequence might be, it does not appear to have adapted to spread other than from where it was isolated from.

There was a second isolate that was assigned the same lab number 259214_S2_2014, possibly from a different site. These two samples however only have an 80% correlation by pairwise alignment and the second sample falls within the GI-19 lineage (Figure 13(g)).

The viruses of 208982_2013, 226827_HF_2013, 220197_2013 and 221316_2013 grouped closer together than that of 222042_2013 and 526250_H2_2018 (Figure 13(h)). The first group of samples appeared to be more related (99.01%) to samples isolated from China (MH397196 and MH427486) collected in 2017, Brazil (KY626045) collected in 2016, and Pakistan (KY588135) from 2015. While no published data was available for the isolates from China, the Pakistan and Brazilian strains were vaccine strains of Ma5 and Mass41 (Dimitrov et al., 2017 and Brandão et al., 2017).

Samples 222042_2013 and 526250_H2_2018 appeared to be more closely related (99.01%) to Genbank submissions of China (MK937831) of 2019, U.S.A. (KX529712) collected in 2015, India (KF188436) from 2012 as well as strains from Brazil (JX182790 and JX182773) isolated in 1978 and 1987 respectively. No published data was available for the isolates from China and the U.S.A. The complete H120 genome from India (KF188436) was classified as a vaccine strain (Kamble et al., 2016). The Brazilian strains mentioned here, also had limited classification information available (Santos et al., 2012). It was unclear to deduce from the abstract if the isolates mentioned formed part of the Massachusetts-like group or the Brazilian isolates the study had focused on. The closely relationship between the reference FJ888351 strain and 222042_2013 and 526250_H2_2018 also indicates that this isolate was most likely a derivative of the H120 vaccine strains used within South Africa.

Figure 13(h). Maximum-likelihood phylogenetic tree of the GI-1 lineage, re-rooted for an enlarged view of isolates 226827_HF_2013, 220197_2013, 221316_2013, 222042_2013, and 526250_H2_2018. Study isolates are highlighted in gold, genome classification reference samples are highlighted in purple and field viruses retrieved from Genbank are in black.

Figure 13(i). Maximum-likelihood phylogenetic tree GI-1, re-rooted for an enlarged view of isolates 198999_B9_2012, 229498_H1_2013, and 242100_H9_2013. Study isolates are highlighted in orange, genome classification reference samples are highlighted in green and field viruses retrieved from Genbank are in black.

Figure 13(i) represents the isolates 198999_B9_2012, 229498_H1_2013, and 242100_H9_2013. These had a close identity match (99.15%) to isolates from China (MW436704, MF447703, KX107793, KX107693, KX129907, KJ425512) that date from 2011 to 2020. The only available published data was available for sequence KJ425512. This isolate was determined to be like that of the H120 vaccine being used (Chen et al., 2015).

Nine isolates namely, 347477_H2_2015, 336344_H8_2015, 346613_H4_2015, 351290_H2_2015, 229015_2013, 235104_2013, 348520_2015, 318879_2015, and 282913_H2_2014 (Figure 13(j)) appeared to be more related (99.01%) to samples collected from Thailand (MG191030), 2016, China (FJ829873, KJ425488 and KJ425500) collected in 2007, 2011 and 2013. MG191030 was found to be closely related to that of circulating vaccine strains (Munyahongse et al., 2020). There appears to be no published data for FJ829873, but KJ425488 and KJ425500 was also determined to be like that of the H120 vaccine strain (Chen et al., 2015).

The last cluster of the representative phylogenetic tree of GI-1 (Figure 13a), held 45 isolates all seemingly more closely related to each other, and Massachusetts strains isolated from China (KJ425490, KJ425486, KJ425498 and KJ425506) from 2011-2014, a strain from India (MG763935), 2013 and Thailand (MG191070 and MG191020), 2016. The isolates from China were all more closely related to vaccine strains (Chen at.al., 2015). Unfortunately, no published data was available for the strain from India. Furthermore, the isolates from Thailand were also vaccine derived strains (Munyahongse et al., 2020). Based on this data it could be assumed that all the abovementioned isolates were also closely related to the H120 vaccine strain rather than field isolates.

Figure 13(j). Maximum-likelihood phylogenetic tree re-rooted for an enlarged view of isolates 347477_H2_2015, 336344_H8_2015, 346613_H4_2015, 351290_H2_2015, 229015_2013, 235104_2013, 348520_2015, 318879_2015, and 282913_H2_2014 in the GI-1 lineage. Study isolates are highlighted in red; genome classification reference samples are highlighted in blue and field viruses retrieved from Genbank are in black.

The GI-1 lineage is one of the most well-known studied lineages of IBV (Valastro, et al., 2016). It is the most widely distributed genetic group due to the extensive use of vaccines derived from one of the groups strains (Valastro, et al., 2016). There is supporting data that the Mass/H120-type strain has been circulating within South Africa since at least 1981 (Knoetze et al., 2014) and it was also one of the first vaccines to be registered in South Africa for use against IBV (Table 1.). This lineage was identified as the second most isolated lineage of this study and covered a total 21.6% of sequences. Isolates from this group were most dominantly isolated from 2012 – 2016. Whether there was a change in vaccination regimes or a change in pathogenicity within the GI-1 lineage remains to be determined. The lack of isolation of this lineage from 2017- 2020, could reflect improvement of vaccination programs or more effective or updated vaccines being used in the industry, but is most probably the lack of isolation requests and the interference of AI outbreaks during 2017. Most sequences within this group appear to have a link to the attenuated H120 reference sequence from the Netherlands (FJ88351), and published sequences also proven to be vaccine derived strains. This suggests that the isolates within this group could either be vaccine strains re-isolated or viruses adapted from vaccine strains. Vaccination programs differ to an extent for each farm. In most cases chicks are exposed to IBV at a day old and most often with the H120 strain. Chickens are then vaccinated with IBV 3-4 times in rearing and again in lay at 4-to-eight-week intervals (Bisschop, S.P.R., personal communication, 2023).

4.3.2 Lineage GI-11 (UFMG/G – Brazil-like strains)

Figure 14. Maximum-likelihood phylogenetic tree of lineage GI-11 IBV. Study isolates are highlighted in purple, genome classification reference samples are in light blue, field viruses retrieved from Genbank are in black.

All isolates sequenced in this study that fall within the GI-11 lineage appeared to form their own cluster at the bottom of the phylogenetic tree but were still closely related to the Brazilian strains. The closest relative was that of a Brazil strain collected in 2011, with accession number: JX559803. Samples 222760_2013 and 282913_H3_2014 had the lowest percentage sequence to JX559803, yet this strain remained their closest relative. Other closely related isolates also from Brazil included: GU383108, GU383110, MK241814 JX559788, JX559806, JX559808 and JX559810. The identity percentage ranged between 89.53% and 92.38%. Chacón, et al., 2011 and Fraga et al., 2013, reported that these isolates form part of the so-called previously characterized Brazilian BR-I genotype. The Brazilian IBV variants were discovered to be more than 25% different from vaccine genotypes, which likely explains the low effectiveness of commercial IBV vaccines (Wei et al., 2008; Chacon et al., 2011).

South Africa and Brazil share common isolations of genotypes GI-1 (Mass) and GI-13 (793/B) (Arshad et al., 2017). Most likely similar vaccines are also used within Brazil as within South Africa. The BR-I genotype, however, is unique to that of Brazil (Chacón et al., 2023) and similarities between the South African strain and this specific strain could suggest an initial introduction of the BR-I genotype with adaption to along with other isolates circulating within the South African environment.

Even with the lower percentage correlation between the South African strains and that of the Brazilian strains, BLAST homology searches did not reveal any other related lineages within the first 100 sequences. The reason for the low identity percentage range can only be determined through further analysis by conducting full genome sequencing.

The GI-11 lineage is recognized as an indigenous South American lineage (Valastro, et al., 2016). 3.6% of samples from this study were found to be related to this group and form a cluster rather than being distributed throughout the phylogenetic tree. Another closest relative within this group is also that of JX182772 that forms part of the study done by Santos et al., 2012. Samples in this study were also isolated over several years suggesting a single introduction event and followed by transmission and circulation between South African flocks. This virus was also not confined to a single province but distributed across Gauteng, North West, Western Cape,

KwaZulu-Natal, and Mpumalanga. South Africa does not have a vaccine registered for this strain which suggests that all these isolates are field isolates. South Africa is also known to import most of its poultry meat products from Brazil (SAPA, 2021) as well as the live importation of birds during 2015 (Trading Economics, 2023). Species imported included chickens, ducks, geese, turkeys, and guineas. This could also be linked to the source of the spread of this strain. There does not appear to be any other studies suggesting that the GI-11 strain may be a problem within South Africa. Internal studies conducted by pharmaceutical companies have yet to produce any related data to this specific lineage (personal communication, Botha, C., Coetzer, M., Dawson, S., Malan H., 2023). To my knowledge, this is the first report of this genotype in South Africa.

4.3.3 Lineage GI-13 (793/B-like strains)

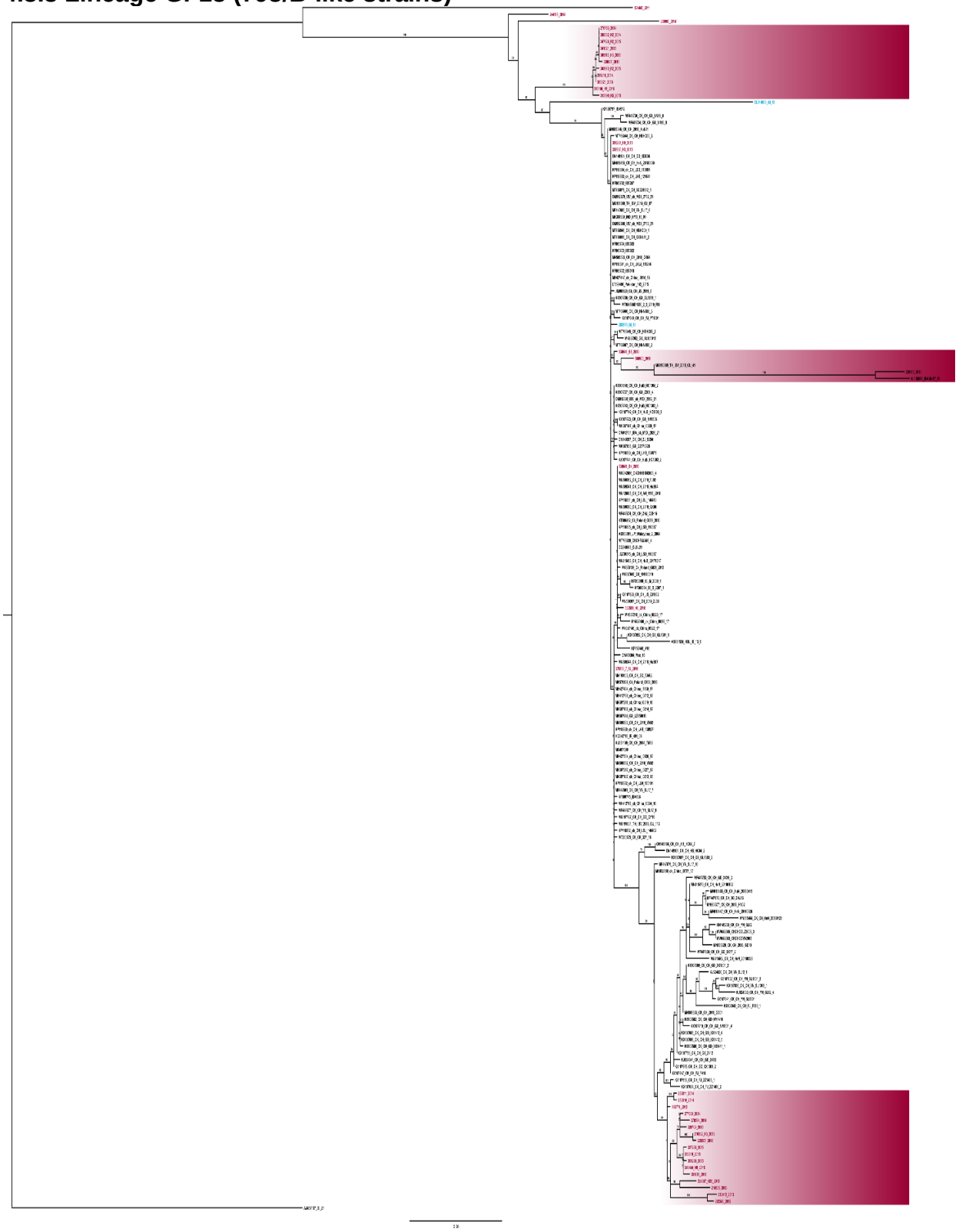

Figure 15(a). Maximum-likelihood phylogenetic tree of lineage GI-13 IBV. Study isolates are highlighted in red, genome classification reference samples are in light blue, field viruses retrieved from Genbank are in black.

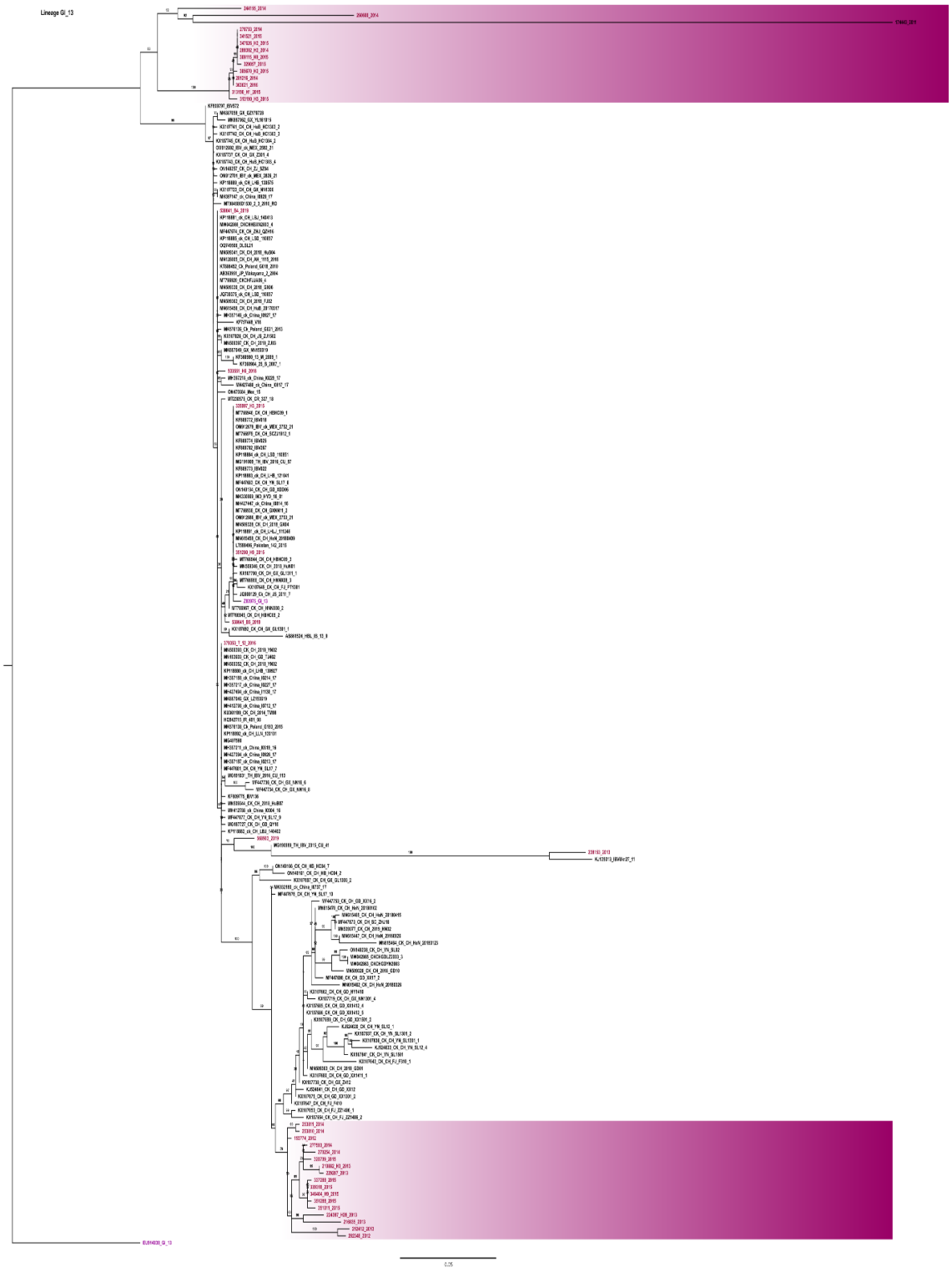

Figure **15(b)**. Maximum-likelihood phylogenetic tree of lineage GI-13 rooted with more closely related reference sequence (EU914938) for a better visualization. Study isolates are highlighted in purple, genome classification reference samples are in purple, field viruses retrieved from Genbank are in black.

Figure 15(c). Maximum-likelihood phylogenetic tree of lineage GI-13, highlighting isolates in the trees to follow (Figure 15d, 15e, and 15g), with more closely related reference sequences for a better visualization. Study isolates are highlighted in red, genome classification reference samples are in light blue, field viruses retrieved from Genbank are in black.

