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# Evolutionary and temporal dynamics of emerging influenza D virus in Europe (2009–22)

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## Abstract

Influenza D virus (IDV) is an emerging influenza virus that was isolated for the first time in 2011 in the USA from swine with respiratory illness. Since then, IDV has been detected worldwide in different animal species, and it was also reported in humans. Molecular epidemiological studies revealed the circulation of two major clades, named D/OK and D/660. Additional divergent clades have been described but have been limited to specific geographic areas (i.e. Japan and California). In Europe, IDV was detected for the first time in France in 2012 and subsequently also in Italy, Luxembourg, Ireland, the UK, Switzerland, and Denmark. To understand the time of introduction and the evolutionary dynamics of IDV on the continent, molecular screening of bovine and swine clinical samples was carried out in different European countries, and phylogenetic analyses were performed on all available and newly generated sequences. Until recently, D/OK was the only clade detected in this area. Starting from 2019, an increase in D/660 clade detections was observed, accompanied by an increase in the overall viral genetic diversity and genetic reassortments. The time to the most recent common ancestor (tMRCA) of all existing IDV sequences was estimated as 1995–16 years before its discovery, indicating that the virus could have started its global spread in this time frame. Despite the D/OK and D/660 clades having a similar mean tMRCA (2007), the mean tMRCA for European D/OK sequences was estimated as January 2013 compared to July 2014 for European D/660 sequences. This indicated that the two clades were likely introduced on the European continent at different time points, as confirmed by virological screening findings. The mean nucleotide substitution rate of the hemagglutinin-esterase-fusion (HEF) glycoprotein segment was estimated as  $1.403 \times 10^{-3}$  substitutions/site/year, which is significantly higher than the one of the HEF of human influenza C virus ( $P < 0.0001$ ). IDV genetic drift, the introduction of new clades on the continent, and multiple reassortment patterns shape the increasing viral diversity observed in the last years. Its elevated substitution rate, diffusion in various animal species, and the growing evidence pointing towards zoonotic potential justify continuous surveillance of this emerging influenza virus.

**Key words:** cattle; epidemiology; influenza D virus; virorelevance; swine; zoonosis; virus evolution; molecular clock.

## Introduction

Influenza D virus (IDV) was discovered in 2011 (Hauser et al. 2013) and classified within the *Orthomyxoviridae* family in 2016 under the *Deltainfluenzavirus* genus. This family includes three other genera of flu viruses: *Alphainfluenzavirus* (comprising influenza A viruses, IAVs), *Betainfluenzavirus* (comprising influenza B viruses, IBVs), and *Gammainfluenzavirus* (comprising influenza C viruses, ICVs). All influenza A, B, and C viruses infect humans and different animal species and represent a group of viruses with a complex ecology. Indeed, IAV can infect birds and mammals, such as swine (Chauhan and Gordon 2022), horses (Sack et al. 2019),

dogs (Borland et al. 2020), marine mammals (Webster et al. 1981; Fereidouni et al. 2014), and bats (Tong et al. 2012; Tong et al. 2013). Importantly, it is also responsible for annual influenza epidemics in humans, together with IBV, and pandemics with high fatality in the past (Kilbourne 2006). IBV and ICV mainly infect humans, but IBV was also detected in seals (Osterhaus et al. 2000) and ICV in pigs (Kimura et al. 1997) and cattle (Zhang et al. 2018; Nissly et al. 2020). The segmented genome of *Orthomyxoviridae* viruses enables them to undergo genetic reassortment when two viruses of the same genus infect the same cell (Lowen 2018; Trifkovic et al. 2021). The exchange of intact genes is a frequent evolutionary

mechanism for influenza viruses, giving rise to chimeric genomes that can result in an increase in viral fitness and cross-species transmission, as reported for the H1N1 IAV pandemic in 2009 (Smith et al. 2009). ICV is the most genetically similar virus to IDV, sharing approximately 50 per cent overall amino acid identity (Haue et al. 2013). ICV and IDV genomes are both organized into seven genomic segments. However, transmission electron microscopic tomography revealed that ICV and IDV virions tend to package eight ribonucleoprotein complexes (Nakatsu et al. 2018), similar to IAV and IBV. Both ICV and IDV only possess one surface glycoprotein named hemagglutinin-esterase-fusion (HEF), which is responsible for viral receptor recognition and binding to the host cell. The genomic segment coding for HEF is the most variable and is, therefore, the most frequently used in phylogenetic analyses for molecular typing of different strains.

