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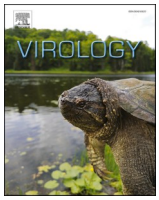

Unveiling the genetic landscape of infectious laryngotracheitis virus in Switzerland: Evidence for vaccine-like and wild-type strains

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ABSTRACT

Infectious laryngotracheitis (ILT) is a respiratory disease affecting chickens worldwide. Unlike many countries, Switzerland does not vaccinate against ILT. This study analysed ILT samples from 21 natural outbreaks in Switzerland using restriction fragment length polymorphism (RFLP) and multiple gene sequencing. Chicken embryo origin (CEO) and tissue culture origin (TCO) vaccine strains were included as references. Both vaccine strains were distinguishable, and 14 out of 21 samples resembled the CEO vaccine. Additionally, four distinct non-vaccine-like groups were identified. Sequencing of three genes from selected Swiss samples and those from neighbouring countries revealed four phylogenetic clades. Notably, four Swiss field strains formed two unique clades, not closely related to vaccine strains or ILTV from neighbouring countries. Overall, RFLP results were supported by sequencing data. This study demonstrates the presence of both vaccine-like and wild-type ILT viruses in Switzerland, where vaccination is *de facto* prohibited.

1. Introduction

Infectious laryngotracheitis (ILT) is a respiratory disease that mainly affects chickens, but can also impact pheasants, peafowl and turkeys (Crawshaw and Boycott, 1982; Portz et al., 2008). It is caused by Iltovirus gallidalpha1, also known as infectious laryngotracheitis virus (ILTV), a member of the genus Iltovirus in the Alphaherpesvirinae subfamily of Orthoherpesviridae (Lefkowitz et al., 2018). Typical signs of ILT include nasal discharge, conjunctivitis, and expectoration of bloody mucus, which can lead to dyspnoea and, eventually to death by suffocation. Depending on the virus strain, mortality among infected chickens ranges from 10 % to 70 %. The disease is usually accompanied by reduced egg production and lower weight gain, leading to severe economic losses in the poultry industry worldwide (Garcia and Spatz, 2019).

Different types of vaccines exist to combat the disease (García and Zavala, 2019), but primarily live attenuated vaccines are used. Attenuation of ILTV is achieved by sequential passages in chicken embryos (chicken embryo origin; CEO) (Samberg et al., 1971) or by multiple passages in tissue culture (tissue culture origin; TCO) (Gelenczei and Marty, 1964). However, live attenuated vaccines may regain virulence through bird-to-bird passages (Guy et al., 1991), with revertant CEO vaccine strains potentially exhibiting higher pathogenicity and greater transmission potential than wild type ILTV strains (Perez-Contreras et al., 2021).

Viral vector vaccines offer an alternative with no risk of reversion but are often used alongside live attenuated vaccines due to slower onset of immunity and reduced efficacy against ILTV replication (García and Zavala, 2019). Vaccination against ILTV is widespread globally. In Switzerland, however, not only the detection of the virus, but also the detection of ILTV antibodies inevitably leads to the culling of the affected flock, so that vaccination against ILTV is *de facto* not permitted in Switzerland (ANONYMOUS, 2023). A few outbreaks of ILT occur every year in Switzerland; almost exclusively in backyard chicken flocks and only rarely in commercial poultry. The low incidence of ILT outbreaks, especially in commercial flocks, may be related to biosecurity measures, and strict animal welfare regulations including the minimum space per animal and the maximum flock size (a maximum of 18000

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Table 1
ILTV samples used for PCR-RFLP and sequencing.

Sample ID	No. of samples	Sample year	Origin	Material	Animal type
Nobilis® ILT (CEO)	1	n/a	n/a	Vaccine	n/a
LT-IVAX® (TCO)	1	n/a	n/a	Vaccine	n/a
13-T0396-1/2	2	2013	CH, Basel-Country	Trachea	Fancy fowl
17-T0583-1/2[a]	2	2017	CH, Thurgau	Trachea	Laying hen
18-T0050	1	2018	CH, Grisons	Trachea	Fancy fowl
18-T0339-1/2	2	2018	CH, Zurich	Trachea	Fancy fowl
18-T0506	1	2018	CH, Thurgau	Trachea	Fancy fowl
19-V0046	1	2019	CH, Geneva	Trachea	Fancy fowl
19-T0190	1	2019	CH, Glarus	Trachea	Fancy fowl
19-T0500[a]	1	2019	CH, Ticino	Trachea	Laying hen
20-T0026	1	2020	CH, St. Gallen	Trachea	Fancy fowl
20-T0305	1	2020	CH, Ticino	Trachea	Fancy fowl
20-V0139	1	2020	CH, Vaud	Swab	Fancy fowl
20-V0167	1	2020	CH, Aargau	Swab	Fancy fowl
21-T0041-1/2	2	2021	CH, Appenzell Outer Rhodes	Trachea	Fancy fowl
21-T0048-1/2[a]	2	2021	CH, Grisons	Trachea	Laying hen
21-T0057-1/2	2	2021	CH, Jura	Trachea	Fancy fowl
21-V0195	1	2021	CH, St. Gallen	Swab	Fancy fowl
21-T0316	1	2021	CH, Grisons	Trachea	Laying hen
21-T0346	1	2021	CH, Thurgau	Trachea	Fancy fowl
21-T0466-1/2	2	2021	CH, Berne	Trachea	Fancy fowl
21-T0506	1	2021	CH, Thurgau	Trachea	Fancy fowl
22-T0009	1	2022	CH, Berne	Trachea	Laying hen
21-AT1	1	2021	AT, Carinthia	DNA	Laying hen
21-AT2	1	2021	AT, Salzburg	DNA	Laying hen
20-AT3	1	2020	AT, Styria	DNA	Laying hen
20-AT4	1	2020	AT, Styria	DNA	Organic broilers
20-AT5	1	2020	AT, Lower Austria	DNA	Laying hen
20-AT6	1	2020	AT, Lower Austria	DNA	Laying hen
22-DE1	1	2022	DE, Greater Berlin area	DNA	n/a
19-DE2	1	2019	DE, Greater Berlin area	DNA	n/a
20-DE3	1	2020	DE, Greater Berlin area	DNA	n/a
19-DE4	1	2019	DE, Greater Berlin area	DNA	n/a
07-IT3	1	2007	IT, Lombardia	AF	n/a
07-IT2	1	2007	IT, Lombardia	AF	n/a
16-IT1	1	2016	IT, Piemonte	AF	n/a

[a] Commercial poultry. AT: Austria, CH: Switzerland, DE: Germany, IT: Italy. AF: allantoic fluid; n/a: not applicable/available.

laying hens may be kept per farm) and the consequent culling of flocks where ILTV was detected (ANONYMOUS, 2024a, 2024b, 2023).