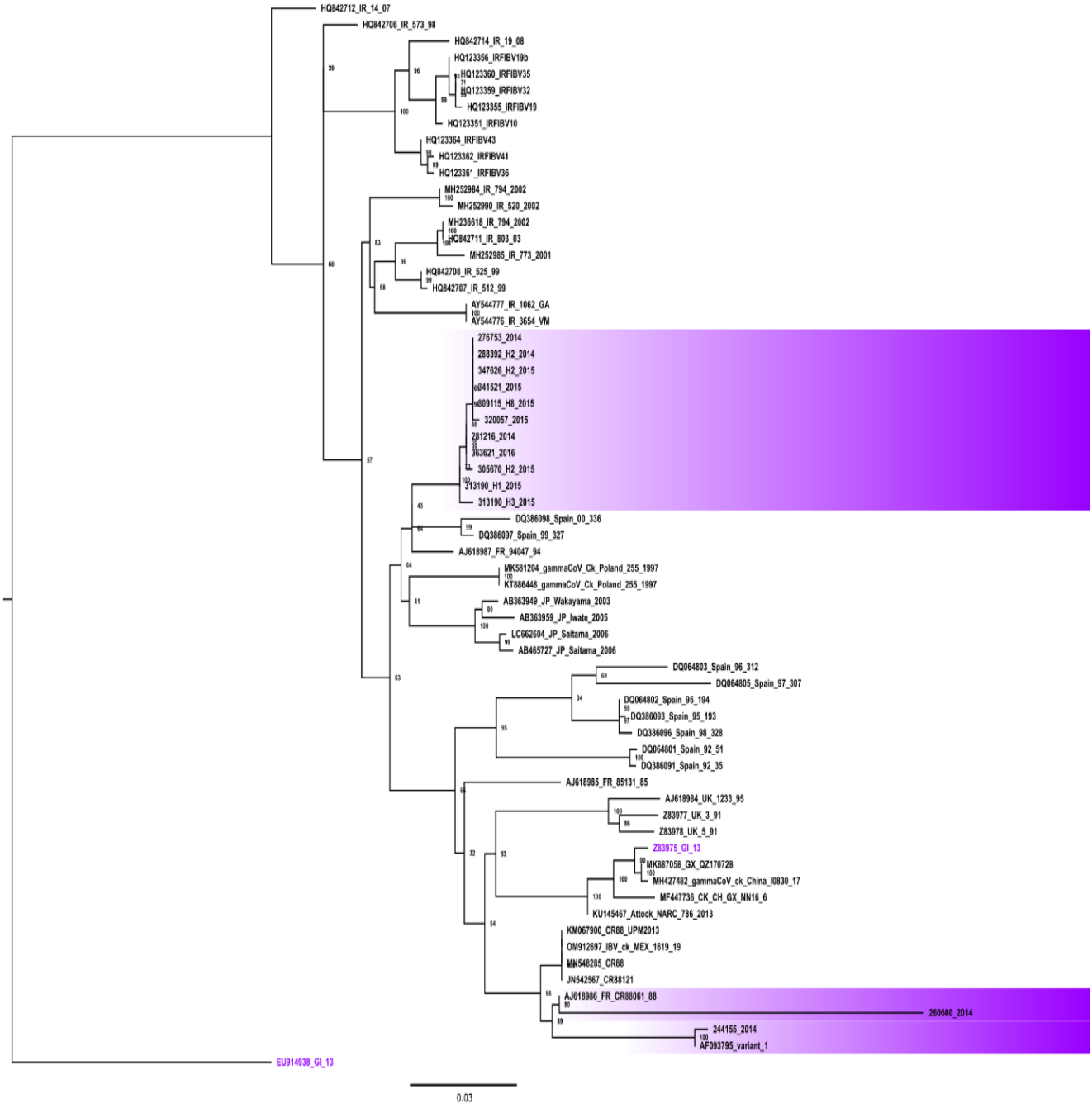

Figure 15(d). Maximum-likelihood phylogenetic tree of the lineage GI-13, re-rooted for an enlarged view of isolates 244155_2014, 260600_2014, 27753_2014, 288392_H2_2014, 347626_H2_2015, 341521_2015, 309115_H8_2015, 320057_2015, 305670_H2_2015, 281216_2014, 363621_2016, 313190_H1_2015, 313190_H3_2015. Isolates are shaded in purple; genome classification reference samples are highlighted in purple font and field viruses retrieved from Genbank are in black font.

Isolates 244155_2014, 260600_2014, 27753_2014, 288392_H2_2014, 347626_H2_2015, 341521_2015, 309115_H8_2015, 320057_2015, 305670_H2_2015, 281216_2014, 363621_2016, 313190_H1_2015, 313190_H3_2015 appeared to have formed one of two separate clades from other isolates in this lineage. Isolates 244155_2014 and 260600_2014 also branch off separately from the second clade formed by isolates 27753_2014, 288392_H2_2014, 347626_H2_2015, 341521_2015, 309115_H8_2015, 320057_2015, 305670_H2_2015, 281216_2014, 363621_2016, 313190_H1_2015, and 313190_H3_2015 (Figure 15(d)).

Isolate 244155_2014 has a close relation (96%) to isolates from Poland (MT984600) collected in 2013, Israel (JQ946056, AF093795, AY793382, AY789965, and AY7899952) submitted to Genbank in 2012, and 2001, and those isolated in 1996. Not much information on the origin of the isolate from Poland appeared to be available but MT984600 appeared to be a recombinant of Mass, Ma5, Connecticut, Conn46, Cal557, L1148, 1148-A and 4/91 (Bali et al., 2021). Limited data was available on the Israel isolate JQ946056 but AF093795 is a field isolate that falls into group 793/B (Callison, Jackwood and Hilt, 2001) with a 91% similarity. Isolates AY793382, AY789965 and AY899952 were field isolates proven to form part of the so-called “Variant 1” group as set out by Callison, Jackwood and Hilt, 2001. They were closely related 4/91 and 793/B from the UK (Gelb et al. 2005). Based on this information 244155_2014 is most likely to be a field strain of the 793/B-like strains.

Isolates 260600_2014, 27753_2014, 288392_H2_2014, 347626_H2_2015, 341521_2015, 309115_H8_2015, 320057_2015, 305670_H2_2015, 281216_2014, 363621_2016, 313190_H1_2015, and 313190_H3_2015 share common related Genbank references through BLAST homology (90%). This included the French isolate, AJ618986, from which the CR88 live vaccine was derived (Cavanagh et al., 2005). Other related Genbank strains were from Spain (DQ386097) submitted in 2008, Iran (HQ842708) collected in 1999, Malaysia (KM067900) collected in 2013, and Mexico (OM912697) collected in 2019. The Spanish was a field isolate (DQ386097) that formed part of “Cluster 2” of Spain Genotype I that classified in the 4/91-type strains (Dolz et al., 2008). Unfortunately, no published data could be found regarding the isolates from Iran and Malaysia. OM912697 was classified as a vaccine variant strain of 793/B and 4/91 strains (Kariithi et al., 2022). 260600_2014

and the other isolates from this group were most likely to be vaccine variants of the 793/B strain.

Isolates 253811_2014, 253810_2014, 193774_2012, 277560_2014, 270254_2014, 320709_2015, 210882_H3_2013, 229207_2013, 337288_2015, 351288_2015, 351288_2015, 346464_H9_2015, 351011_2015, 224387_H28_2013, 216935_2013, 21241_2013, and 202348_2012 formed the second cluster in the phylogenetic tree of the GI-13 lineage (Figure 15(e)). Most of the closely related 4/91 (94-96%) sequences from Genbank originated from China (MK032180, MH397178, MF447676, KX107719, KX107689, MN615470, and MW042863) and dated from 2013 – 2020. MW042863 was isolated collected in 2020, well after that of the isolates found in this study. It was, however, classified as part of the 4/91 genotype (Lian et al., 2021). No published data could be linked to any of the other isolates from China. AF093793 also appeared to be closely related (96%) to isolates within this cluster during the BLAST homology, which was the attenuated vaccine strain of 793/B from the U.K (Callison, Jackwood and Hilt, 2001). This cluster also only contains most of the earlier sequences of this group isolated whereas the previous cluster mentioned contained those isolates of later years. While not a close bootstrap value at 67% to the prototype strain used during this study (EU914938) for the GI-13 lineage, this clusters origin was probably more related to the original strain.

The two separate clusters could be due to two different variants of 793/B-like vaccines being used in the country.

The sequence of isolate 238153_2013 (Figure 15(f)) branched off separately from any other isolates and had a 100% bootstrap value to the partial sequence of the Ukraine strain (KJ135013) isolated in 2011 and a similarity of 94.20% through BLAST homology to the full genome. Unfortunately, no further information is available on this sequence and no published date could be found besides other sequences showing also showing similarities to the Ukraine isolate such as the strain from China (KP036502) (Zhang et al., 2015). No relationship could be linked however to KP036502 and 238153_2013. The next closest relatives (89.09%) of 238153_2013 were that of the isolate collected in Thailand in 2015 (MG190989) and India (AB861524) collected in 2013. MG190989 was identified as part of the GI-13 lineage and formed part of the isolates closely related to 4/91 vaccine strains

(Munyahongse et al., 2020). Given the relatedness to seemingly field type 4/91 strains and lower percentage rate to the vaccine strain specifically, it can be suggested that 238153_2013 was an official field isolate. Whole genome sequencing and further analysis would be needed to confirm this hypothesis.

Figure 15(e). Maximum-likelihood phylogenetic tree of the GI-13 lineage re-rooted for an enlarged view of isolates 253811_2014, 253810_2014, 193774_2012, 277560_2014, 270254_2014, 320709_2015, 210882_H3_2013, 229207_2013, 337288_2015, 351288_2015, 351288_2015, 346464_H9_2015, 351011_2015, 224387_H28_2013, 216935_2013, 21241_2013, and 202348_2012. Isolates are shaded in pink; genome classification reference samples are highlighted in pink and field viruses retrieved from Genbank are in black.

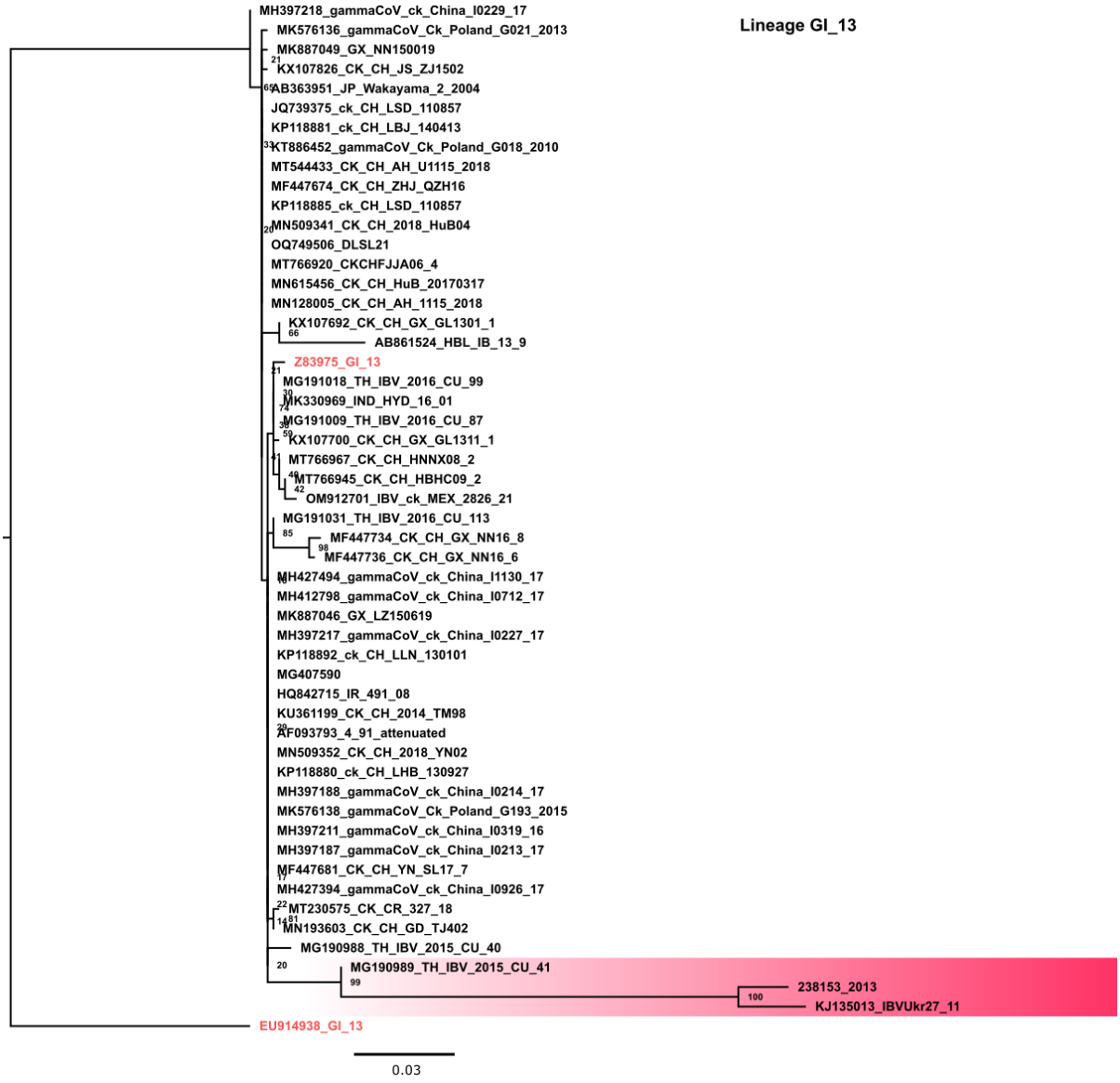

Figure 15(f). Maximum-likelihood phylogenetic tree re-rooted for an enlarged view of isolate 238153_2013 in the GI-13 lineage. Isolates are shaded in red; genome classification reference samples are highlighted in red and field viruses retrieved from Genbank are in black.

Figure 15(g). Maximum-likelihood phylogenetic tree re-rooted for an enlarged view of isolate 174443_2011 in the GI-13 lineage. Isolates are highlighted in bright pink; genome classification reference samples are highlighted in dark pink and field viruses retrieved from Genbank are in black.

The Isolate 174443_2011 does not appear to fit on any phylogenetic tree (Figure 15(g)). Initially it appeared to be linked to the GI-14 lineage but later after analysis the isolate sequence was analyzed using the online BLAST platform. The closest relative was a sample collected from Iran in 1999, accession number: HQ842708. No published data was found for this sequence. The serotype of this sample was determined to be more closely related to 793/B strains (82.35%), which forms part of the GI-13 lineage. Interestingly, this sample was also related (100% bootstrap value) to the reference strain MZ325299, used in this study from the novel GI-31 lineage (Bali, et al., 2022).

174443_2011 could also possibly be a unique strain that developed but did not spread any further in later years. Its impact on the industry was probably less significant than any other outbreak. Further sequencing and analysis would be beneficial to determine the origin or recombination of this virus.

Isolates 538641_B4_2019, 533551_H6_2018, 379353_7_12_2016, 335997_H6_2015, 351290_H9_2015, 538641_B5_2019, and 568903_2019 appear to not form part of the two separate clusters found in the phylogenetic tree. BLAST homology revealed that 538641_B4_2019, 335997_H6_2015, 351290_H9_2015, 538641_B5_2019, and 568903_2019 were genetically (94%-95%) similar to the sequences from Thailand (MG191018 and MG19009), India (MK330969), and China (MH427447) all isolated in 2016. Unfortunately, no published data was available for the sequences from India and China. The isolates from Thailand proved to be closely related to the complete genome of a 4/91 vaccine (KF377577) (Munyahongse et al., 2020).

Isolate 533551_H6_2018 was 95% related to samples from China (MN193603, MK887058, and MN509344) all isolated between 2017 and 2018. The other isolate was from Thailand (MN615456) isolated in 2016 and formed part of the cluster related to the complete genome of a 4/91 vaccine (KF377577) (Munyahongse et al., 2020). One strain from isolated from China, MK887058, also was found to be more closely related to the UK strain from 1991 (AF093794) (Fan et al., 2019 and Li et al., 2012).

Lastly isolate 379353_7_12_2016 had similar identity percentage (95%) to strains of from China (MN193603, MK887046 and MH427494) isolated between 2015 and

2017 and MK887046 was classified in the 4/91 group (MK887046) (Fan et al., 2019). The other sequence that was closely related was one from Poland (MK576138) dated to 2015. The 2015 strain formed part of a few sequences with the highest similarity to the sequence of the 4/91 strain most used in Poland (Domanska-Blicharz, Lisowska and Sajewicz-Krukowska, 2020).

GI-13's origin, first described in the UK in 1991, can be dated back to an isolate from Morocco in 1987, originally called the 793B type (also known as 4/91 and CR88). The first CR88 virus strain was first isolated in France in 1985. It is a widespread strain that can be found across the globe (Valestro et al., 2016). Vaccines are commercially available for this strain, and it was also the third most common strain identified in this study. Not all the pharmaceutical companies were approached to request vaccine registration information for this study and the number of 4/91 vaccines are most likely to not be limited to a single brand available for purchase. Most of the sequences isolated from this group appears to be related or derivatives of vaccines. Isolate 244155_2014 was most probably a cause for concern as a field isolate and its close identity to other pathogenic strains of the GI-13 lineage. Further investigation into the introduction points and origin would be beneficial. The two unique isolates, 174443_2011 and 238153_2013, were most likely field isolates the link between 238153_2013 and the Ukrainian strain (KJ135013) could possibly be through importation of grains from the Ukraine used as feed products for poultry, and there might be a possibility that grain could be contaminated with bird faeces. South Africa does not appear to obtain any live chickens or meat products from the Ukraine (Trading Economics, 2023). This could have been a once-off coincidental occurrence as it appears to be the only sequence with such a similarity to this specific strain (Pillay, Y., 2022). Sample number 174443_2011 does not quite fit anywhere within a specific lineage and branches off separately. Its closest relative is that of a unique isolated classified into a novel GI-31 lineage, represented by MZ325299. This isolate could have possibly been from a recombination event between a 793/B vaccine strain and, as mentioned in Bali *et al.* (2022), the Nigerian GI-26 reference strains. The authors, however, failed to prove any recombination through additional analyses and thus dubbing it a unique strain (Bali et al., 2022). Any further conclusions require further investigation.