So far, IDV or anti-IDV antibodies have been detected worldwide and in multiple hosts such as cattle (Chiapponi et al. 2016; Murakami et al. 2016; Luo et al. 2017; Oliva et al. 2019), swine (Foni et al. 2017; Zhai et al. 2017) and feral swine (Ferguson et al. 2018), sheep and goats (Quast et al. 2015; Oliva et al. 2019), horses (Nedland et al. 2018), camelids (Salem et al. 2017; Murakami et al. 2019), and hedgehogs (Oliva 2019), contrasting with the host range of other influenza viruses. IDV was first isolated from a swine displaying influenza-like illness symptoms (Haue et al. 2013); however, epidemiological studies revealed a higher prevalence in cattle compared to other species (Oliva et al. 2019; Gaudino et al. 2021), suggesting bovine as the primary virus host. Cattle were never suspected of being a host for influenza viruses (Sreenivasan et al. 2019), but recent studies on ICV and IDV prevalence in cattle suggested otherwise (Zhang et al. 2018; Nissly et al. 2020). Altogether, these data advocate for an underestimation of the role of cattle as a host for influenza viruses. IDV was also detected in bioaerosols in a poultry farm in Malaysia in 2018 (Bailey et al. 2020), raising new questions about an even wider host range. Increasing evidence suggests IDV spillover into the human population (White et al. 2016; Bailey et al. 2018; Borkenhagen et al. 2018; Choi et al. 2018; Trombetta et al. 2019) with yet unknown consequences for public health.

IDV origin remains unknown, and evolutionary analyses estimated the most recent common ancestor (tMRCA) of both ICV and IDV about 1,304–1,539 years ago (Sheng et al. 2014; Su et al. 2017). So far, two major circulating IDV clades (designated as D/OK and D/660) have been described in North America and Europe based on HEF diversity, and multiple reassortment events between these two clades were also detected (Collin et al. 2015; Chiapponi et al. 2019; Saegerman et al. 2022). In Europe, one genetically divergent clade was described in France in 2012 and Ireland in 2014, represented by D/bovine/France/2986/2012 and D/bovine/Ireland/007780/2014 strains (Ducatez 2015; Flynn et al. 2018). Divergent local clades are also present in other countries on other continents, such as in Japan and California (Murakami et al. 2016; Murakami et al. 2020; Huang et al. 2021). In addition to Italy, Ireland, and France, IDV or anti-IDV antibodies were also detected in Luxembourg (Snoeck et al. 2018), the UK (Dane et al. 2019), Switzerland (Studer et al. 2021), Sweden (retrospective serological survey of IDV among cattle in Sweden—Zohari, pers. comm.), and Denmark (Goetze et al. 2022). The clade D/OK was detected during the last decade of virological surveillance in Europe (2012–9) (Ducatez 2015; Chiapponi et al. 2016; Foni et al. 2017; Flynn et al. 2018). The D/660 clade was only recently detected in Italy for the first time in 2019 (Chiapponi et al. 2019), suggesting a more recent introduction of this latter clade. The current extent

of IDV infection spread on the continent, and its genetic diversity, is still poorly understood. In addition, the limited number of IDV sequences available so far has prevented the scientific community from the development of an official genotyping system for clade assignment of different IDV sequences. To better understand IDV prevalence in Europe, surveillance through molecular screening of bovine and swine clinical samples was carried out in several European countries in the last decade. New cohorts were tested for the presence of IDV in this study. Genetic population analyses and phylogenetic reconstruction based on published and newly generated sequences were carried out to assess the evolutionary dynamics of the novel pathogen on the continent and to estimate the date of emergence of the main lineages in Europe.

## Materials and methods

### IDV molecular screening

In France, RNA extraction was performed on 140 µl of the clinical sample with the QIAamp viral RNA minikit (Qiagen), following the manufacturer protocol, and stored at –80°C. IDV screening in clinical samples was performed by Quantitative reverse transcription PCR (RT-qPCR) using primers (0.8 µM of final concentration) and hydrolysis probe (0.2 µM of final concentration) as described in Haue et al. (2013) using the QuantiNova probe RT-PCR kit (Qiagen, Germany). The RT-qPCR reactions were carried out on a LightCycler ninety-six real-time PCR system (Roche, Switzerland) with the following cycling conditions: 45°C for 30 min, 95°C for 15 min, followed by forty cycles at 95°C for 5 s, and 60°C for 30 s. In Italy, IDV molecular screening was carried out as described in Faccini et al. (2017). In Luxembourg, RNA extraction was performed with the QIAamp viral RNA minikit (Qiagen). The presence of IDV in clinical samples was tested by real-time RT-PCRs by using the primers (0.4 µM of final concentration) and probe (0.15 µM of final concentration) as described in Haue et al. (2013) using the QuantiTect probe RT-PCR kit (Qiagen). Cycling conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by forty-five cycles at 95°C for 15 s, and 60°C for 40 s.