It is uncertain whether recent outbreaks in Switzerland stem from vaccine strain spillovers or wildtype strains. Previous studies have shown that wildtype and vaccine strains can be distinguished by restriction fragment length polymorphism (RFLP) analysis (Chang et al., 1997; Kirkpatrick et al., 2006; Oldoni and García, 2007). Some studies from Italy, Switzerland and the US found that outbreaks were mainly caused by vaccine-related strains (Moreno et al., 2010; Neff et al., 2008; Oldoni et al., 2008; Oldoni and García, 2007), while others from Brazil and Australia found wildtype strains, not closely related to vaccine strains, were the primary cause of outbreaks (Chacón et al., 2010; Kirkpatrick et al., 2006). Furthermore, studies from Australia have shown that vaccine strains, although attenuated, can recombine and form virulent viruses, that continuously cause outbreaks (Agnew-Crumpton et al., 2016; Lee et al., 2012). The aim of this study was to gain an overview of different ILTV strains circulating in Switzerland and comparing them to vaccine strains.

2. Materials and methods

2.1. Samples

In this study 21 ILTV clinical samples collected from natural outbreaks in Switzerland were analysed (Table 1). Three outbreaks occurred in commercial laying hens and 18 outbreaks in hobby chickens. The samples were collected from November 2013 to January 2022 in the Section of Poultry and Rabbit Diseases, Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Switzerland. Patient samples (tracheal swabs and tracheae) that tested positive for ILTV in routine diagnostics in a real-time PCR assay (Callison et al., 2007) were stored at −20 °C until further processing. As reference, two vaccine strains were included: a CEO vaccine (Nobilis® ILT, Intervet International B.V., Boxmeer, The Netherlands; Serva strain) and a TCO vaccine (LT-IVAX®, Schering-Plough Animal Health, San Augustin, Spain; strain unknown). The CEO vaccine (Nobilis® ILT) is used in the neighbouring countries. The TCO vaccine (LT-IVAX) is not used in Europe and thus it was not expected to see any strains resembling this vaccine type. Furthermore, ILTV positive samples (or their DNA) from neighbouring countries were kindly provided by Dörte Lüschow (Freie Universität Berlin, Germany), Ana Moreno (Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Italy), and Ivana Bilic (University of Veterinary Medicine Vienna, Austria). Samples from neighbouring countries were genotyped exclusively by multiple gene sequencing. Samples from Switzerland were genotyped by both multiple gene sequencing and PCR-RFLP.

2.2. DNA extraction

DNA was extracted directly from tracheaes, tracheal swabs, or commercial vaccines. Trachea samples were homogenized using TissueLyser II (Qiagen, Hilden, Germany). DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA concentration was measured using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA was stored at −20 °C.

2.3. PCR

All oligonucleotide primers were synthesized by Microsynth,

Table 2
Primers used in the analyses of ILTV samples.

Primer name	Target genome region	Sequence (5' – 3')	Expected product size (kb)	Annealing temperature (°C)	Restriction enzymes	References
ORFB-TK F	ORF B-TK	TCT GCG ATC TTC GCA GTG GTC AG	4.7	58	*Fok*I	Kirkpatrick et al. (2006)
ORFB-TK R		TGA CGA GGA GAG CGA ACT TTA ATC C				
ICP4 F	ICP4	AAC CTG TAG AGA CAG TAC CGT GAC CC	5.0	57	*Hae*III, *Hin*P1I, *Alw*I, *Ava*I	Chang et al. (1997)
ICP4 R		CCA TTA CTA CGT GAC CTA CAT TGA GCC				
ICP18.5 F	ICP18.5 (UL36)	TCG CTT GCA AGG TCT TCT GAT GG	5.9	57	*Hae*III	Kirkpatrick et al. (2006)
ICP18.5 R		AGA AGA TGT TAA TTC ACA CGG ACA C				
UL47gG F	UL47/gG	TCT TGA ATG ACC TTG CCC CAT	2.9	58	*Hae*III, *Msp*I	Oldoni and García (2007)
UL47gG R		ACT CTC GGG TGG CTA CTG CTG				
TKseq F	TK	GTA ATA TCG CCA GAA TGA GG	1.4	50	n/a	this study
TKseq R		GTA TTT GCT CGC CTT TCA G				
ICP4seq3 F	ICP4	CAC CAG AAA GCT TCA CGT TTC TTC	4.7	55	n/a	this study
ICP4seq3 R		GAG CAC GCA ACC AGA AGT AAA TTG				
UL36seq2 F	UL36	CCG AGC TCC AAT GCT TCG AGA TCG	7.9	56	n/a	this study
UL36seq2 R		GGC TCA GGA TTT CCA TCC TCC GAC				

n/a: not applicable/available.

Balgach, Switzerland. Primers were selected from previously published work or were designed in this study (Table 2).

Amplifications for PCR-RFLP analysis were performed using 50 µl reaction mixture containing 1x High Fidelity PCR buffer, 200 µM of each dNTP, 2 mM $MgSO_4$, 250 nM of each primer, 1 U Platinum® Taq DNA High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 5 µl template DNA as previously published by Kirkpatrick et al. (2006). For all reactions an initial denaturation was performed at 94 °C for 1 min, followed by 40 cycles of 94 °C for 45 s, annealing temperatures of 58 °C (ORFB-TK and UL47/gG) or 57 °C (ICP18.5 and ICP4) for 1 min and extension at 68 °C for 6 min (ORFB-TK and ICP4), 7 min (ICP18.5), and 3.5 min (UL47/gG) (Table 2). The final extension was performed at 68 °C for 10 min.

Amplifications for sequencing were performed using 50 µl reaction mixture containing 25 µl 1x GoTaq®Long PCR Master Mix (Promega, Madison, WI, USA), 0.5 mM (TK) or 1 mM (ICP4, UL36) $MgCl_2$, 500 nM, 400 nM, or 300 nM of each primer targeting UL36, TK, or ICP4, respectively and 1–2 µl template DNA. Initial denaturation was performed at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C, 50 °C, and 56 °C for ICP4, TK and UL36 respectively for 30 s, and extension at 66 °C for 5.5 min (ICP4), 2 min (TK), and 8.5 min (UL36) (Table 2). The final extension was performed at 72 °C for 10 min (ICP4 and UL36), or 5 min (TK).