4.3.4 Lineage GI-19 (QX-like strains)

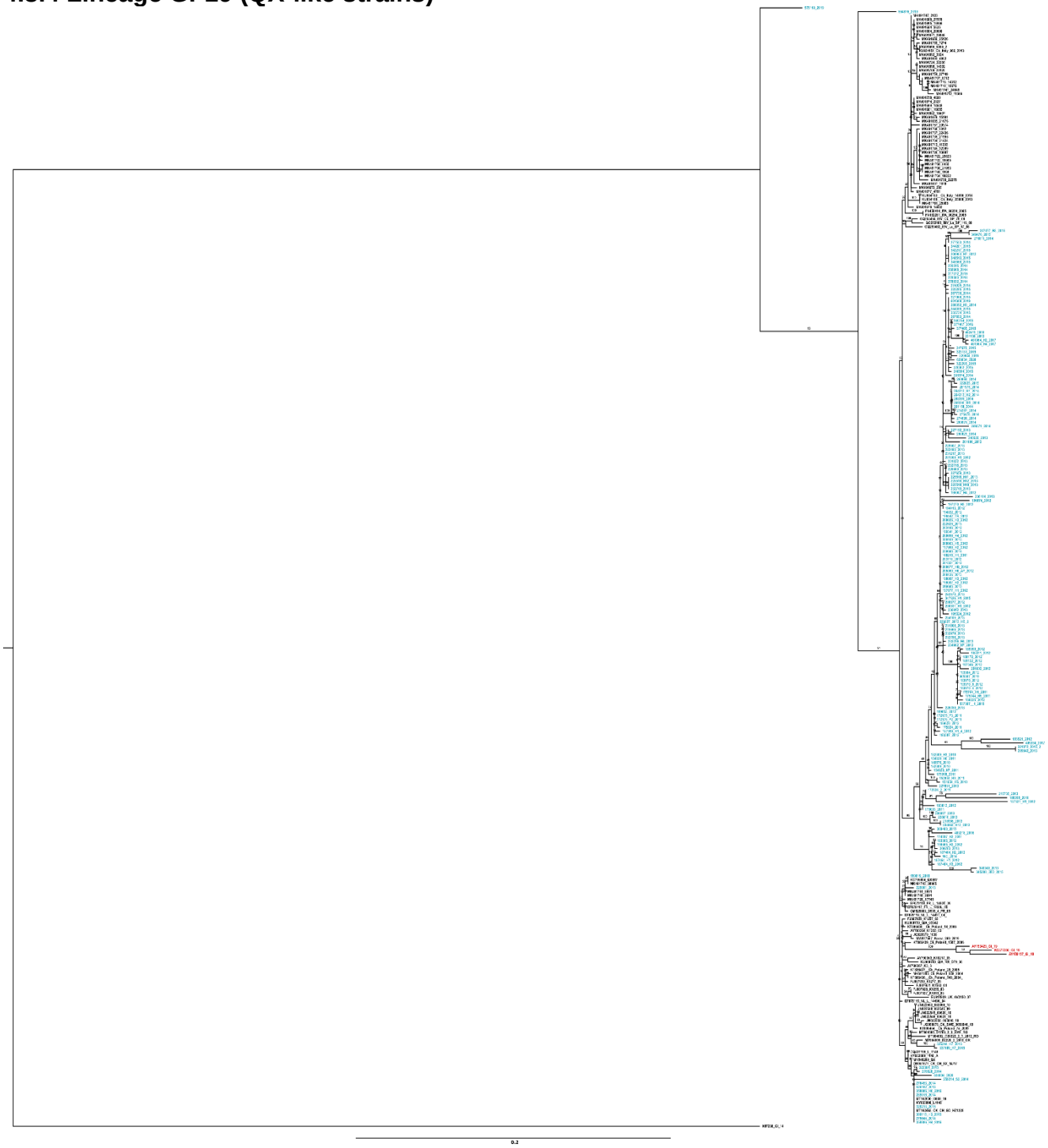

Figure 16(a). Maximum-likelihood phylogenetic tree of lineage GI-19 IBV. Study isolates are highlighted in light blue, genome classification reference samples are in red, field viruses retrieved from Genbank are in black.

Figure 16(b). Maximum-likelihood phylogenetic tree of GI-19 lineage rooted with more closely related reference sequences (KC577395 and AY189157) for a better visualization. Study isolates are highlighted in light blue, genome classification reference samples are in red, field viruses retrieved from Genbank are in black.

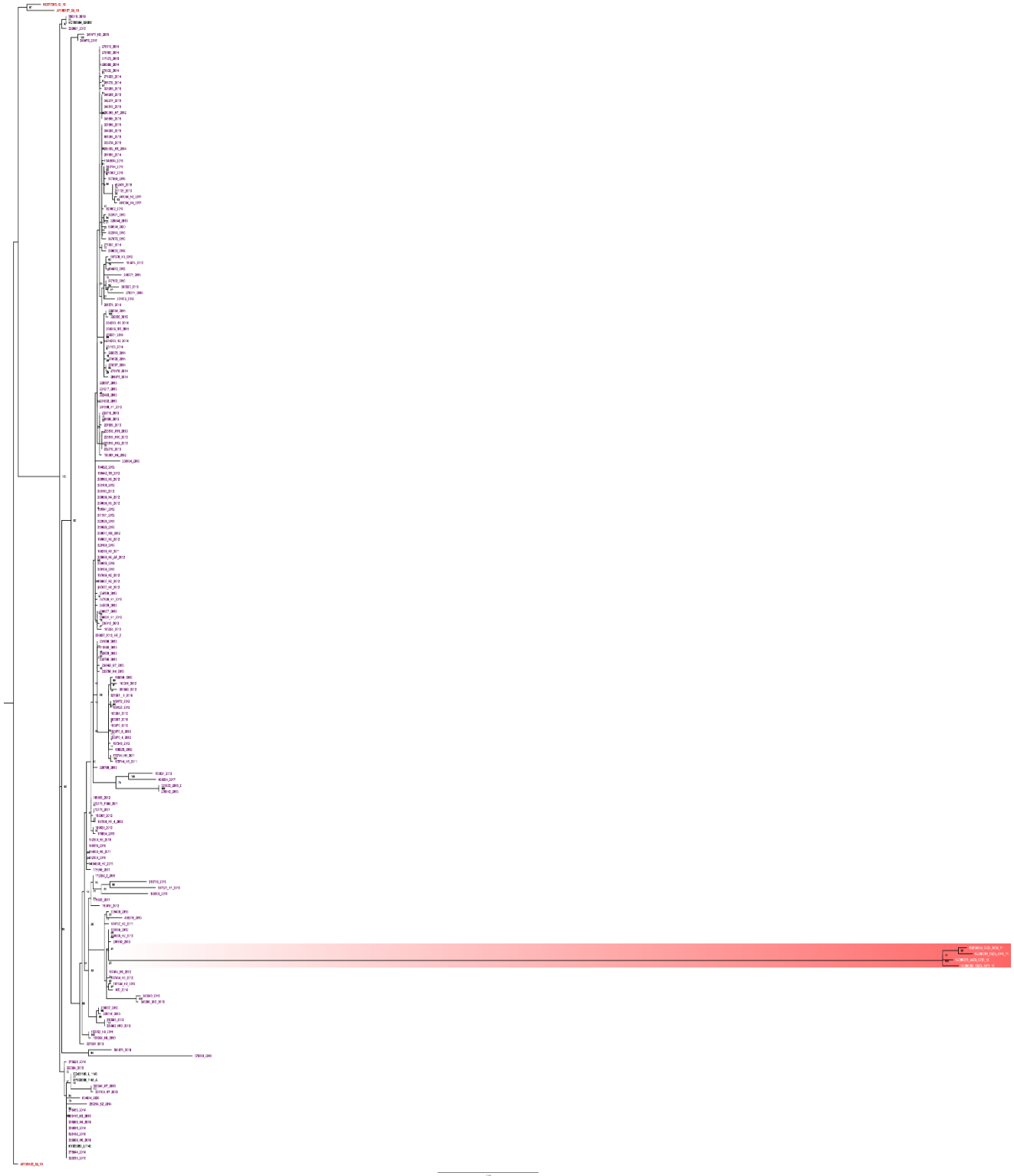

Figure 16(c). Maximum-likelihood phylogenetic tree of lineage GI-19 that includes previously identified ZA QX-like strains from Knoetze et al., 2014 (KJ200284, KJ200281, KJ200282, and KJ200273) shaded in red. Study isolates are highlighted in purple font, genome classification reference samples are in red font, field viruses retrieved from Genbank are in black font.

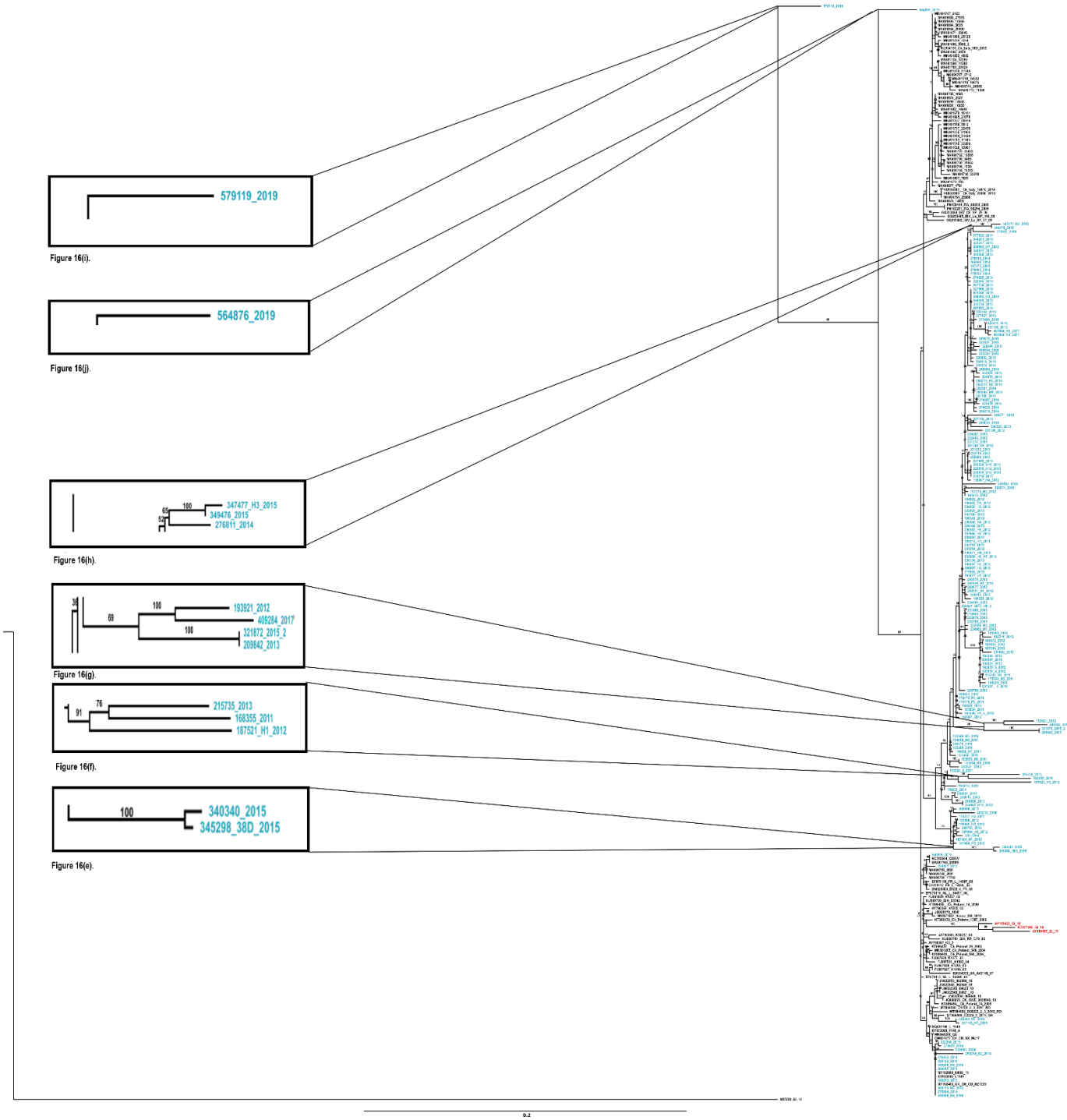

Figure 16(d). Maximum-likelihood phylogenetic tree of GI-19 lineage highlighting isolates in the trees to follow (Figure 16(e), 16(f), 16(g), 16(h), 16(i), 16(j)), with more closely related reference sequences for a better visualization. Study isolates are highlighted in light blue, genome classification reference samples are in red, field viruses retrieved from Genbank are in black.

Figure 16(e). Maximum-likelihood phylogenetic tree of GI-19 lineage re-rooted for an enlarged view of isolates 340340_2015 and 345298_38D_2015. Isolates are shaded in purple; genome classification reference samples are highlighted in purple and field viruses retrieved from Genbank are in black.

Most of the samples that identified as QX-like strains in South Africa appeared to form their own separate cluster related more closely to each other rather than those QX-strains from either Asia or Europe. Reference sequences (AF193423, AY189157, KC77395) in this study used for the phylogenetic tree assembly originated from China. Previously identified ZA QX-like strains from Knoetze et al., 2014 (KJ200284, KJ200281, KJ200282, and KJ200273) also appeared to branch off separately, similar to the H120-like strains. There appeared to be no close relativeness between the samples from this study and samples from Knoetze et al., 2014. Whole genome sequencing of samples from this study might provide better insight when compared.

While the entire “South African” cluster is an interesting observation in itself it is not unheard of since they appear to evolve separately following an initial introduction and are known to cluster by country (Franzo et al., 2017 and Domanska-Blicharz, Lisowska and Sajewicz-Krukowska, 2020). Visually this can also be seen with Italian strains through the study conducted by Franzo et al., 2019. A few selected isolates also branched off separately within this cluster. Isolates 340340_2015 and 345298_38D_2015 (figure 16(e)) were more closely related (96%) to sequences from South Korea (KU900739, KU900744 and HM486962) collected between 2003-2006, Greece (OM525804) collected in 2005 and Italy (MK491728) collected in 2016. According to Hong et al., 2017, KU900739 and KU900744 appeared to be recombinant viruses of the KM91 (JQ977698) (Korean QX-like strain), and QX-like strains more from North America and Europe rather than those QX-like strains from China. These were however introduced from other countries and did not originate in South Korea. No published data was available for the other isolates to draw any conclusions from.

Isolates 166355_2011, 187521_H1_2012, and 215735_2013 (Figure 16(f)) showed similarity (94-95%) to viruses from South Korea (JQ920393 and JQ920391) isolated in 2009, Spain (GQ253482) isolated in 2008, the Netherlands (EF079115) isolated in 2004, and isolate 187521_H1_2012 specifically most related (94%) to the UK strain (EU914939) isolated in 2007. JQ920391 was identified as one of the recombinant viruses and was found to have putative parents of a Korean QX-like strain of the same study (JQ920386), similar to the KM91 (JQ977698) strain, and those QX-like strains from China (Mo, et al., 2013). While information on the isolate from Spain and

are not published, the strain from the Netherlands (EF079115) was that of a QX-like field isolate (Worthington, Currie, and Jones, 2008). The strain from the UK was linked to the first report of QX-like strains within the UK isolated from a backyard flock (Gough et al., 2008).

Isolates 193921_2012, 209842_2013, 321872_2015_2, and 409284_2017 (Figure 16(g)) had 97% similarities to samples from the Netherlands (EF079115) and France (EF079117 and EF079118) collected in 2004 and 2005 respectively, and the original progenitor field strain from the UK (KY933089) - 1148-A (Listorti et al., 2017). 193921_2012 also had 97% similarity to Italian strains (MK491747, MK491748 and MK491751) collected in 2017, while 409284_2017 additionally had a 97% similarity to a strain from Poland (KT886454) isolated in 2009. The Netherlands strain as well as the isolates from France form part of the same study and were noted as field QX-like strains (Worthington, Currie, and Jones, 2008). The Italian strains also formed part of a group that were classified as field isolates similar to that of the QX-like strain (Franzo et al, 2019). Lastly the strain from Poland (KT886454) was classified as closely related to European strains from Sweden and Italy and one from South Africa and Sudan and also exhibited recombination with 4/91 and QX-like strains from China (Domanska-Blicharz, Lisowska and Sajewicz-Krukowska, 2020).

Isolates 276811_2014, 347477_H3_2015, and 349476_2015 (Figure 16(h)) have similar (97% nucleotide identity) sequences to previously mentioned isolates from Italy (MK491751 and MK491748), France (EF079117), South Korea (KU900744) and the UK (KY933089). An additional sequence closely related strain from France (OM525803) was isolated in 2005 formed part of this BLAST homology. The sequence was found to have had a similarity of 99.85% to that of the originally identified QX-strain (MN548289) (da Silva, Jude, and Gallardo, 2022).