### Virus isolation

In France, attempts of virus isolation were made for samples with the lowest C<sub>q</sub> values on 70–80 per cent confluent human rectal tumour 18G (HRT-18G) (ATCC CRL-11663) cells and swine testis cells (ATCC CRL-1746) in twenty-four-well plates at 37°C and with 5 per cent of CO<sub>2</sub>. For viral isolation, two passages with 5 days of incubation per passage were performed in Dulbecco's modified Eagle's medium (Dutscher, France) in the presence of tosylsulfonil phenylalanyl chloromethyl ketone (TPCK) trypsin (1 µg/ml; Thermo Fisher Scientific, MA), amphotericin B (2.5 µg/ml; Sigma-Aldrich), BM-cyclin (15 µg/ml; Sigma-Aldrich), ciprofloxacin (10 µg/ml; Sigma-Aldrich, MO), and 1 per cent of penicillin-streptomycin (10,000 U/10 mg/ml, Pan Biotech, France). In Luxembourg, virus isolation was attempted on swine testis cells in 25-cm<sup>2</sup> flasks and six-well plates with or without TPCK trypsin, and no isolate was recovered after two blind passages. Sequencing was thus performed directly on the original material. In Italy, samples positive by real-time RT-PCR were tested for virus isolation in HRT-18G, without trypsin added to the medium, as previously described (Foni et al. 2017). Viral isolation was attempted by three passages with 5 days of incubation per passage, and viral growth of IDV was confirmed by hemagglutination test and by IDV sandwich virological enzyme-linked immunosorbent assay (ELISA) performed as described in Moreno et al. (2019).

## IDV complete genome sequencing

In France, seven French isolates were amplified using primers as described in [Ducatez \(2015\)](#) by a one-step RT-PCR kit (Qiagen). Amplicons were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) and sequenced with Sanger technology (Eurofins GATC). Obtained contigs were aligned with BioEdit v7.1 using ClustalW, and an additional fifteen IDV were sequenced using Illumina HiSeq sequencer. Contigs were assembled with D/bovine/France/5920/2014 as reference using Burrows-Wheeler Alignment tool v.0.7.12-r1039 implemented on Galaxy workbench ([Giardine et al. 2005](#)). In Italy, isolates (if available) or clinical samples were sequenced by the next-generation sequencing technique on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) as previously described ([Chiapponi et al. 2016](#); [Chiapponi et al. 2019](#)). Contigs from Italian strains were assembled using CLC Genomic Workbench v. 11 (Qiagen, Hilden, Germany) with D/swine/Oklahoma/1334/2011 as assembly reference. The strain from Luxembourg was amplified by overlapping nested RT-PCRs using one-step RT-PCR kit (first round; Qiagen), Platinum® Taq DNA Polymerase (nested; Life Technologies, Merelbeke, Belgium), and a combination of previously published ([Ducatez 2015](#)) and newly designed primers (sequences available upon request; detailed PCR conditions in [Snoeck et al. \(2013\)](#)). After purification, amplicons were sequenced in both directions on an ABI 3130 Avant capillary sequencer (Applied Biosystems) as previously described ([Snoeck et al. 2013](#)). Contigs of the IDV strain from Luxembourg were assembled using SeqScape v2.5 (Applied Biosystems) and D/swine/Italy/199724-3/2015 or D/swine/Italy/254578/2015 (for PB2 gene only) as references. The sequences generated in this study were deposited in GenBank and comprised thirty-five IDV sequences in cattle in France, Italy, and Luxembourg collected over years 2018–22, including twenty whole genomes (see [Supplementary Table S1](#) for details).

## Data set curation, phylogenetic reconstruction, and reassortment detection

All publicly available IDV full-length coding sequences were downloaded from the National Center for Biotechnology Information GenBank. Partial sequences or sequences derived from synthetic constructs were excluded from the analysis. Sequences were aligned with Clustal Omega ([Sievers et al. 2011](#)) on European Molecular Biology Laboratory (EMBL)-EBI search and sequence analysis tools ([Madeira et al. 2019](#)). The alignment for HEF comprised 142 sequences from years 2010 to 2022, and the number of sequences in the analysis for the other segments was 104 covering years 2010–20. In particular, the data set contained 133 bovine and 9 swine sequences derived from North America, China, and Europe. Phylogenetic trees were calculated for each genome segment with the maximum likelihood (ML) method using the nucleotide substitution model with the lowest Bayesian information criterion scores, as identified for each alignment in MEGA-X v10.1.7 software ([Kumar et al. 2018](#)). Tree robustness was assessed by 1,000 bootstrap replicates. The mean distance between different genetic groups was also carried out in MEGA-X v10.1.7 software, using the maximum composite likelihood method with gamma distribution rate (shape parameter = 1). Codon positions included were 1st + 2nd + 3rd + Noncoding. Reassortment events between the two main clades were inferred based on phylogenetic incongruence between tree topologies. Within-clade reassortments were searched using the Genetic Algorithm of Recombination Detection method ([Kosakovsky Pond et al. 2006](#)) in the Datamonkey server of HyPhy v2 ([Kosakovsky Pond, Frost, and Muse 2005](#); [Delpont et al. 2010](#)).