All PCR products were separated and visualized by capillary electrophoresis with QIAxcel® (Qiagen, Hilden, Germany) using the QIAxcel Screening Kit and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). DNA concentration was measured using an Epoch 2 Microplate Spectrophotometer (Biotek, Winooski, VT, USA) or a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. RFLP analysis

PCR products were digested separately with the respective restriction endonucleases (New England Biolabs, Ipswich, MA, USA) at 37 °C for 3 h 500 ng of DNA were used for each reaction. The ORFB-TK fragment was digested with *Fok*I, the ICP18.5 fragment with *Hae*III, the ICP4 fragment with *Hae*III, *HinP1*I, *Alw*I and *Ava*I and the UL47/gG fragment with *Hae*III and *Msp*I. The resulting DNA fragments were analysed by capillary electrophoresis with QIAxcel® using the QIAxcel® DNA High Resolution Kit (Qiagen, Hilden, Germany). Size and alignment markers were chosen according to manufacturer's recommendations. To accurately determine fragment sizes, the restriction patterns obtained for each amplicon and restriction enzyme used were analysed with the QIAxcel® ScreenGel software (Qiagen, 2011). The bands as observed in the "gel view" were checked using the peaks in the "electropherogram view". The specific patterns of the CEO vaccine strain (Nobilis® ILT) were each assigned the letter "A". Based on the RFLP patterns, genetic distances (GD) of the samples were calculated for each restriction enzyme and gene using Jaccard formula (Jaccard, 1908): $GD = 1 - N_{XY}/(N_{XY} + N_X + N_Y)$ with N_{xy} = number of bands common in sample x and y, N_x = number of bands exclusively present in sample x, N_y = number of bands exclusively present in sample y. For the generation of a distance matrix, the overall genetic distances (δ) for each sample pair (for all genes and restriction enzymes used) was calculated as follows: $\delta = \sum_x^n GD_x/n$ with n = total number of RFLP patterns per sample (n = 8) and x = the respective GD per restriction enzyme. The distance matrix was converted to an UPGMA tree (dendrogram) using the UPGMA command with standard settings in the R-package phangorn (Schliep, 2011).

2.5. Sequencing

The choice for TK, ICP4, and UL36 was based on previous studies, which identified them as suitable targets for differentiation of ILTV

strains (Chacón et al., 2015; Choi et al., 2016; Moreno et al., 2010). Their amplification products were diluted to a final concentration of 20 ng/µl in Tris-EDTA buffer and sent to Microsynth, Balgach, Switzerland, for Nanopore sequencing.

2.6. Phylogenetic analysis

Raw Sanger sequences were cut using the ICP4 (locus_tag GaHV1_gp76), TK (GaHV1_gp17) and UL36 (GaHV1_gp30) from the complete genome of Gallid herpesvirus 1 (Accession NC_006623; (Thureen and Keeler, 2006)) as reference. The same loci were used as query in a blast search to identify ICP4, TK, and UL36 in complete genomes followed by sequence selection with Samtools (Danecek et al., 2021). Separate or concatenated sequences were aligned using MAFFT (Katoh et al., 2002), with standard settings and outputs were stored as clustal alignments. Trees were calculated using IQtree 2.0 (Minh et al., 2020), with the option "-m MFP" for extended model selection followed by tree inference activated. Trees were visualized in iTOL (https://itol.embl.de/) as midpoint rooted trees.

2.7. Mapping

The place of the respective outbreaks was taken from the corresponding patient reports and was geo-coded to determine the latitude and longitude using a webtool (https://www.koordinaten-umrechner.de). The data were entered into an Excel spreadsheet with the case number and ILTV group of the respective isolate and was then imported into Datawrapper (https://www.datawrapper.de/) to create a map using the Locator Map function.

3. Results

3.1. PCR-RFLP

The target genes (ICP18.5, ICP4, UL47/gG, ORFB-TK) analysed by PCR-RFLP were selected on the basis of their suitability for differentiating various ILTV strains (Chang et al., 1997; Kirkpatrick et al., 2006; Oldoni and García, 2007). Digestion of ICP18.5 with *Hae*III produced three different patterns (Fig. 1A, Table 3). Digestion of ICP4 with *Hae*III, *HinP1*I, *Alw*I and *Ava*I produced three, two, four and four different patterns respectively (Fig. 1B, C, D, E, Table 3). Digestion of UL47/gG with *Hae*III and *Msp*I produced two and three different patterns respectively (Fig. 1F and G, Table 3). Digestion of ORFB-TK with *Fok*I produced two different patterns (Fig. 1H, Table 3).

According to the RFLP analysis the samples could be assigned to six different groups (Table 3). The CEO vaccine Nobilis® ILT and samples from 14 outbreaks were assigned to group 1. The TCO vaccine LT-IVAX® was the only virus assigned to group 2. It differs from group 1 in four patterns (detected for ICP4 digested with *Hae*III, *HinP1*I, *Alw*I and *Ava*I). Two samples from one outbreak (21-T0048-1/2) were the only samples