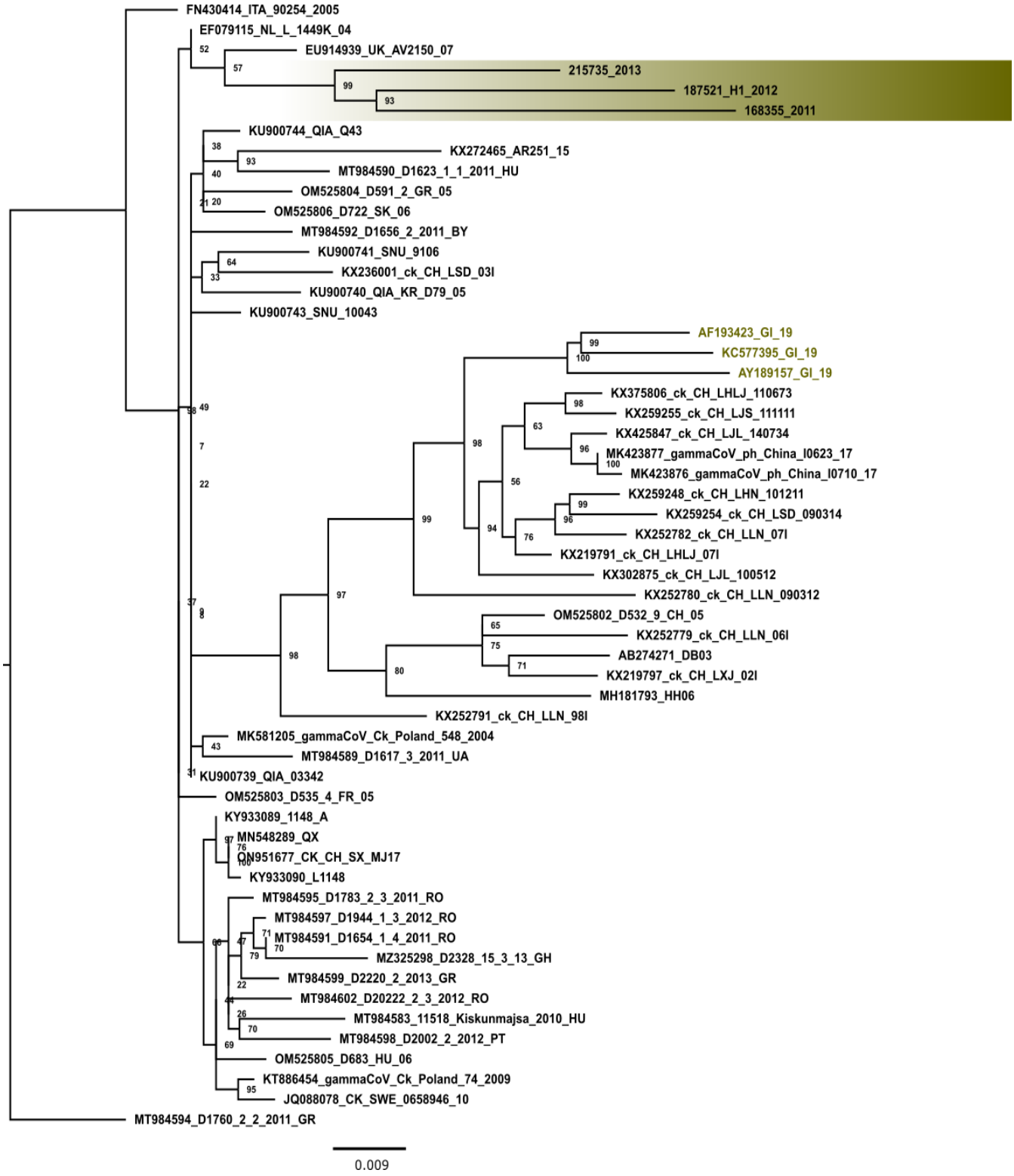

Figure 16(f). Maximum-likelihood phylogenetic tree of the GI-19 lineage, re-rooted for an enlarged view of isolates 166355_2011, 187521_H1_2012, and 215735_2013 in the GI-19 lineage. Isolates are shaded in green; genome classification reference samples are highlighted in green and field viruses retrieved from Genbank are in black.

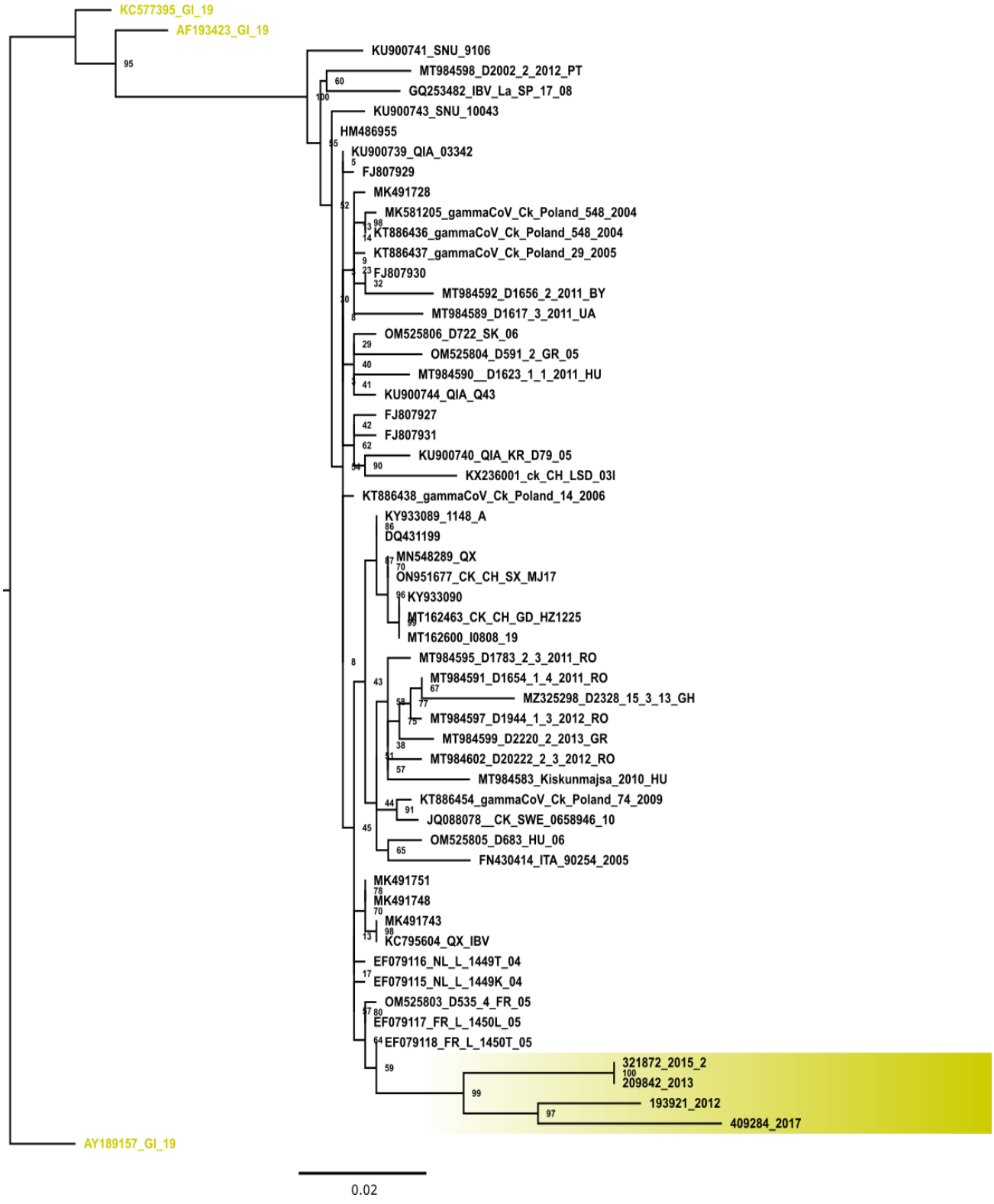

Figure16(g). Maximum-likelihood phylogenetic tree of the GI-19 lineage, re-rooted for an enlarged view of isolates 321872_2015_2, 209842_2013, 193921_2012 and 409284_2017. Isolates are shaded in yellow; genome classification reference samples are highlighted in yellow and field viruses retrieved from Genbank are in black.

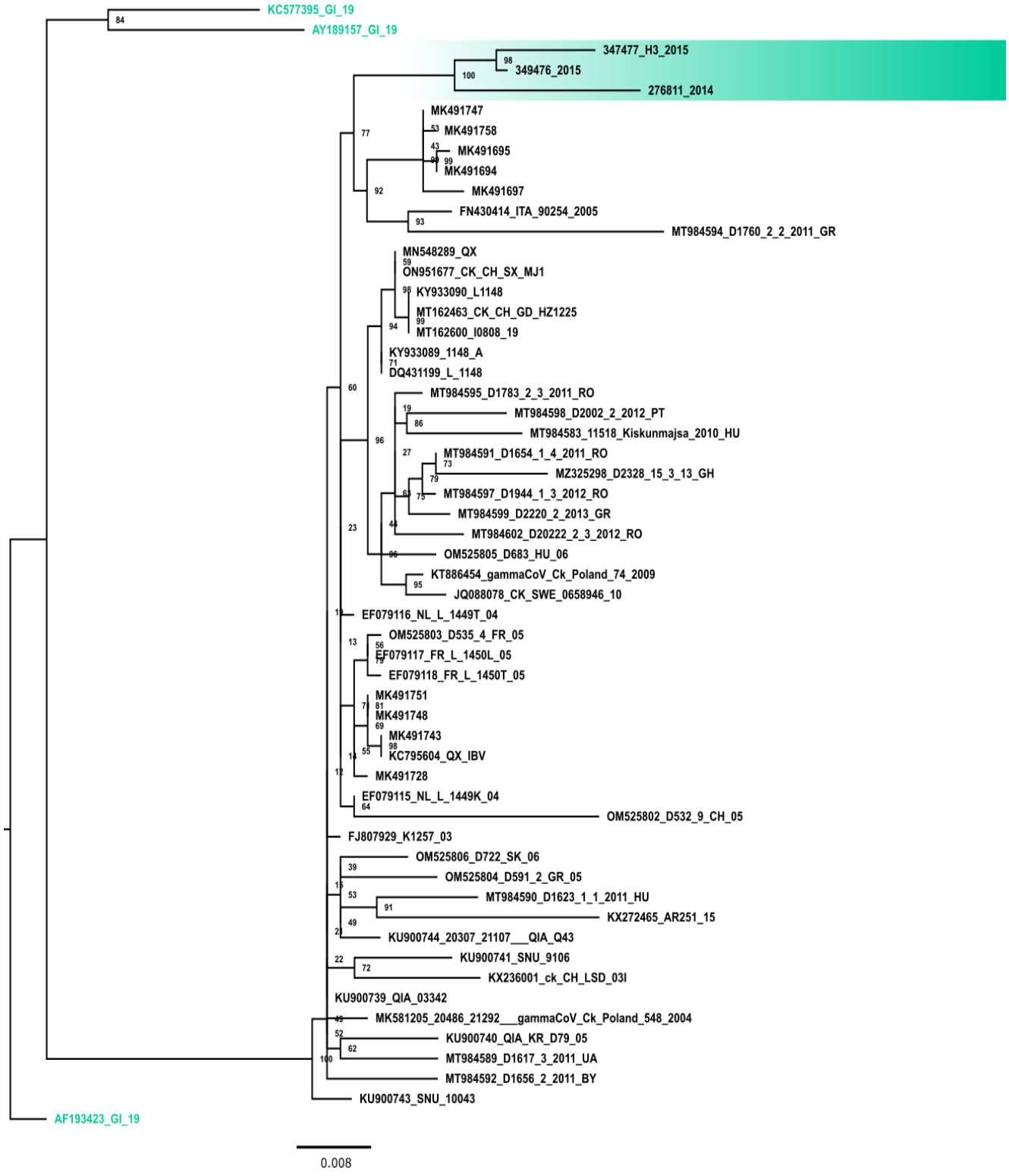

Figure 16(h). Maximum-likelihood phylogenetic tree of the GI-19 lineage re-rooted for an enlarged view of isolates 276811_2014, 347477_H3_2015, and 349476_2015. Isolates are shaded in light blue; genome classification reference samples are highlighted in light blue and field viruses retrieved from Genbank are in black.

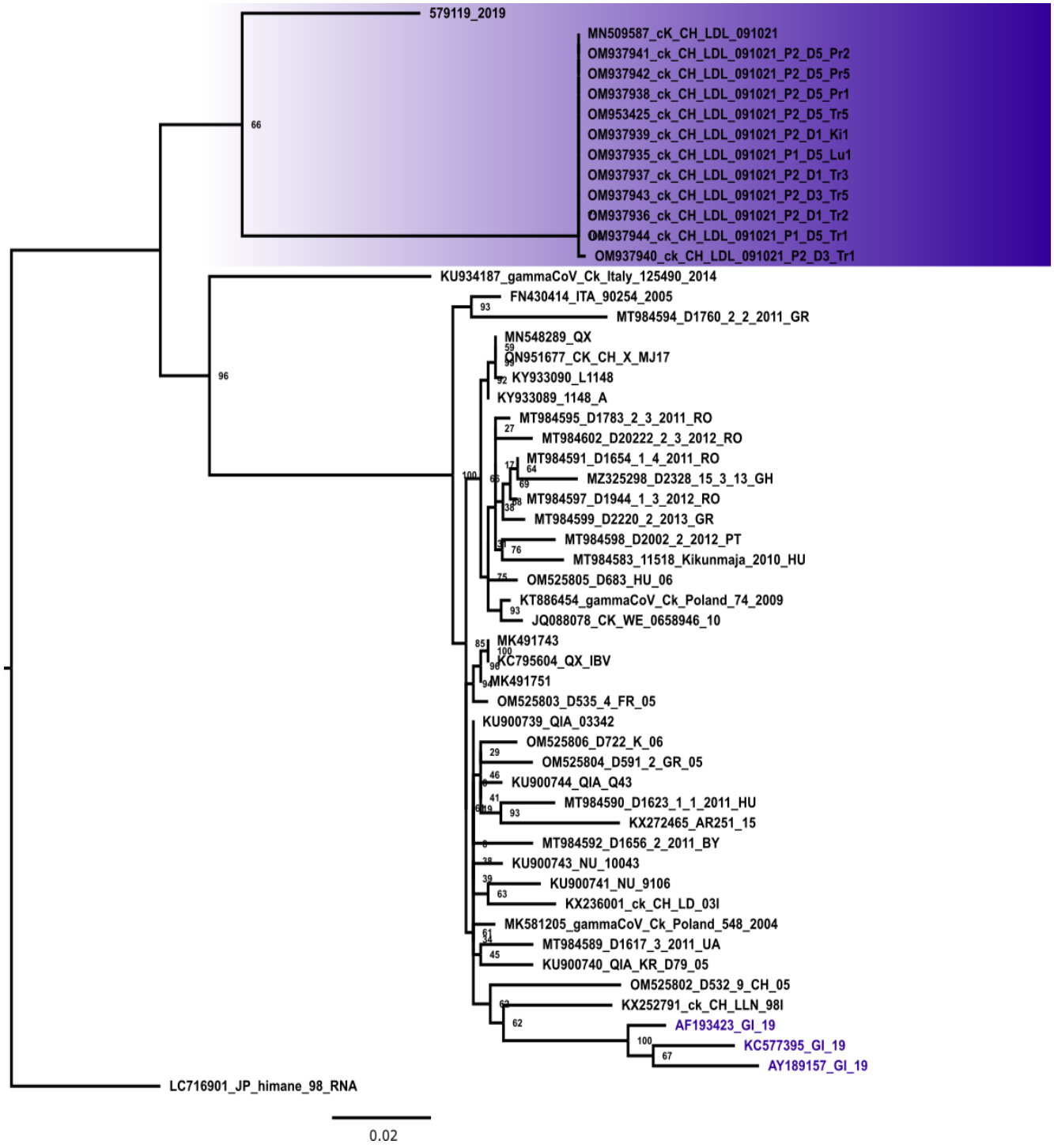

Figure 16(i). Maximum-likelihood phylogenetic tree of the GI-19 lineage re-rooted for an enlarged view of isolates 579119_2019. Isolate is shaded in blue with closest related field viruses retrieved from Genbank in black; genome classification reference samples are highlighted in dark blue. All other field viruses retrieved from Genbank are in black.

Figure 16(j). Maximum-likelihood phylogenetic tree of the GI-19 lineage re-rooted for an enlarged view of isolates 564876_2019. Isolate is shaded in light green with closest relating field virus retrieved from Genbank in black; genome classification reference samples are highlighted in light green and field viruses retrieved from Genbank are in black.

579119_2019 (Figure (i)) only had a 91% identity to strains from Italy (KU934187 and MK491743) isolated in 2014 and 2016 respectively and furthermore a 90% identity to isolates from China (MN509587 and OM937944) from 2009 and 2022. Isolate MK491743 forms part of the study conducted by Franzo et al., 2019. The isolate from Italy (KU934187) had a recombination pattern of two QX-like strain inserts as well as that of a 793/B-like strain (Moreno et al., 2017). No published data was obtained for isolates from China.

564876_2019 (Figure (j)) only have a 94% similarity to previously mentioned isolates from Italy (MK491751, MK491748, and MK491743), South Korea (KU900744), but also to the vaccine strain L1148 (KU900744) derived from the virulent 1148-A strain from the UK. The Bootstrap value from the phylogenetic tree suggests an 87% similarity to that of MK032178 isolated in Chine in 2017, but again no published data appears to be available for this strain.