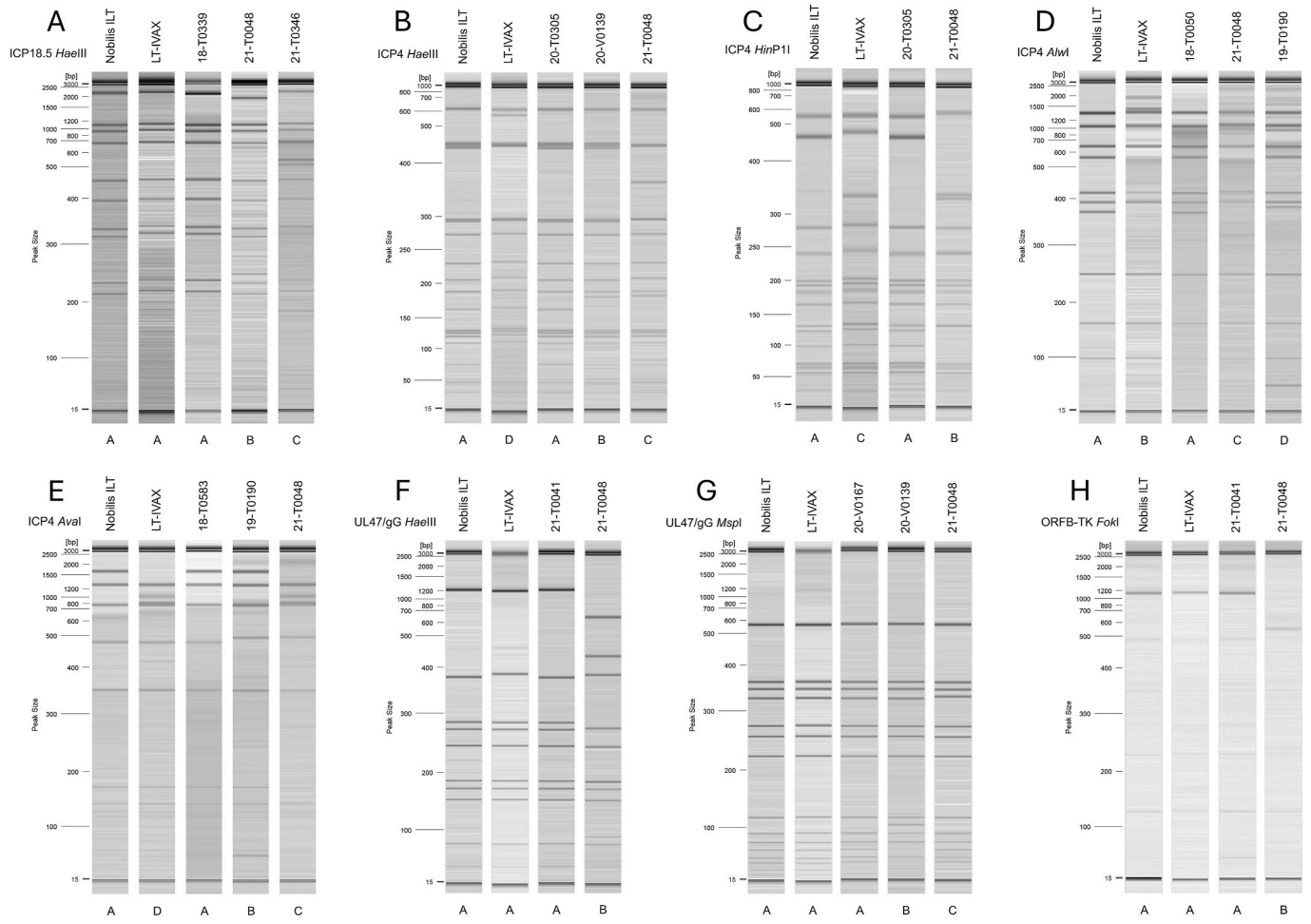

Fig. 1. Capillary electrophoresis of PCR fragments generated by digestion of PCR products. ICP18.5 digested with *Hae*III (A), ICP4 digested with *Hae*III (B), *Hin*P1I (C), *Alw*I (D) and *Ava*I (E), UL47/gG digested with *Hae*III (F) and *Msp*I (G), ORFB-TK digested with *Fok*I (H).

Table 3
PCR-RFLP patterns and corresponding RFLP groups and phylogenetic clades.

PCR product	ICP18.5	ICP4				UL47/gG		ORFB-TK		
Restriction enzyme	*Hae*III	*Hae*III	*Hin*P1I	*Alw*I	*Ava*I	*Hae*III	*Msp*I	*Fok*I		
Sample ID	RFLP pattern								Combined	Group
Nobilis® ILT[a]	A	A	A	A	A	A	A	A	AAAAAAAA	1
LT-IVAX®[a]	A	D	C	B	D	A	A	A	ADCBDAAA	2
13-T0396-1/2	A	A	A	A	A	A	A	A	AAAAAAAA	1
17-T0583-1/2	A	A	A	A	A	A	A	A	AAAAAAAA	1
18-T0050	A	A	A	A	A	A	A	A	AAAAAAAA	1
18-T0339-1/2	A	A	A	A	A	A	A	A	AAAAAAAA	1
18-T0506	A	A	A	A	A	A	A	A	AAAAAAAA	1
19-V0046	A	A	A	A	A	A	A	A	AAAAAAAA	1
19-T0190[a]	A	B	A	D	B	A	A	A	ABADBAAA	4
19-T0500[a]	A	A	A	A	A	A	A	A	AAAAAAAA	1
20-T0026	A	A	A	A	A	A	A	A	AAAAAAAA	1
20-T0305[a]	A	A	A	A	A	A	A	A	AAAAAAAA	1
20-V0139[a]	A	B	A	D	B	A	B	A	ABADBABA	5
20-V0167	A	A	A	A	A	A	A	A	AAAAAAAA	1
21-T0041-1/2	A	A	A	A	A	A	A	A	AAAAAAAA	1
21-T0048-1[a]**/2**	B	C	B	C	C	B	C	B	BCBCCBCB	3
21-T0057-1/2	A	A	A	A	A	A	A	A	AAAAAAAA	1
21-V0195	A	A	A	A	A	A	A	A	AAAAAAAA	1
21-T0316[a]	A	A	A	A	A	A	A	A	AAAAAAAA	1
21-T0346[a]	C	A	A	A	A	A	A	A	CAAAAAAA	6
21-T0466-1/2	A	B	A	D	B	A	A	A	ABADBAAA	4
21-T0506	A	B	A	D	B	A	A	A	ABADBAAA	4
22-T0009[a]	A	B	A	D	B	A	A	A	ABADBAAA	4

[a] Included in the sequencing.

in group 3. Compared to group 1 they differ in every single pattern. Group 4 encompasses samples from four outbreaks. These samples differ from group 1 samples in three patterns (detected for ICP4 digested with *Hae*III, *Alw*I, and *Ava*I). Group 5 consists of one sample, differing from group 1 in five patterns (detected for ICP4 digested with *Hae*III, *Alw*I, and *Ava*I and UL47/gG digested with *Msp*I) but from group 4 only in one pattern (UL47/gG digested with *Msp*I). The isolate assigned to group 6 differs from group 1 in one pattern (detected for ICP18.5 digested with *Hae*III).

If two samples from the same outbreak were tested (13-T0396, 17-T0583, 18-T0339, 21-T0041, 21-T0048, 21-T0057, 21-T0466), identical patterns were obtained in all cases.

3.2. Cluster analysis of ILTV PCR-RFLP data

The six RFLP groups generated by combining the eight RFLP patterns were distributed into four main clusters determined by analysing their genomic distance (Fig. 2). The first cluster consisted of closely related RFLP group 1 and 6. The second cluster was composed of RFLP group 2 encompassing only the TCO vaccine LT-IVAX®. The third cluster comprised RFLP group 3, to which only isolate 21-T0048 was assigned. A fourth cluster encompassed RFLP group 4 and 5.

3.3. Geographical mapping

Remarkably, examining the geographical distribution of different

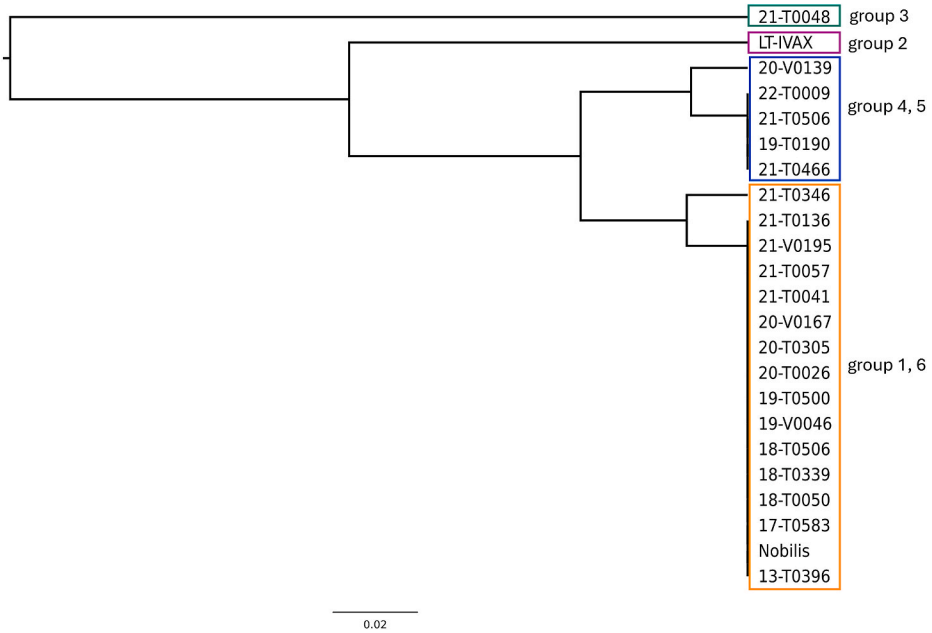

Fig. 2. Dendrogram based on cluster analysis of PCR-RFLP pattern combination of nine groups of Swiss ILTV isolates and two vaccine strains. Boxes mark the different RFLP groups. Yellow, group 1 and 6; violet, group 2; green, group 3; blue, group 4 and 5.

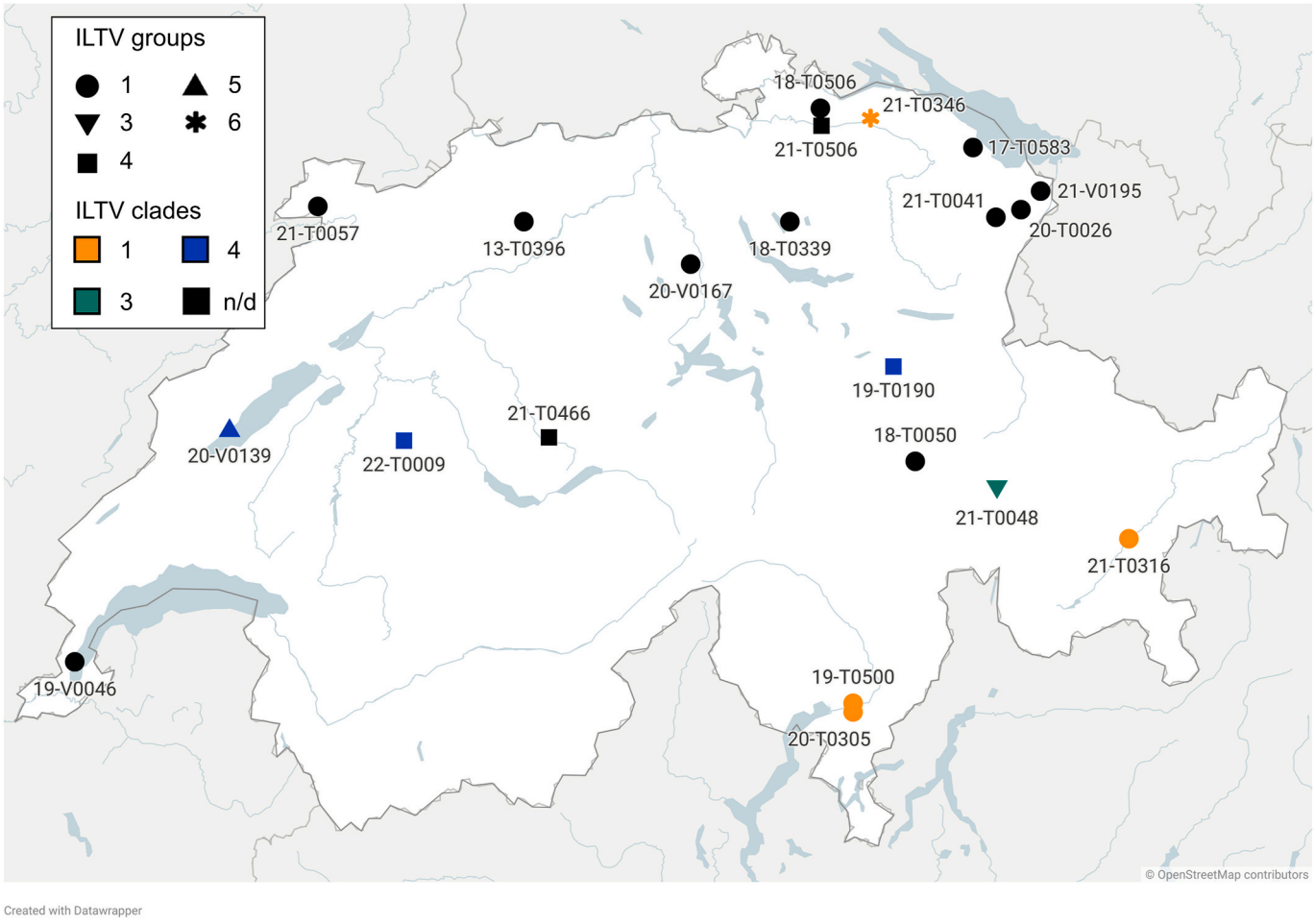

Fig. 3. Geographical distribution of ILTV types in Switzerland.

RFLP groups in Switzerland reveals that RFLP group 1 samples (CEO-like strains) are frequently found along the border regions (Fig. 3).

3.4. Phylogenetic analysis

The phylogenetic tree created by concatenating and aligning all three sequenced genes (ICP4, UL36, TK) revealed four major clades (Fig. 4A). Clade 1 contains the CEO vaccine Nobilis® ILT, RFLP group 1 and RFLP group 6 samples and all samples from neighbouring countries. The sequences from clade 1 differ in a maximum of 11 single nucleotide polymorphisms (SNPs; 20-AT3, 20-AT4, 20-AT5, 20-AT6) which represents a nucleotide sequence identity of 99.92 % compared to Nobilis®-ILT. LT-IVAX®, a TCO vaccine, is the only member of clade 2, and thus did not cluster with Nobilis® ILT nor with any of the field samples. Clade 3 encompasses sample 21-T0048 (RFLP group 3), which is genetically most distant and clustered separately from all other field samples and the two vaccine strains. The samples from RFLP group 4 (19-T0190 and 22-T0009) and RFLP group 5 (20-V0139) are closely related and clustered in clade 4.