Isolates 322394_2015, 278628_2014, 259214_S2_2014, 276453_2014, 289015_2014, 275644_2014, 320213_2015, 309115_H3_2015, 358886_H6_2016, 358886_H4_2016, 526182_2018, and 634004_2020 form a cluster separately from those in the so called “South African” cluster. Homology results revealed that all of these isolates are related (96-97%) to the L1148 vaccine strain (KU900744). The timing of these isolates correlates with when the first QX vaccine was registered in South Africa in 2013 (Table 1).

The GI-19 lineage is encountered worldwide. What was first identified in China, Japan and Korea was soon reported as QX-like viruses in other countries such as Russia, Africa, and the Middle Eastern countries. It is speculated that the GI-19 virus originated in China long before being reported (Franzo et al., 2017). This could be due to a low report rate of IBV during the time in China or possibly a less virulent strain IBV that had gained more virulence factors over the years to produce the current QX-like viruses that had caused many waves of infection (Franzo, et al. 2017). Most of the isolates from this study fall (50.1%) within this lineage. Before pharmaceutical companies were approved to sell their QX vaccines within South Africa in 2013, the virus was introduced into South Africa as early as 2010 if not earlier. The phylogenetic tree of the GI-19 appears to hold a separate cluster of QX

strains based solely on the isolates from this study. Franzo et al. (2017), explained a similar pattern observed GI-19 strains cluster together according to country. Interestingly even though the QX strain originated from China, the South African strains appear to be more closely related to those QX-like strains isolated from Europe rather than Asia. Two distinct clades are visible from the phylogenetic tree analysis. One creating a “South African” cluster which contains most viruses from this group (approximately 90%) and based on BLAST homology, were most likely field isolates. The second group that was more closely related (approximately 97%) to the vaccine strain L1148 (KU900744) from the UK. Two isolates branch off separately from both groups (579119_2019 and 564876_2019). Homology results suggest possible recombinant events for sample 579119_2019 and possibly 564876_2019, however, WGS and further analysis would be necessary to prove this.

4.3.5 Lineage GI-23 (Var-2-like strains)

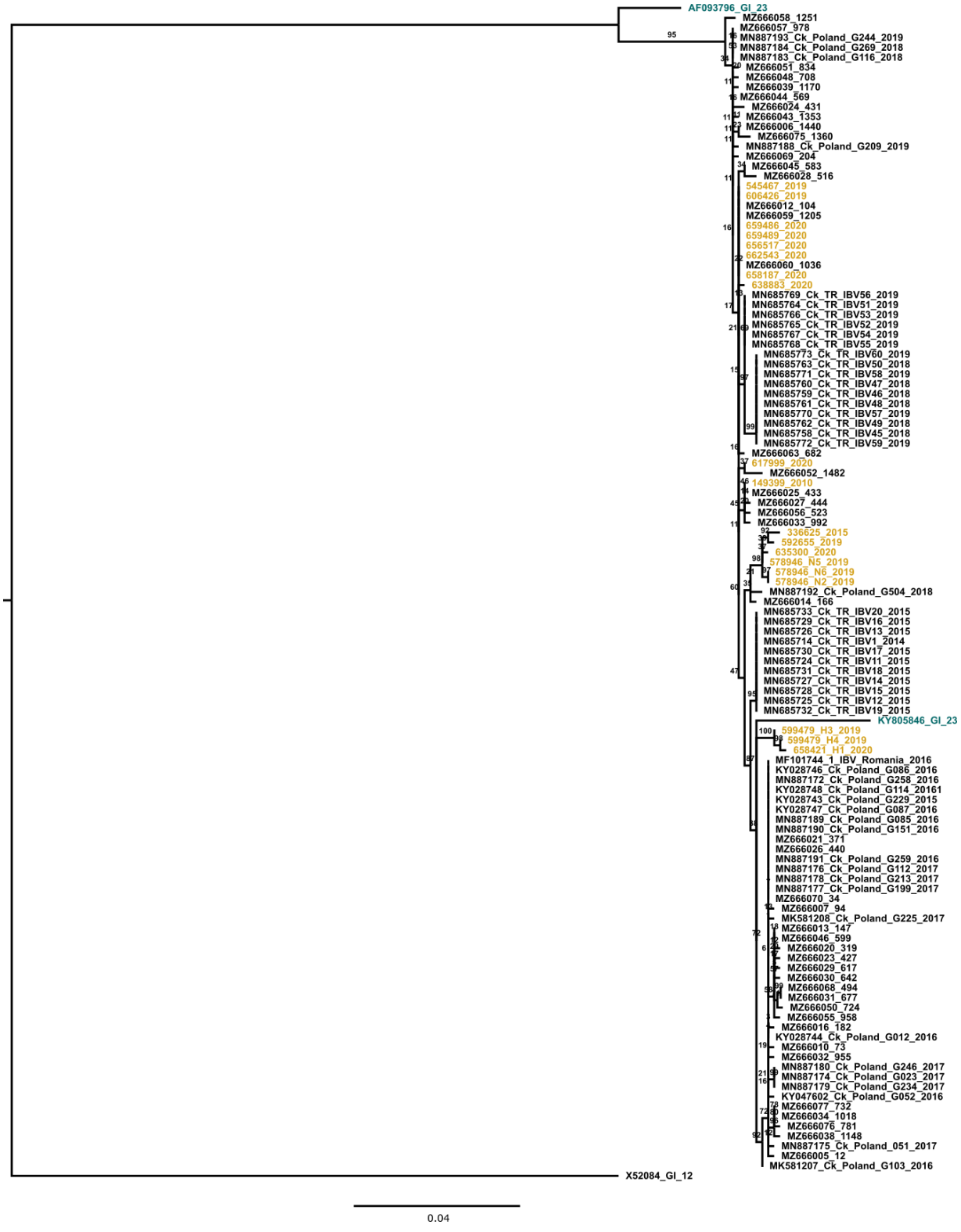

Figure 17(a). Maximum-likelihood phylogenetic tree of lineage GI-23 IBV. Study isolates are highlighted in green, genome classification reference samples are in yellow, field viruses retrieved from Genbank are in black.

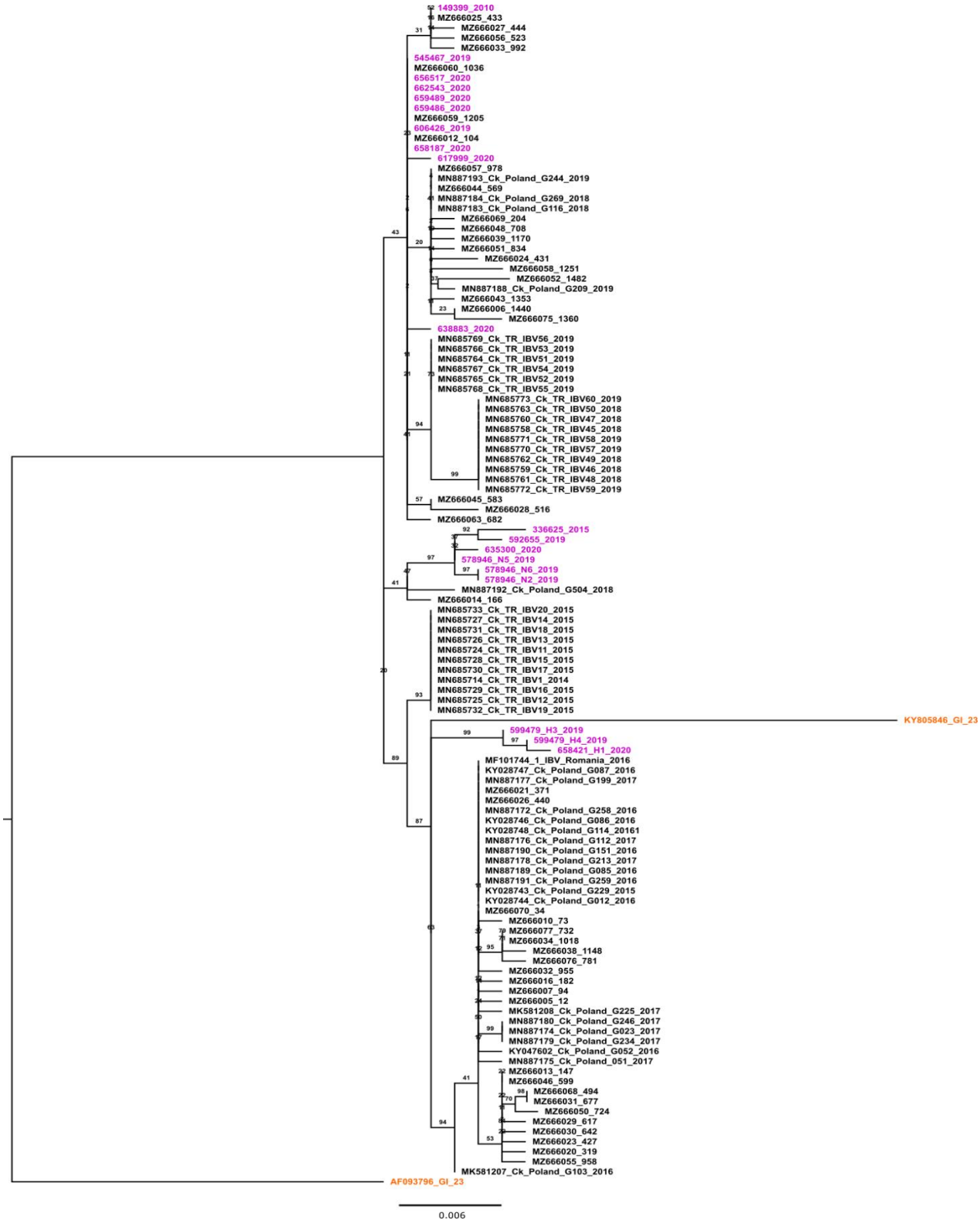

Figure 17(b). Maximum-likelihood phylogenetic tree rooted with more closely related reference sequence (AF093796) for a better visualization of lineage GI-23. Study isolates are highlighted in pink, genome classification reference samples are in orange, field viruses retrieved from Genbank are in black.

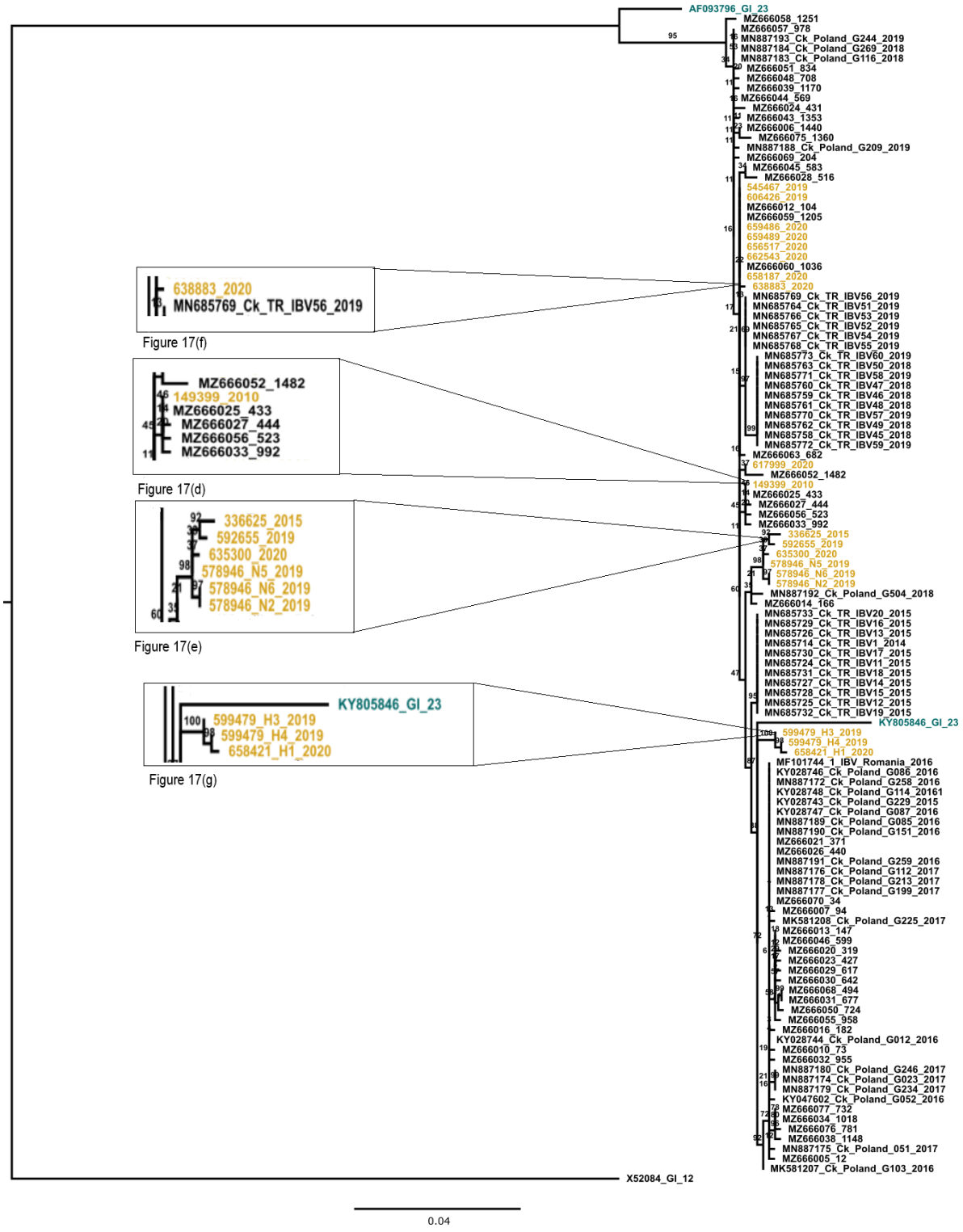

Figure 17(c). Maximum-likelihood phylogenetic tree of the GI-23 lineage highlighting isolates in the trees to follow (Figure 17(d), 17(e), 17(f), 17(g)), with more closely related reference sequences for a better visualization. Study isolates are highlighted in light blue, genome classification reference samples are in red, field viruses retrieved from Genbank are in black.

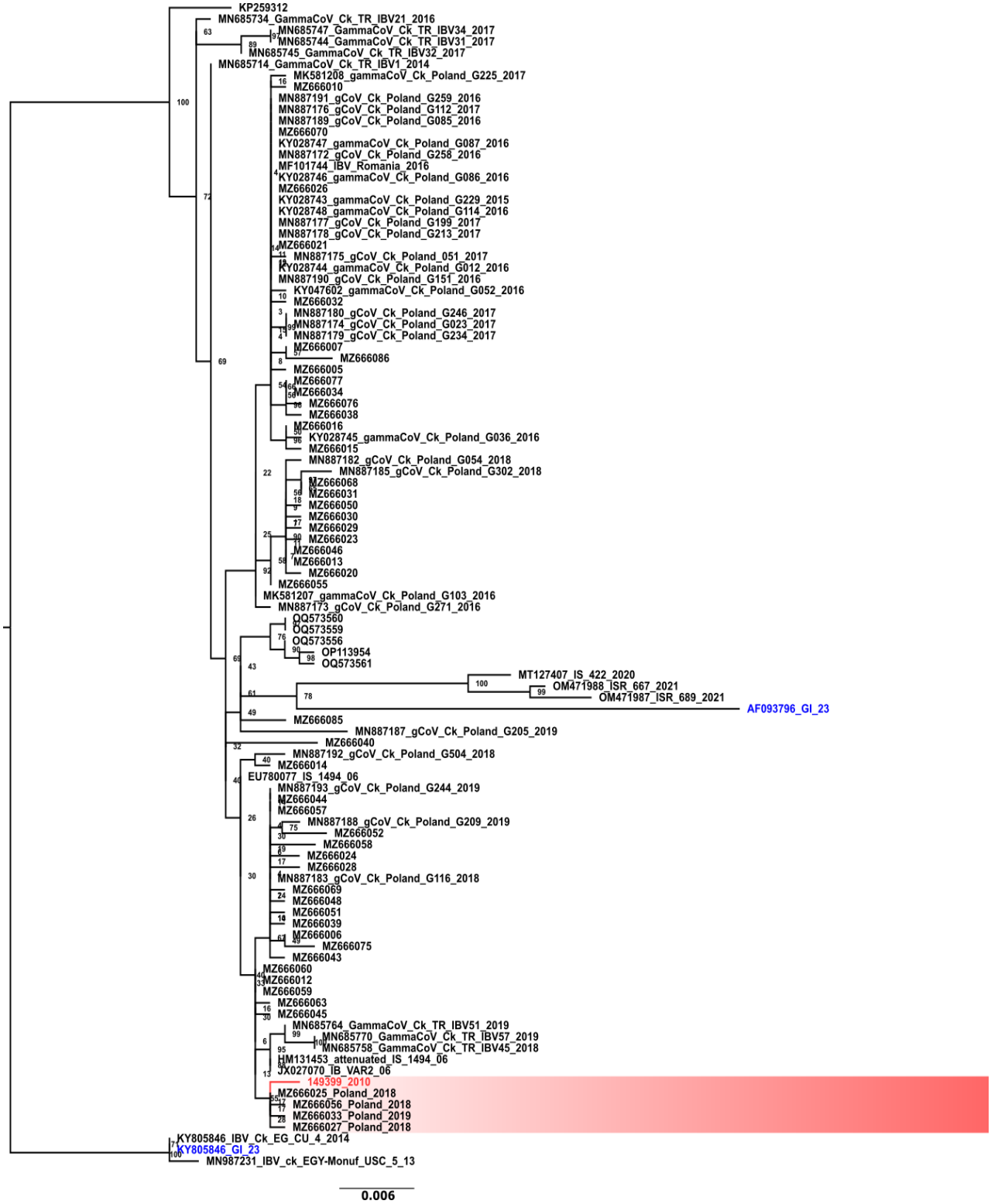

Figure 17(d). Maximum-likelihood phylogenetic tree of the GI-23 lineage re-rooted for an enlarged view of isolates 149399_2010. Isolates are shaded in red; genome classification reference samples are highlighted in blue and field viruses retrieved from Genbank are in black.

Isolate 149399_2010 (Figure 17(d)) according to its sampling date, could have possibly been one of the first known isolates of the Var-2-like strains to start circulating within South Africa. The isolate itself is more closely related (99%) to isolates collected during the 2018-2019 period in Poland (MZ666025, MZ666060, MZ666059 and MZ666056). Another isolate that was included was from Turkey (MN685764) isolated in 2019. A little further down the list also at an identity match of 99% is an isolate collected from Israel in 2006 along with its attenuated version (EU780077 and HM131453 respectively). There was no published data available for the isolates from Poland or Turkey to draw any conclusions from, and although similarly no published data was available for the isolates isolated from Israel, the sequence EU780077 is most obviously a field strain from which the attenuated version, HM131453 was made from. Given the date of the isolate and the fact that vaccines were only registered for this strain in 2015 (Table 1), isolate 149399_2010 was most likely a field strain.

Five years later, sample 336625_2015 emerged, while also closely related to similar viruses from Poland it was also more closely related to the field strain of EU780077 isolated from Israel in 2006. The isolates 578946_N5_2019, 578946_N6_2019, 578946_N2_2019, 592655_2019, and 635300_2020 (Figure 17(e)) formed a separate cluster along with isolate 336625_2015 and produced similar results.

Isolate 638883_2020 (Figure 17(f)) produced similar results through BLAST homology to that of isolate 149399_2010. It also produced a 96% similarity to that of isolate 149399_2010 when compared by pairwise alignment. Based on the timeline cannot be said for certain if this isolate was more closely related of vaccine or field strain.

Another group that appeared to form their own cluster based on the phylogenetic tree were isolates, 599479_H13_2019, 599479_H4_2019, 658421_H1_2020 (Figure 17(g)). These were closely related to isolates from Poland (MK581207) and Romania (MF101744) isolated in 2016. MK581207 was one of eight isolates classified in the GI-23 lineage. It was also one of two isolates chosen from this lineage for further analysis to determine if the isolates had resulted from recombination events. MK581207 and another strain proved to have recombined with QX-like strains from Europe and China (Domanska-Blicharz, Lisowska and Sajewicz-Krukowska, 2020).

The Romanian strain MF101744 formed part of a surveillance program within Romania and obtained four isolates that classified as Var-2-like strains and was closely related to the isolates from Poland in the study conducted by Domanska-Blicharz, Lisowska and Sajewicz-Krukowska, 2020 (Franzo et al., 2017).

GI-23 spread dramatically across South Africa after 2019. Two isolates from this group however confirms the presence of the lineage as early as 2010 and 2015. Why the two earlier viruses (149399_2010 and 336625_2015) did not start an original outbreak of this pathogenic strain, remains unknown. GI-23 is a lineage that was considered unique to the Middle East (Valestro et al., 2016). It was first identified in 1998 but has since then spread to several other countries including Brazil (Trevisol, et al., 2023). The sequences from this study group all had closely related (99%) sequence identities to those sequences isolated from Poland circulated in 2018-2019. The fact that 149399_2010 is more closely related to that of Polish strains isolated years later could suggest a common source of infection or common introduction route that follows both South Africa and Poland. It could also possibly mean overrepresentation of sequences from Poland were submitted to Genbank, creating a bias comparison.

The phylogenetic analysis does appear to create three different clusters of the GI-23 lineage but through BLAST homology all isolates appeared to be similar with the odd closely related sequences difference. Based on the BLAST homology, however, it is most likely that all the samples within this group were that of field isolates and not re-isolated vaccine strains. Full genome sequencing could reveal what differences there are between early viruses and the later epidemic strains, certain introductory points as well as confirm whether all isolates that fall within this lineage were field or vaccine strains.

Figure 17(e). Maximum-likelihood phylogenetic tree of the GI-23 lineage re-rooted for an enlarged view of isolates 635300_2020, 578946_N5_2019, 578946_N6_2019, 578946_N2_2019, 336625_2015 and 592655_2019. Isolates are shaded in yellow; genome classification reference samples are highlighted in gold and field viruses retrieved from Genbank are in black.

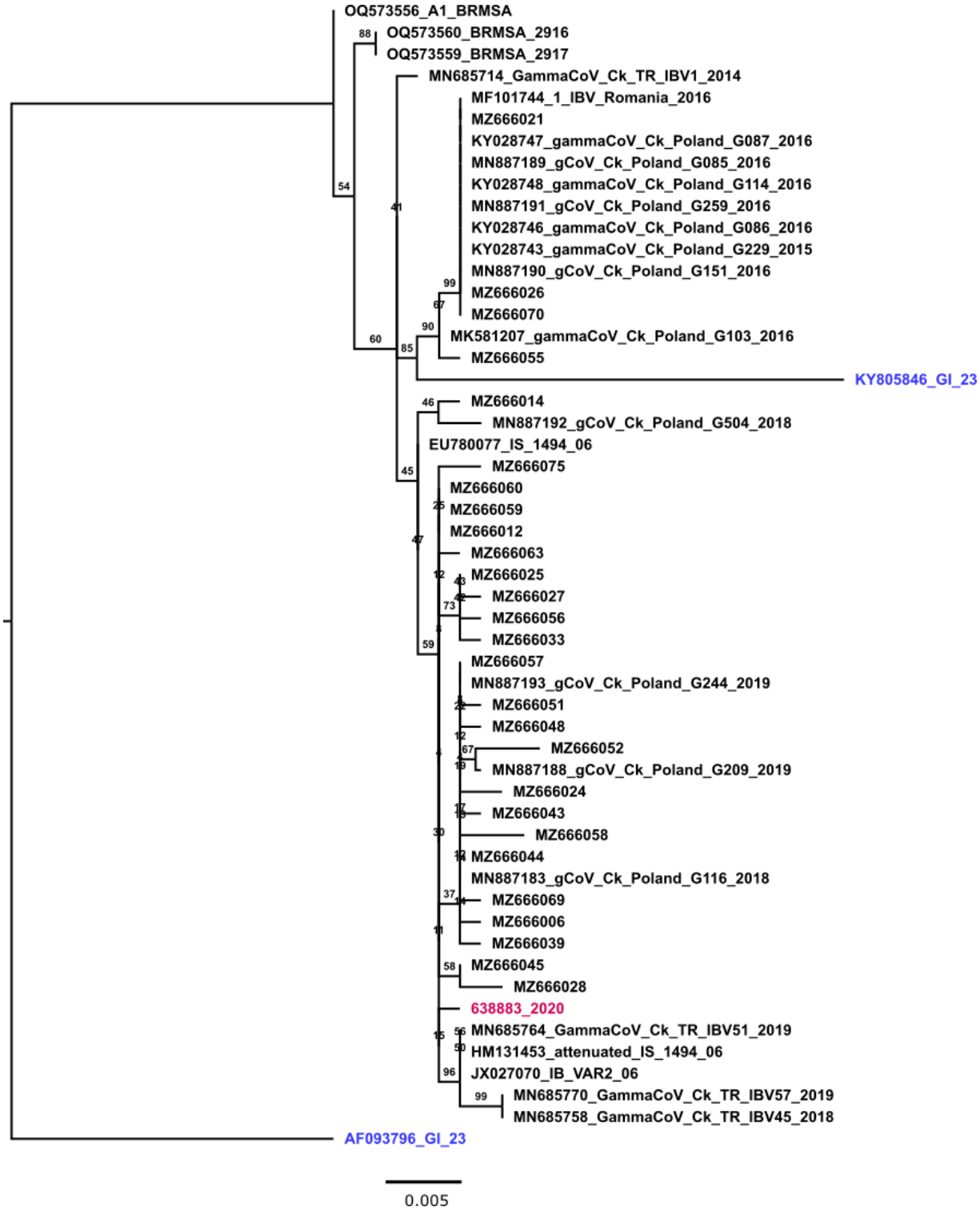

Figure 17(f). Maximum-likelihood phylogenetic tree of the GI-23 lineage re-rooted for an enlarged view of isolates 638883_2020. Isolates are highlighted in pink; genome classification reference samples are highlighted in blue and field viruses retrieved from Genbank are in black.

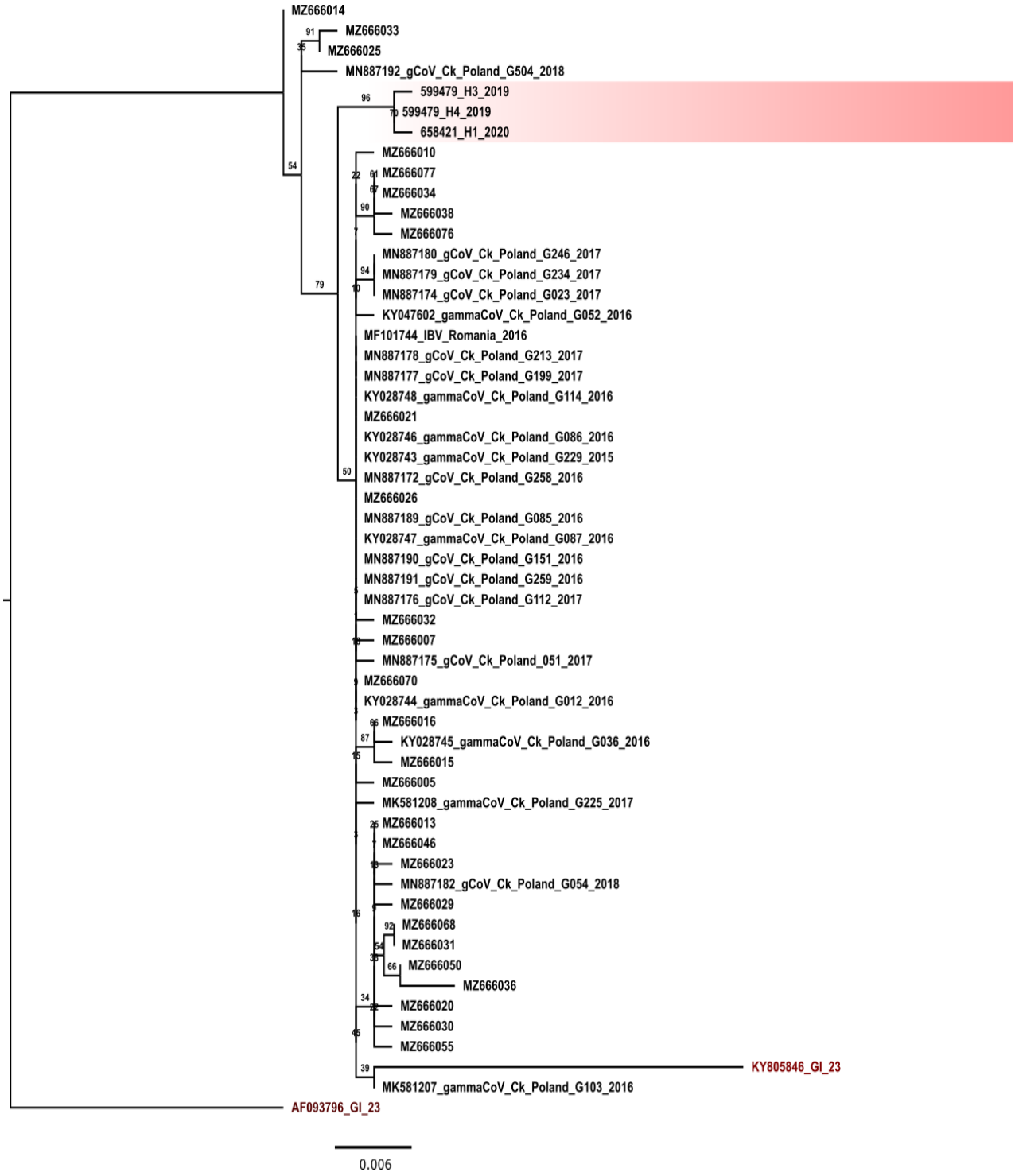

Figure 17(g). Maximum-likelihood phylogenetic tree of the GI-23 lineage re-rooted for an enlarged view of isolates 599479_H13_2019, 599479_H4_2019, 658421_H1_2020. Isolates are shaded in red; genome classification reference samples are highlighted in maroon and field viruses retrieved from Genbank are in black.

4.3.6 Lineage GIV-1 (Delaware-like strains)

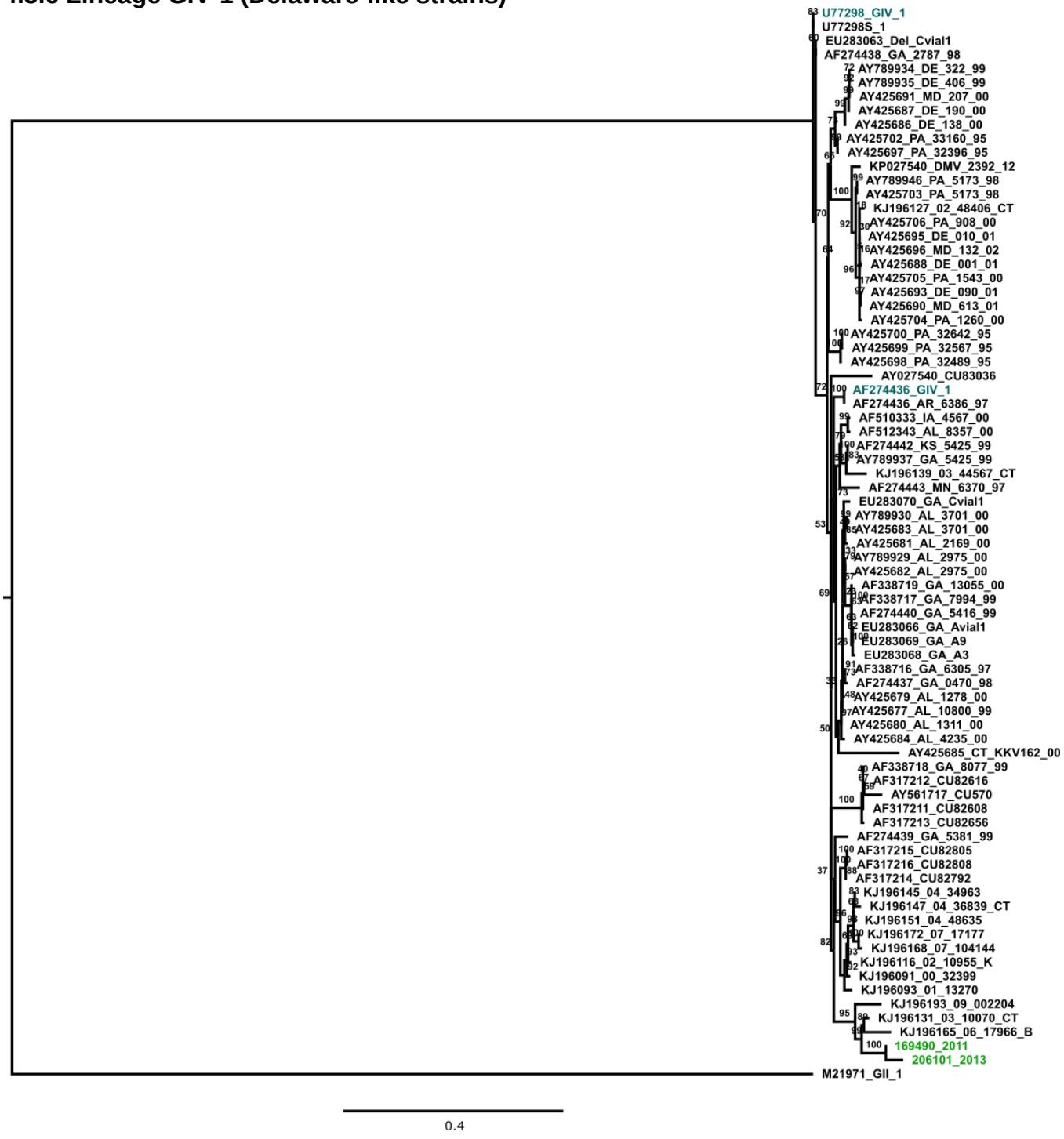

Figure 18(a). Maximum-likelihood phylogenetic tree of lineage GIV-1 IBV. Study isolates are highlighted in green, genome classification reference samples are in blue, field viruses retrieved from Genbank are in black.