The separate phylogenetic analysis of the three individual genes, revealed that sample 21-T0048 consistently forms a separate clade for each gene analysed, making it the least genetically related to the other strains (Fig. 4B, C, D). Based on the ICP4 nucleotide sequence (Fig. 4B) the samples are divided into four clades that are very similar to those in the phylogenetic analysis of the three concatenated genes (ICP4, UL36, TK; Fig. 4A). The UL36 analysis resulted in the most diverse clustering of ILTV samples (Fig. 4C). Although four major clades are apparent, the grouping within these clades differs from the ICP4 analysis and the concatenated sequences. Notably, the two vaccine strains cluster in one clade, however, they are not identical. The TK gene exhibits the highest nucleotide sequence identities but sample 21-T0048 remains the most distant (4 SNPs, 99.63 % nucleotide identity as compared to Nobilis®-ILT), forming a separate clade (Fig. 4D).

When aligning the three concatenated gene sequences with the respective sequences of ILTV strains from other countries worldwide (Supplementary Table 1, Fig. 5), the Swiss wild type strain 21-T0048 assigned to clade 3 clusters with a Canadian field isolate (CA-AB-S20) and a Russian field isolate (RU-2009-1634AN-20), but also with Australian- originated CEO vaccine strains (SA2 and A20). The other Swiss wild type strains (19-T0190, 20-V0139, 22-T0009) from clade 4 clustered with various wildtype ILTV from USA (US_J2), Canada (CA-QC-2175807), Australia (AU_CSW-1, AU_V199), and Peru (PE_V-FAR043). The Swiss clade 1 strains clustered either with CEO revertant strains (19-T0500, 20-T0305, 21-T0346) or CEO vaccine like strains (21-T0316). The classification made here refers to the genotyping introduced by Oldoni and Garcia based on their PCR- RFLP analyses (Oldoni and García, 2007): Genotypes I, II, III include TCO vaccine and TCO vaccine-like viruses, genotype IV corresponds to CEO vaccine and CEO vaccine-like viruses and genotypes VI - IX represent wild-type viruses.

4. Discussion

In this study, ILTV samples from Switzerland were genetically characterized. The samples were collected over a period of almost 10

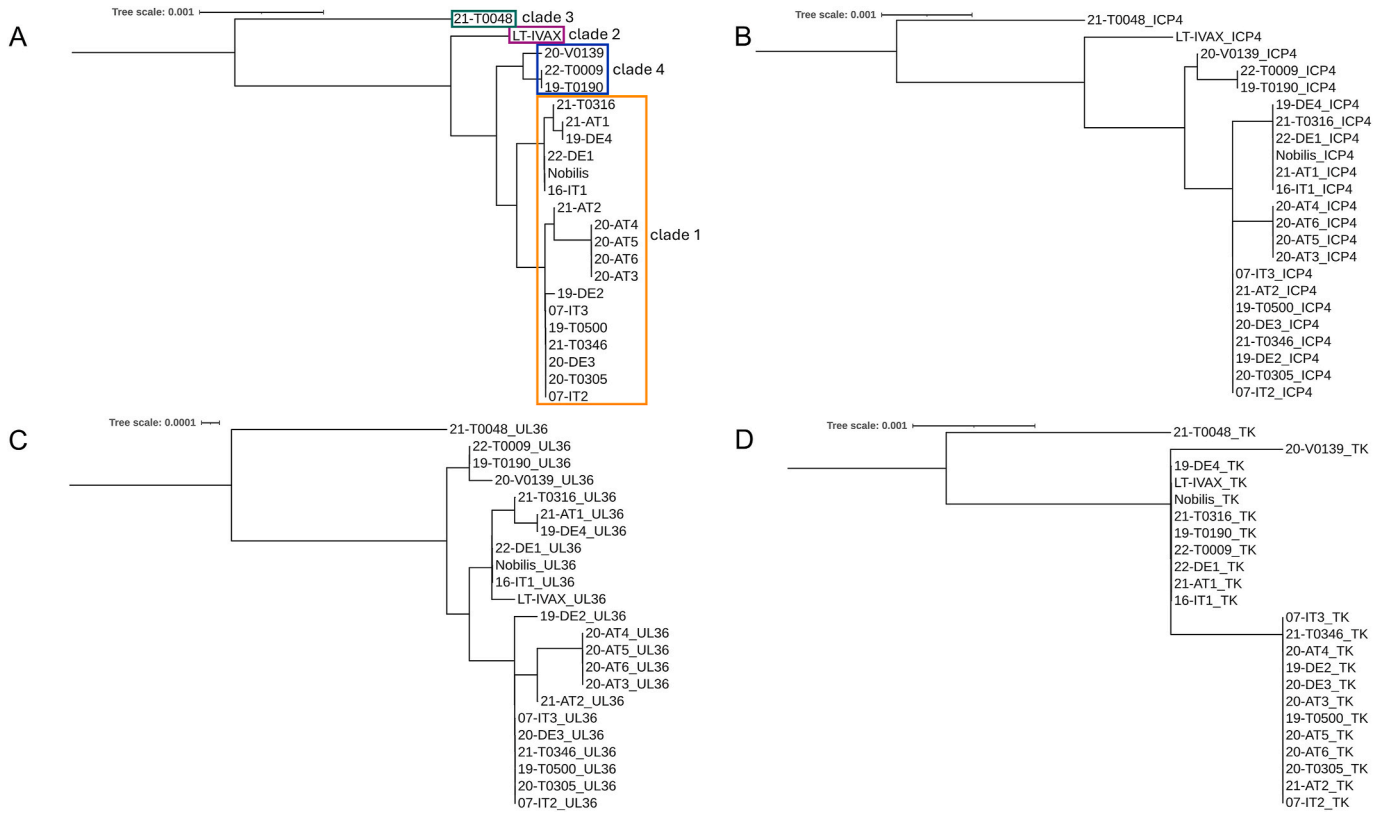

Fig. 4. Midpoint-rooted trees of aligned concatenated nucleotide sequences of all three concatenated genes (A) and sequences of ICP4 (B), UL36 (C), TK (D) separately of selected Swiss isolates, two vaccine strains and isolates from neighbouring countries. Boxes mark the different clades. Yellow, clade 1; violet, clade 2; green, clade 3; blue, clade 4.

years (2013–2022) and analysed using PCR-RFLP and multiple gene sequencing. As reference two vaccine virus strains were included. Both vaccine strains (CEO and TCO) could be differentiated in our study and were assigned to group 1 and 2. The restriction analysis of the ICP4 fragment of LT-IVAX® and Nobilis® ILT with *HinP1*I as also examined by Chacón et al. yielded comparable results (Chacón et al., 2010). However, they found no differences in the restriction pattern when *Hae*III was used, in contrast to the results presented here. Because the two largest fragments only differ by just about 40 base pairs, this discrepancy is likely due to the method of fragment separation. Chacón et al. separated the fragments using a 0.8% agarose gel, whereas we used a capillary electrophoresis system, which allows better separation.

The RFLP analysis of the samples from 21 ILT outbreaks in Switzerland revealed five different groups with two thirds (n = 14) of the samples belonging to CEO vaccine-like group 1. The other seven samples were assigned to four different groups (4x group 4, 1x group 3, 5, 6) which were classified as non-vaccine-like by RFLP analysis. After RFLP-based distance calculations it is apparent that RFLP group 1 and 6 and RFLP group 4 and 5 are genetically close. This was also confirmed by the sequence analysis (Fig. 4), which revealed four main clades and resembled the clustering of the four main groups from the dendrogram (Fig. 2). Clade 1 corresponds to RFLP group 1 and 6, encompassing Nobilis® ILT and CEO vaccine-like strains that were all closely related, but not identical to Nobilis® ILT. This clade also includes all samples from neighbouring countries. As in the dendrogram, the RFLP group 6 isolate 21-T0346 clustered with RFLP group 1 isolate 19-T0500, both being part of clade 1. These two samples only differed in the RFLP analysis of the ICP18.5 fragment digested with *Hae*III. Since ICP18.5 was not included in the sequence analysis, this difference was not apparent. As in the RFLP analysis, LT-IVAX® also formed its own group based on the sequence analysis (group 2, clade 2 respectively). None of the field samples are closely related to this TCO vaccine strain, which can be explained by the fact that LT-IVAX® is not used in Europe (García and Zavala, 2019). The only sample in clade 3 (21-T0048) was genetically most distant from all other samples which is consistent with the RFLP analysis where it showed different patterns for all analysed fragments and restriction enzymes used. Interestingly, this isolate originates from one of a total of three outbreaks in commercial holdings. The other two samples (17-T0583, 19-T0500) from commercial poultry were assigned to RFLP group 1 or cluster 1. The two RFLP group 4 samples (19-T0190, 22-T0009) and the RFLP group 5 sample (20-V0139) together formed clade 4, whereby both RFLP group 4 samples are identical and the RFLP group 5 sample forms a separate branch. This also correlates with the RFLP analysis, where group 4 and 5 differed only in one restriction pattern (*Msp*I) of the UL47/gG fragment.

RFLP analysis and multiple gene sequencing complement each other well. The great advantage of the sequence analyses lies in the precise differentiation of the individual strains within a group, which makes it evident that most of the vaccine-like samples are only similar and not identical to Nobilis® ILT. This is consistent with a study from Italy, in which the field samples likewise could only be distinguished from the CEO vaccines by partial sequencing (Moreno et al., 2010). Two of the Italian samples were additionally included in the multiple gene sequencing in this study, in which they also belong to clade 1 (CEO vaccine-like) and cluster with two samples from Switzerland (21-T0346, 19-T0500). In addition, the sequencing of two Swiss samples (19-T0500, 20-T0305) from Ticino revealed that they were identical for the genes examined (ICP4, TK, UL36). The two outbreaks occurred only seven months apart and at a geographical distance of just 3 km, which suggests that the same ILTV strain was responsible for both outbreaks.

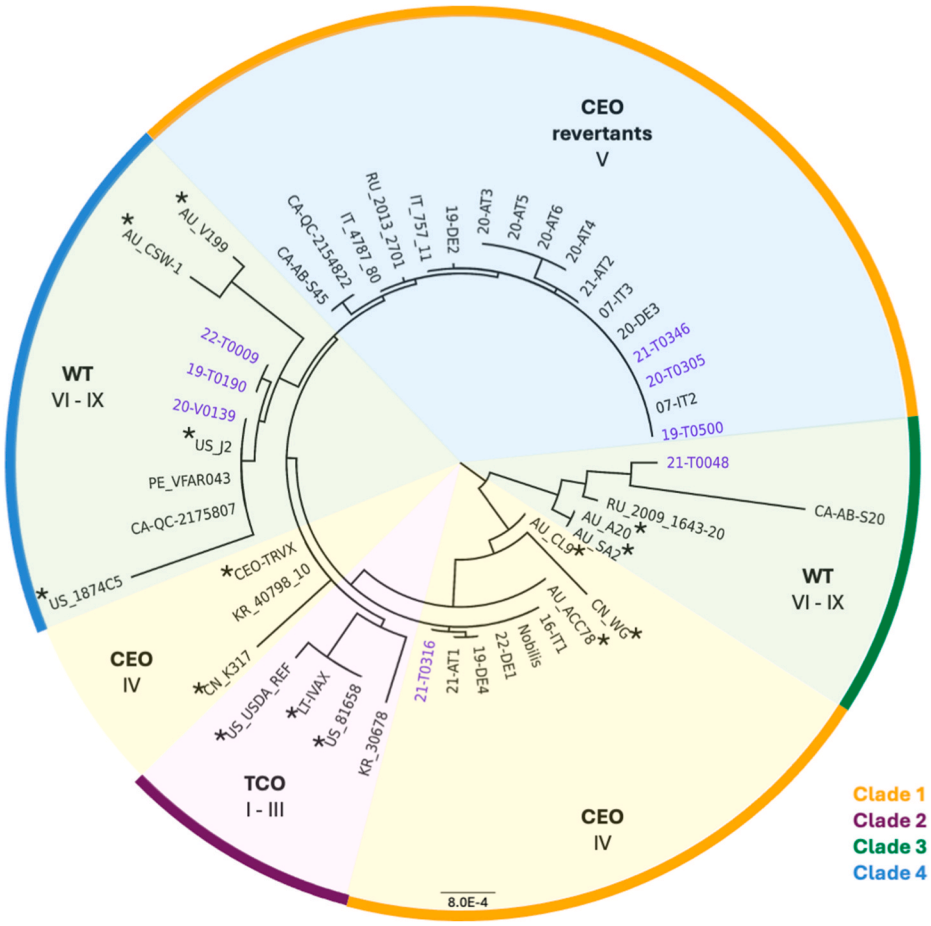

Fig. 5. Circular midpoint-rooted trees of aligned concatenated nucleotide sequences of all three concatenated genes (ICP4, UL36, TK) including the respective sequences from ILT viruses worldwide. Ring: Clades as assigned in this study (Yellow, clade 1; violet, clade 2; green, clade 3; blue, clade 4). Shaded parts of the circle correspond to the classification introduced by Garcia and Oldoni and previously used by Perez Contrera (Pink, TCO like strains incl. genotype I, II, III, Yellow, CEO like strains incl. genotype IV; Blue, CEO revertants incl. genotype V; Green, wildtype incl. genotype VI – IX). Swiss strains are written in blue. Asterisks mark strains, which are classified by further genotyping systems (Supplementary Table 1).