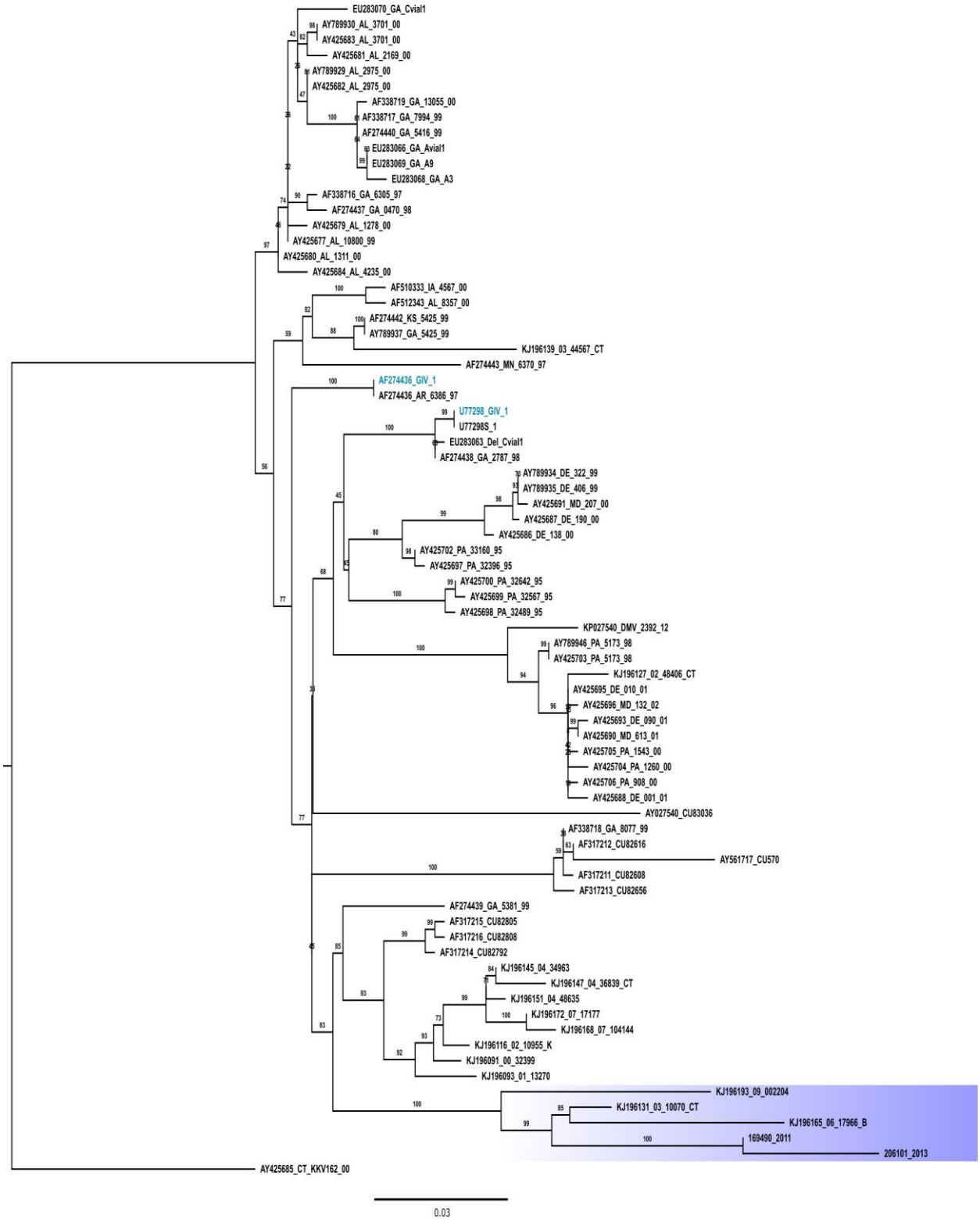

Figure 18(b). Maximum-likelihood phylogenetic tree of the lineage GIV-1, rooted with more closely related sequence (AY425685) for a better visualization. Study isolates are shaded blue, genome classification reference samples are blue, field viruses retrieved from Genbank are in black.

The two unique viruses, 169490_2011 and 206101_2013, in this group are 97% related to each other when compared by pairwise alignment. They were isolated two years apart and no other isolates of the same lineage had been isolated after 2013. Their corresponding sequences when using the BLAST tool are closest to those isolated from Canada in the years 2002-2009. The highest identity percentage, however, ranged between 93% and 95%. The sequence identity to the commercial DE072 vaccine strain, EU359660, ranged between 91 and 92%.

These viruses were also isolated from broiler flocks in two different provinces, one from the North West and one from Mpumalanga. According to Valestro et al., (2016), the Delaware variant of this lineage is a cluster restricted to the U.S.A. Other viruses also within this lineage which is closely related is the previously designated GA98 (Valestro et al., 2016) as well as viruses that were recovered from layer flocks with reduction in egg production (Valestro et al., 2016). The two isolates from South Africa are more closely related to the GA98 version within this lineage and not the Delaware strain that is restricted to the U.S.A. region. The closest relating viruses at 95% sequence identity were those isolated from Canada during the 2002-2009 period. This specific strain appears not to have spread much further in South Africa, nor has it caused any further outbreaks on other farms within the regions they were isolated. Why the two viruses were isolated so far apart remains to be determined. Full genome sequencing could reveal more epidemiological information.

4.3.7 Lineage GVI-1 (TC07-2-like strains)

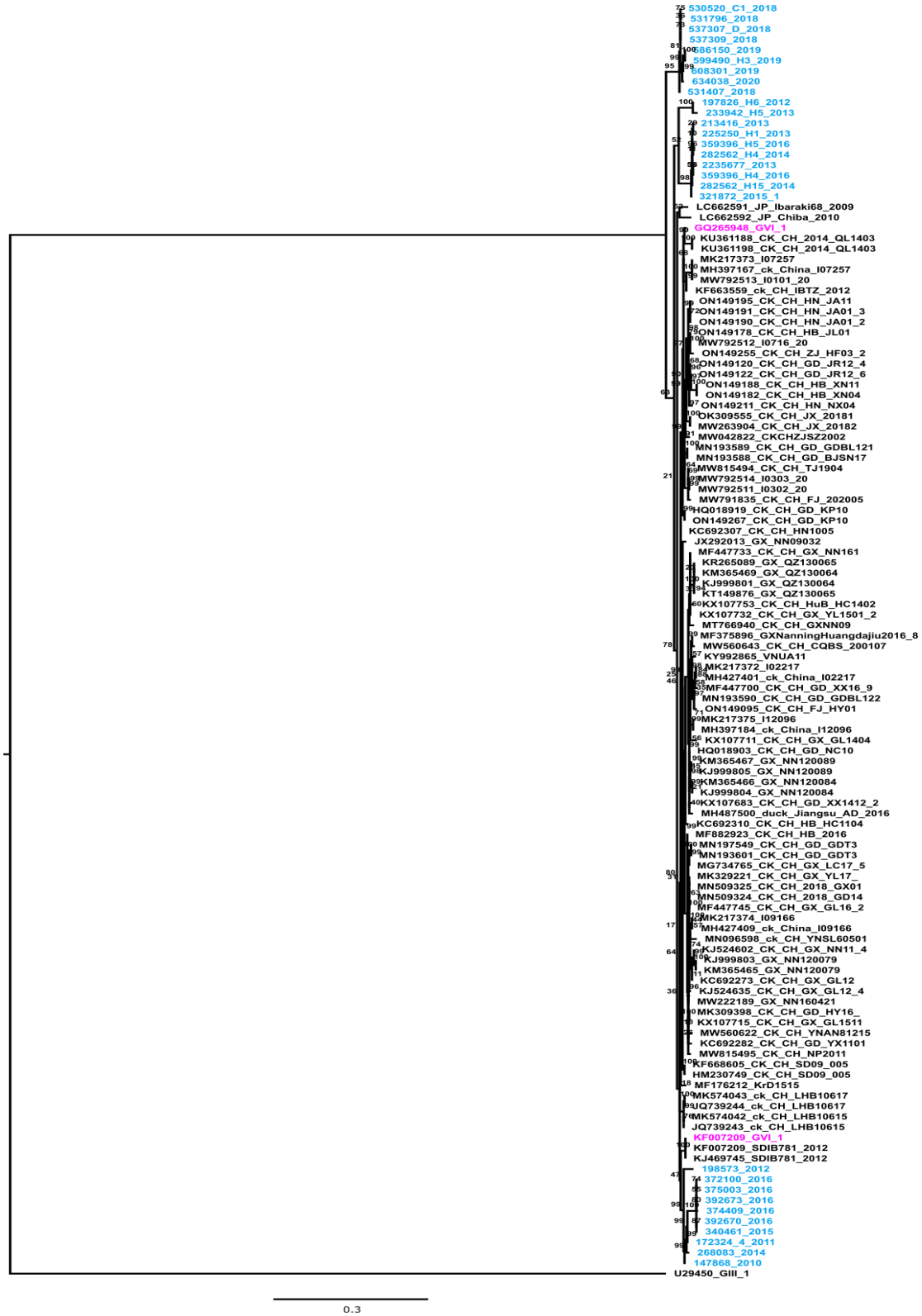

Figure 19(a). Maximum-likelihood phylogenetic tree of lineage GVI-1 IBV. Study isolates are highlighted in light blue, genome classification reference samples are in pink, field viruses retrieved from Genbank are in black.

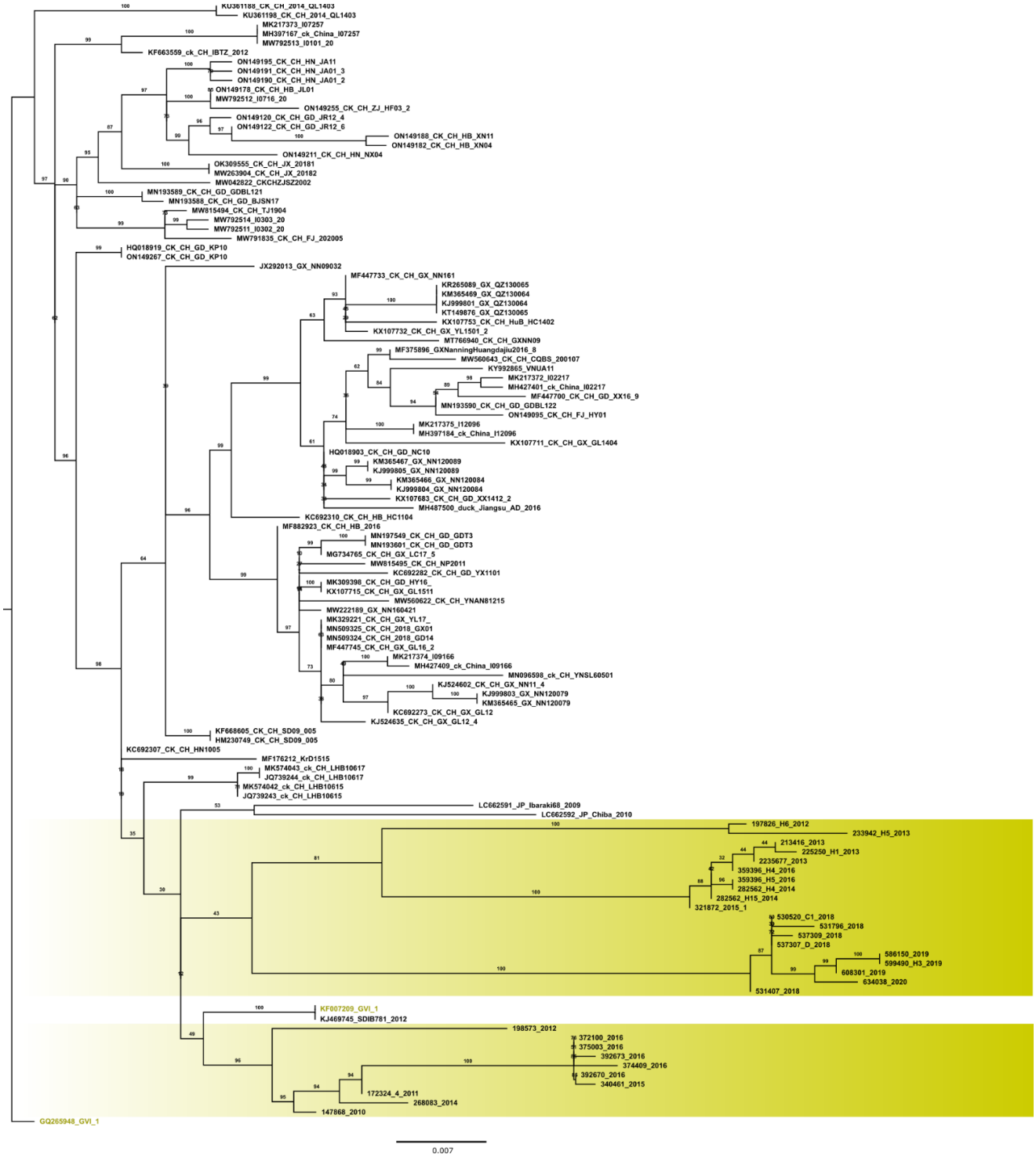

Figure 19(b). Maximum-likelihood phylogenetic tree of the lineage GVI-1, rooted with more closely related reference sequence for a better visualization. Study isolates are shaded yellow, genome classification reference samples are in yellow font, field viruses retrieved from Genbank are in black font.

The South African isolates appear to form a single cluster, but some variation within this cluster is also apparent. The closest sequences when using the BLAST tool was isolate JQ739243, KC792307, and KF007209, which was isolated in China between 2010 and 2012. Another closely related strain was one from South Korea (MF176212) isolated in 2015. The complete genome of JQ739243 is under the accession number MK574042. The nucleotide sequence identity ranged between 94% - 95%.

GVI-1 is a lineage previously believed to be native to Asia (Valestro et al., 2016), but a more recent study has revealed that the GVI-1 might have originated from IBV isolates from Colombia CO8089L and CO8091L (Ren, et al., 2019). This however is yet to be confirmed. It is a fact that this virus has indeed spread to at least five countries including Colombia, China, Korea, Japan, and Vietnam (Ren, et al., 2019). This study also confirms that South Africa and Namibia might be included in the group of countries to which it has spread to. According to the results obtained at least one isolate of the TC07-2/GVI-1 strain was isolated per year excluding the year of an AI outbreak in 2017. Overall, the most isolates from this lineage were isolated from Namibia a neighbouring country to South Africa, however, in the year 2016 the most isolates of this lineage were isolated in South Africa and only two isolates from Namibia were from this lineage in that year. No vaccine for this strain is currently registered in South Africa (personal communication, Aitchison, H., 2023). The strain has been detected in some epidemiological studies of pharmaceutical companies but not all (personal communication, Botha, C., Coetzer, M., Dawson, S., Malan H., 2023). Namibia, and South Africa's Gauteng and North West provinces appear to be the regions where this variant most commonly circulated. TC07-2 caused some problems in broiler birds in the North West province 10 years ago with symptoms of tracheal plugs and later mortalities at 28-days of age (personal communication, Aitchison, H., 2023). This was observed during a vaccination drive with a cloned form of NDV vaccine (personal communication, Aitchison, H., 2023). GVI-1 does not appear to cause severe disease on its own within flocks (personal communication, Aitchison, H., 2023) but persistence within the environment could possibly lead to recombination of viruses and new pathogenic traits.

Table 10. Number of samples isolated per year, according to lineage.

Lineage	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
GI-1			19	16	16	22	3		2		
GI-11		1	1	5	4		1				1
GI-13		1	2	7	8	15	2		3	1	
GI-19	4	18	44	37	32	31	5	4	1	6	2
GI-23	1					1				8	9
GIV-1		1		1							
GVI-1	1	1	2	4	3	2	7		5	3	1

The years 2011 to 2015 had the greatest number of samples isolated compared to other periods. While virus isolation in SPF embryonated eggs is a great tool for growing poultry viruses, it is not the most cost-effective method for diagnostic purposes. Unless there is a useful reason, like vaccine development, farmers generally do not prioritise this test in their schedule. Pharmaceutical companies conduct their own surveillance on coronaviruses and even though data does not get published their vaccines are based on the general distribution they find within their studies. From personal observations, the data gathered by vaccine companies is usually also not obtained through virus isolation but rather based on data retrieved though PCR results and sequencing. Information gained from this data is shared nationally and internationally in congresses via posters or presentations, but not necessarily published in peer-reviewed journal articles.

The GI-1 lineage showed an increase in isolations from 2012 onwards reaching a peak in 2015. The most GI-19 samples were isolated in 2012 (before official registration of its vaccine) with a decrease in incidence (as detected by virus isolation) occurring between 2013 and 2015. The GI-11 lineage had a peak in isolation in the years 2013 and 2014 but was scarce thereafter. GI-13 isolation increased from 2011 onwards with a peak in 2015. This correlates with the registration of its vaccine from 2013 onwards. This coincides with the previous findings that most of the isolates from this group was most likely that of vaccine strains.

The increase of isolations for GI-23 occurred from 2019 onwards. The general low number of isolates between 2016 -2018 was due to a decline in the number of virus isolations requests and an AI outbreak in 2017 rather than a decline in IBV viruses circulating throughout southern Africa. There does not appear to be a vaccine registered in South Africa for lineage GVI-1, but the peak of isolations happened in 2016 when there were few VI requests. This variant isolated at least once a year except in 2017 in which South Africa had an outbreak of AI and the focus shifted more towards the isolation of the Influenza A virus instead.

Table 11. Number of samples isolated per province, according to lineage.

	South Africa								Botswana	Eswatini	Namibia	Unknown
Lineage	**Gauteng**	**North West**	**Eastern Cape**	**Western Cape**	**Free State**	**KwaZulu-Natal**	**Limpopo**	**Mpumalanga**				
GI-1	12	32		4	23		1	5				1
GI-11	4	2		1		2		1				3
GI-13	10	8		1	12	1		2			3	2
GI-19	30	67	1	10	56	4		6	1	1		8
GI-23	3	2		2	1	1						10
GIV-1		1						1				
GVI-1	5	4	1	3	4			2			6	4

Figure 20 below shows the distribution of poultry flocks across South Africa in 2021.