Analysis of the RFLP and multiple gene sequencing results, demonstrated that only six outbreaks were caused by non-vaccine or wild-type viruses (RFLP group 3, 4, 5/clade 3, 4). The majority, 15 out of the 21 ILT outbreaks, were attributed to CEO vaccine-like strains. In terms of the geographical distribution of the samples analysed, outbreaks caused by vaccine-like strains were predominantly found in areas close to the borders, while outbreaks caused by wild-type viruses occurred in the centre of Switzerland. An explanation for the predominance of CEO vaccine-like strains could be private importation of hobby chickens/fancy fowl from neighbouring countries that are using live attenuated vaccines. Once in Switzerland, these live vaccine viruses can spread to unvaccinated poultry. Another possible explanation is the illegal importation and use of these vaccines. In addition, it is possible that ILTVs are indirectly transmitted across borders *via* inanimate vectors such as equipment or vehicles (Beaudette, 1937; Mallinson et al., 1981). The spread of ILTV through the transportation of infected poultry can be largely ruled out, as infected flocks are immediately culled on the farm in question. In addition, there is a time limit that determines the maximum permitted transportation time, and thus the distance to the next slaughterhouse is limited. It should also be considered that the vaccine viruses were introduced into Switzerland in the past *via* one of the routes described above and have been circulating in the Swiss chicken population ever since. This hypothesis is supported by an earlier study (Neff et al., 2008) which found that 43 out of 48 samples from Switzerland from 1973 to 2007 were considered vaccine-like in both, RFLP analysis and sequence analysis of the TK gene. However, they could not differentiate between the CEO vaccine Nobilis® ILT and the TCO vaccine LT-IVAX® and the five (~10 %) non-vaccine-like viruses from the study by Neff et al. all belonged to the same group, which may be due to the sole analysis of the TK gene. In contrast, we found six (~30 %) non-vaccine-like viruses that could be assigned to three different RFLP groups or two clades and both vaccine viruses clustered in separate RFLP groups and different clades. This discrepancy in typing can be explained by the higher resolution power of the present study, which in total analysed 5 different genes, three by RFLP and three by sequencing (one gene was the same for RFLP and sequencing), compared to a single gene used by Neff et al. The introduction or evolution of new, non-vaccine-like viruses is possible too, but since re-analysis of the old Swiss samples is not possible, it will be difficult to prove.

While both vaccine-like and wild-type ILT viruses are present, pursuing a "non-vaccination strategy" continues to make sense even if ILTV vaccines are used in neighbouring countries. In Switzerland, ILT outbreaks are rare in commercial layer flocks, completely absent in broilers, and typically occur in small backyard holdings. However, it is important to consider that the actual number of ILTV outbreaks in hobby farms might be significantly higher, as only cases sent to laboratories for testing are diagnosed. Since vaccines are usually sold in large quantities, the question remains whether a cost-benefit analysis would be in favour of vaccinating small backyard flocks.

Comparison of the Swiss ILTV strains with ILT viruses from other countries largely supports the classification presented in this study. The CEO vaccine-like strains cluster with other CEO vaccine-related ILT

viruses, while the strains identified as wild-type (19-T0190, 20-V0139, 21-T0048, 22-T0009) group with international wild-type viruses. Notably, the two Australian vaccine strains, SA2 and A20, deviate from this pattern and, as previously demonstrated, cluster with some wild-type strains rather than with other CEO vaccine strains (Perez Contreras et al., 2020; Spatz et al., 2019).

However, comparing ILT viruses across different countries is challenging due to the lack of complete genome sequences and a standardized classification method. In the USA and Canada, ILTV genotyping primarily relies on the PCR RFLP system introduced by Oldoni and García, which classifies ILTV strains into nine genotypes (Oldoni et al., 2008; Oldoni and García, 2007). This method evolved into a multi-allelic amplicon-sequencing assay that divides the isolates into seven instead of nine groups, but otherwise agrees with the genotyping results obtained by PCR RFLP by Oldoni and García. In an attempt to provide a simple system to classify ILTV strains worldwide, this assay was further refined into a single allelic amplicon-sequencing assay and classification system which divides the US strains into five genotypes (Spatz et al., 2019), which we also indicated (Fig. 5, Supplementary Table 1). This single allelic amplicon-sequencing assay also aligns quite good with the previously developed genotyping method from Oldoni and Garcia at least when analysing ILTV strains from the USA (Oldoni and García, 2007), but it does not apply very well to Australian strains. For these, a classification system also based on PCR RFLP, but not comparable with the Oldoni and Garcia nomenclature, was introduced in 2006 and expanded to new strains in 2011 (Supplementary Table 1 (Blacker et al., 2011; Kirkpatrick et al., 2006)). Argentina also employed a PCR RFLP-based classification (Craig et al., 2017). Additionally, a multi-allelic amplicon-sequencing assay has been used to classify ILTV strains from various countries worldwide (Choi et al., 2016).

To improve our understanding of the global distribution and epidemiology of various ILTV genotypes, establishing a standardized classification system is essential. With the increasing accessibility of whole genome sequencing, developing a nomenclature based on full-length genome sequences would be beneficial, as this approach offers the highest accuracy for genotyping.

5. Conclusion

In conclusion, the current study shows that in Switzerland, where vaccination against ILT is *de facto* prohibited, both vaccine-like and wild-type ILT viruses are present. A combination of RFLP and multiple gene sequencing proved to be a useful approach for accurately differentiating the ILTV samples. Nevertheless, an attempted comparison with ILTV strains from other studies clearly shows that a uniform system for genotyping ILTV is urgently needed to better compare ILTV strains occurring worldwide.

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CRediT authorship contribution statement

Sonja Hermann: Writing – original draft, Visualization, Investigation, Data curation. **Marc J.A. Stevens:** Writing – original draft, Formal analysis, Data curation. **Brigitte Sigrist:** Investigation. **Ivana Bilic:** Writing – review & editing, Resources. **Sarah Albini:** Writing – review & editing, Resources, Conceptualization. **Nina Wolfrum:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (GPT-3.5) in order to improve readability of the final draft. After using this tool, the authors carefully reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2024.110217.

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