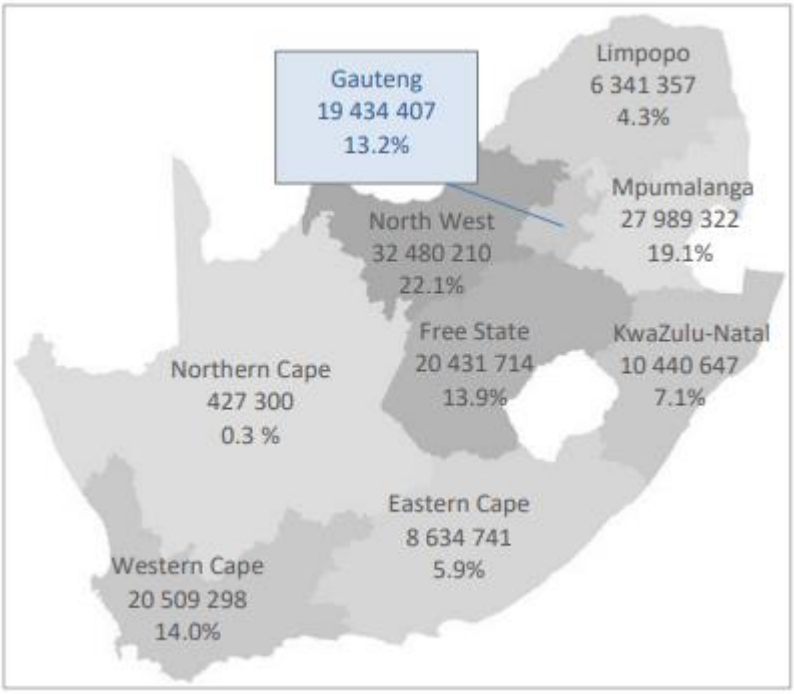

Figure 20. Provincial distribution of the national chicken flock (December 2021) (source: https://www.sapoultry.co.za/wp-content/uploads/2022/03/4Q2021-Provincial-distribution-report.pdf).

31.9% of samples were isolated from the North West, 26.4% from the Free State, 17.6% of samples were isolated from Gauteng, 5.8% from the Western Cape, 4.7 % from Mpumalanga, 2.2% from Kwazulu-Natal, 0.5% from the Eastern Cape Province, and 0.3% from Limpopo. 3.3% were isolate from other southern African countries and 7.7% were of unknown regions. The number of samples per province correlate with the population of national chicken flocks at the time with North West having the most cases and Free State the second most cases. The Western Cape and Mpumalanga have more flocks than that of Gauteng yet more samples were isolated from Gauteng at the time. This could be due to the number of consulting veterinarians in the area referring to a specific laboratory, the distribution of the company's clients at the time and the location of the laboratory.

There does not appear to be a prevalence of certain strains to certain regions, Knoetze et al. (2014) found that more Mass type viruses were circulating within the

KZN region. The 2014 study did have a significantly larger number of samples from this specific region than this study but, in this study, not a single Mass strain was isolated from the KZN region.

Table 12. The distribution of lineages across the different classifications of poultry

Classification	GI-1	G-1	GI-13	GI-19	GI-23	GIV-1	GVI-1
Breeders	1		1	6			2
Broilers	70	6	30	159	6	2	20
Layers	3	4	3	6	3		2
Grandparent			2				1
Rearing	2						
SPF	1			2			
Unknown	1	3	3	11	10		4

80.5% of samples isolated were from broiler flocks, 5.8% from layers, 1.1% from breeders, 0.8% from Grandparent flocks and SPF birds, 0.05% from birds in rearing. 8.85 of samples were from unknown origin. Most clients' samples included in this study is of broiler bias. Broiler birds also make up most of the poultry industry followed by layer birds (SAPA, 2021). The data above reflects much of the same.

South Africa imports a large amount of poultry meats from other countries. According to SAPA in 2021 Brazil accounted for 66.6% of all imports followed by the U.S.A (15.6%), EU countries (8.8%), Argentina (5.5%), Canada (1.5%) and smaller other countries (2% combined).

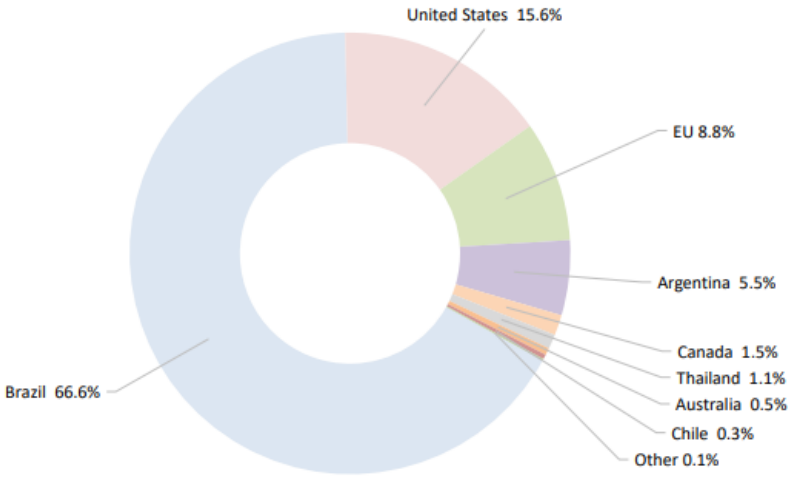

Figure 21. Country of origin 2021 (EU countries grouped) (source: https://www.sapoultry.co.za/wp-content/uploads/2022/03/2021-12-COUNTRY-REPORT.pdf).

Reports of the import of live birds into South Africa from Brazil (Trading Economics, 2023) could play a role in the spread of lineage GI-11 and possibly other IBV strains across the country. Movement of birds also from farm-to-farm and province-to-province aids in the spread of the different genotypes of the virus, and because not everyone follows the same vaccination programmes, an introduction of a different genotype could cause an outbreak of new IBV type on the farm. Biosecurity has improved dramatically since the numerous outbreaks of AI. It is however still not perfect (Bisschop, S.P.R. and Travers, F., personal communication, 2023). IBV biosecurity protocols follow the same route as with any other disease prevention methods (SAPA, 2013). These include, but are not limited to, access control, proper and closed housing systems to prevent wild birds, rodents and insect movement, personal protective clothing (PPE), restriction of movement between houses or the change of PPE between houses and sites, proper disinfection before, during and after placement of birds, limited sharing of equipment between farms and proper disinfecting of equipment before use on a new site or farm (SAPA, 2013). IBV is highly contagious and spreads rapidly among chickens. It is of utmost importance to maintain biosecurity on farms to prevent spread of all diseases (Bisschop, S.P.R. and Travers, F., personal communication, 2023).

How fresh and frozen meat play a role in the introduction of IBV onto farms remains to be determined. It is possible on a farm with multiple animals, these imported

meats or bones could be fed to animals introducing the new strains to the environment. The import data does correlate to an extent for most lineages found within South Africa except for those linked to China, Korea, and Poland. Another explanation about the seemingly closer genetic links between southern Africa's IBV variants and those from China, Korea and Poland could be a lack of sequence data from other countries and a sequence "dump" of countries that continuously monitor their IBV prevalence (Franzo, 2017).

Feed products such as grain and second-hand equipment could also possibly act as fomite introduction points. However small the possibility, migratory birds could also possibly play a role in the spread of the virus, though there is a lack of evidence for this speculation (Franzo, 2017).

Chapter 5

CONCLUSION

The study conducted shows the diverse number of IBV strains circulating within some southern African countries and South African provinces. It has revealed that at least 7 different lineages and 3 different genotypes circulating within the sub-region's poultry flocks. These were identified as GI-1 (Mass/H120-type strains), GI-11 (UFMG/G- Brazil-like strains), GI-13 (793/B-like strains), GI-19 (QX-like strains), GI-23 (Var-2-like strains), GIV-1 (Delaware-like strains), and GVI-I (TC07-2-like strains).

Isolations of the GI-1 lineage were most likely to be vaccine-derived strains with additional mutations or variations gained after circulation. The sequences of the South African H120-like strain from the study conducted by Knoetze et al., 2014, appeared to be uniquely separate from the isolates obtained for this study. The reason for this phenomenon remains unknown and a comparison study on the full genome sequences would be beneficial.

GI-11 isolates were field isolates that may have been introduced through importations of live poultry and poultry products from Brazil. Lineage GI-13 appears to be mixture of both Asian and European 4/91 strains, where vaccine-derived isolates are more likely closely related to the attenuated 4/91 UK strain. While it is possible that a small number of isolates obtained from lineage the GI-19 could be vaccine-derived, the field isolates do appear to have adopted their own unique sequences and form a cluster of South African QX-like strains. Similarly, the QX-like strains from South African sequences previously conducted by Knoetze et al., 2014, were also separated completely from isolates used in this study.

The pathogenicity of lineage GI-23 was quite apparent from 2019 onward. Its introduction to South Africa needs more investigation since Middle Eastern countries are not major exporters of poultry products to South Africa. This variant could have been introduced via another exporter. The common link between the separate isolation of the viruses of the lineage GIV-1 is yet to be found. Isolates from GVI-1 appear to have a direct link to China. China too, is not a major exporter to South Africa and this strain most likely also came through other channels into the country. Illegal swill dumping in harbours has been route for infectious animal diseases to

South Africa from Asia in the past. A few isolates show possible recombination in the small region sequenced here but the uniquely combined viruses did not necessarily create strains that could replicate any further within the environment and were not necessarily contagious enough or able to spread to neighbouring farms and cause major outbreaks. There appears to be a link between certain strains and possibly the importation of poultry products and live birds especially from Brazil, China, and Poland. Further studies would need to be conducted to verify this.

Furthermore, full genome sequencing and interpretations of data is needed to draw conclusions on unique strains and isolates of note.

Recommended isolates for full genome sequencing:

- GI-1: 259214_S1_2014
- GI-11: 222760_2013
- GI-11: 282913_H3_2014
- GI-13: 244155_2014
- GI-13: 238153_2013
- GI-13: 174443_2011
- GI-19: 187521_H1_2012
- GI-19: 579119_2019
- GI-19: 564876_2019
- GI-23: 149399_2010
- GI-23: 336625_2015
- GI-23: 638883_2020
- GIV-1: 169490_2011
- GIV-1: 206101_2013
- GVI-1: 147868_2010
- GVI-1: 172324_4_2011
- GVI-1: 198573_2012
- GVI-1: 634038_2020

One of the short comings of this study is that only isolates from virus isolation were included. Due to the cost of virus isolation, it is not the preferred method for diagnosis and far more samples are submitted for IBV PCR instead. While PCR

alone is beneficial for diagnostic purposes, the original samples are usually discarded leaving only the RNA that might be available for further testing. In some cases, the RNA alone might not have sufficient genetic material for sequencing and if a unique virus or new introduction of IBV it is beneficial to isolate viruses for vaccine development and increasing RNA yield. Another suggestion for surveillance of IBV would be to use isolates that have been obtained from similar organs due to tissue tropism. Surveillance of wild birds in the South African region is also another area that could be explored.

Lastly, continuous surveillance of IBV is necessary to identify any new strains that might emerge as well as any changes in currently circulating strains to gain insight in relevance of vaccines available and to prevent any further disease and economic loss. The assay from this study worked well on the South African strains. The phylogenetic tree analysis and the BLAST homology correlated with each other, and no samples appeared to be misplaced. The method is easy to follow and can be done in-house and sent for Sanger sequencing to a local laboratory. The sample analysis should however be conducted by someone with knowledge of sequence analysis. The method used here could possibly be more cost effective and improve the turnaround time than methods currently being used.

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ANNEXURE 1

Isolate description	Genbank Accession number
201216_2012	OR727107
235198_2013	OR727111
347477_H2_2015	OR727108
377455_2016	OR727109
526250_H2_2018	OR727110
175829_H3_2011	OR742726
210882_H1_2013	OR742727
222760_2013	OR742730
226668_H2_2013	OR742731
226827_HD_2013	OR742729
269246_AF_2014	OR742732
269246_KID_2014	OR742733
538641_B5_2019	OR742734
568903_2019	OR742735
244155_2014	OR742736
174443_2011	OR742737
260600_2014	OR742738
626739_2020	OR742728
168355_2011	OR742765
187521_H1_2012	OR742763
193311_2012	OR742753
201106_2012	OR742758
201602_2012	OR742754
231139_2013	OR742755
236104_2013	OR742766
249371_2014	OR742762
259214_S2_2014	OR742757
276811_2014	OR742761
347675_2015	OR742759
403218_2016	OR742760
461064_H2_2017	OR742756
579119_2019	OR742764
149399_2010	OR742739
336625_2015	OR742740
617999_2020	OR742741
638883_2020	OR742742
169490_2011	OR704347
206101_2013	OR704348
147868_2010	OR742743
172324_4_2011	OR742744

Isolate description	Genbank Accession number
537307_D_2018	OR742747
197826_H6_2012	OR742748
213416_2013	OR742750
225250_H1_2013	OR742751
233942_H5_2013	OR742749
282562_H15_2014	OR742752
374409_2016	OR742745
530520_C1_2018	OR742746

ANNEXURE 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

27 February 2024

LETTER OF APPROVAL

Ethics Reference No	**REC120-21**
Protocol Title	**Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011- 2020**
Principal Investigator	**Mrs C Strydom**
Supervisors	**Prof C Abolnik**

Dear Mrs C Strydom,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Veterinary Sciences Research Ethics committee.

Please note the following about your ethics approval:

1. Please use your reference number (REC120-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application (for Post graduate studies e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. Note: All FVS animal research applications for ethical clearance will be automatically rerouted to the Animal Ethics committee (AEC) once the applications meet the requirements for FVS ethical clearance. As such, all FVS REC applications for ethical clearance related to human health research will be automatically rerouted to the Health Sciences Research Ethics Committee, and all FVS applications involving a questionnaire will be automatically rerouted to the Humanities Research Ethics Committee. Also take note that, should the study involve questionnaires aimed at UP staff or students, permission must also be obtained from the relevant Dean and the UP Survey Committee. Research may not proceed until all approvals are granted.

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

4 November 2021

Approval Certificate
New Application

AEC Reference No.:	REC120-21
Title:	Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011- 2020
Researcher:	Mrs C Strydom
Student's Supervisor:	Prof C Abolnik

Dear Mrs C Strydom,

The **New Application** as supported by documents received between 2021-08-24 and 2021-10-25 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2021-10-25.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	**Number**
Poultry	
Samples Allantoic Fluid	320 (Stored- Historic/Retrospective)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-11-04.
3. Please remember to use your protocol number (REC120-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

19 October 2022

Approval Certificate
Annual Renewal
(EXT1)

AEC Reference No.:	REC120-21 Line 1
Title:	Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011- 2020
Researcher:	Mrs C Strydom
Student's Supervisor:	Prof C Abolnik

Dear Mrs C Strydom,

The **Annual Renewal** as supported by documents received between 2022-09-12 and 2022-09-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-09-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Samples	Approved
Poultry - Allantoic Fluid (Stored-historic/retrospective samples)	320

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-10-19.

3. Please remember to use your protocol number (REC120-21) on any documents or correspondence with the AEC regarding your research.

4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.

6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research

Yours sincerely

Prof Andrew McKechnie
Acting Chairperson: UP-Animal Ethics Committee

ANNEXURE 3

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/8 (2056 LH)

Christine Strydom
Department of Production Animal Studies
Faculty of Veterinary Science, University Pretoria
Old Soutpan Road
Onderstepoort, Pretoria
Tel: 0824271190
E-mail: ts@smtvet.co.za
Christine.f3nn@gmail.com

Dear Christine Strydom,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 13 July 2021, received by us on 16 July 2021, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. This permission is given upon finding the biosecurity of the research project as described to be acceptable to DALRRD;
3. The research project is approved as per the application form dated 13 July 2021 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;

5. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority;
6. Only IBV isolates stored in the Deltamune repository, dating from 2011 to 2020 (as per the application form and the list of samples provided), may be used for this research study, and no field samples may be collected;
7. The extraction of DNA may only be performed at the Poultry Research Laboratory at the University of Pretoria. Only extracted DNA may be removed to SMT Labs;
8. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National *Road Traffic Act,* 1996 (*Act* No. 93 of 1996);
9. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the study;
10. Only a waste disposal company registered for the disposal of biohazardous waste may be used for the removal of all potentially infectious waste from the research project; Records must be kept for five years for auditing purposes;
11. Only RNA extracts may be stored under access control in the Poultry Research Laboratory, as per the application form;
12. Stored samples may not be outsourced for research without prior written approval from the Director: Animal Health.
13. Should samples be used for further research, written approval from the Director: Animal Health must be obtained prior to start of project

Title of research/study: Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011-2020
Researcher: Christine Strydom
Institutions: University of Pretoria, Faculty of Veterinary Science, Department of Production Animal Studies
Permit Expiry date: 30 November 2022
Our ref Number: 12/11/1/1/8 (2056 LH)
Your ref: REC120-21

Kind regards,

Name: Dr Mpho Maja
Reason:
Date: 2021.08.24 11:06:41 CAT

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Marna Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/8 (2725BD)

Christine Strydom
University of Pretoria - Faculty of Veterinary Science - Department of Production Animal Studies
University of Pretoria, Faculty of Veterinary Science,
Department of Production Animal Studies
E-mail: christine.f3nn@gmail.com

Dear Christine Strydom,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) – EXTENSION OF THE EXPIRY DATE

Title of research project / study: "Molecular epidemiology of infectious bronchitis coronavirus in South African poultry flocks from 2011 – 2020"

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 2021-08-24.

1. As requested, the validity of the section 20 approval is extended to 30 November 2023;
2. All other conditions as specified in the Section 20 approval of 2021-08-24 remain in full effect. This includes the validity of laboratory approvals in terms of SANAS and DALRRD.

Kind regards,

DIRECTOR: ANIMAL HEALTH

Date: 2022 -10- 19