## Clade assignment for IDV sequences

As no official system to classify IDV strains into different clades is available to date, we adopted the following criteria to assign IDV strains to a clade using a method similar to those used for the classification of IAVs ([Smith and Donis 2014](#)) or Newcastle disease virus ([Dimitrov et al. 2019](#)):

1. Despite the presence of multiple strains that originated from reassortment events, we classified IDV strains based on the complete coding sequence of the HEF segment (1,992 nucleotides) due to its variability and biological function.
2. The analyses were carried out based on a data set containing sequences from all the different existing clades.
3. The division into different clades was done based on tree topology inferred by the ML method, and the tree topology of the clades was then confirmed by Bayesian Evolutionary Analysis Sampling Tree (BEAST) analysis.
4. A bootstrap value at the defining nodes of monophyletic groups of a minimum of 70 per cent was considered.
5. A monophyletic group was considered an IDV clade when its mean genetic distance to other clades was higher than 2.5 per cent. The mean genetic distance was computed using the maximum composite likelihood method with gamma distribution rate.
6. A genetic group was considered a sub-clade when nested within a major clade of IDV HEF, but the mean genetic distance between the two was lower than 2.5 per cent.

Using this method, we classified the HEF sequences currently available into eight clades and two subclades.

## Evolutionary rate and tMRCA estimation by Bayesian analysis

Complete coding HEF sequences ( $n = 142$ ) were used to estimate IDV evolutionary dynamics. Nucleotide substitution rates and evolutionary time scale of divergence of IDV strains were inferred using the year of sample collection as tip-calibrations in the lognormal relaxed molecular clock method under the Bayesian Markov chain Monte Carlo (MCMC) framework in BEAST v1.8.1 ([Suchard et al. 2018](#)). The Hasegawa–Kishino–Yano nucleotide substitution model and gamma as site heterogeneity were specified under a constant population size coalescent model, based on previous estimates for influenza viruses. The analysis was run across two separate partitions (the first and second codon positions in partition one and the third codon positions in partition 2). The analysis was run for  $10^8$  generations, sampling every 10,000 generations and removing 10 per cent ‘burn-in’. The distribution of priors was assessed using Tracer v1.6 ([Rambaut et al. 2018](#)). The maximum clade credibility trees with the mean tMRCA and their 95 per cent highest posterior density (HPDs) were summarized using the TreeAnnotator program included in the BEAST package and visualized in FigTree v1.4.3. A literature search was done to compare the evolutionary rate of IDV HEF glycoprotein with those of hemagglutinins (HA) of other influenza viruses. The inclusion criteria for IAV, IBV, and ICV evolutionary rate studies were the following: (1) for simplicity, analyses were carried out on influenza sequences only of human origin, (2) analyses were performed on HA segment for IAV and IBV and on HEF for ICV, (3) analyses were performed on data derived only from clinical samples (studies describing the evolutionary rate based on data produced in experimental models were excluded), and (4) evolutionary rates expressed as substitutions/site/year. The studies included in this

**Table 1.** Overview over IDV viro-prevalence in cattle in Europe.

Country	Year of sampling	Region/ department	Type of surveillance	Type of specimen	No. of positive samples/no. of collected samples (per cent)	No. of positive herds/total no. of sampled herds (per cent)	Clade detected	References
FR	2010–4	Saône-et-Loire	Passive	NS, BAL, lung fragments	6/134 (4.5)		D/OK D/France-2012	Ducatez et al. (2015)
FR	2013–4	Occitanie	Passive	NS, BAL	4/140 (2.9)	1/23 (4.3)	D/OK	This study
FR	2018	Côte-d'Or	Passive	NS	3/96 (3.12)		D/OK	This study
FR	2018	Occitanie	Active	NS	64/145 (44.1)	3/3 (100)	D/OK	This study
FR	2018	Occitanie	Active	NS	0/182	0/13		This study
FR	2018	Normandie	Active	NS	16/51 (31.3)			This study
FR	2019	Normandie	Active	NS	0/59			This study
IT	2014–6	Po Valley	Active	NS	52/744 (7.0)		D/OK	Rosignoli et al. (2017)
IT	2014–6	Po Valley	Active	Lung fragments	6/151 (4.0)		D/OK	Rosignoli et al. (2017)
IT	2014–6	Po Valley	Active	BAL	0/22 (0)			Rosignoli et al. (2017)
IT	2014–5	Po Valley	Passive	NS	2/150 (1.3)			Chiapponi, et al. (2016)
IT	2018–9	Po Valley	Passive	NS	92/664 (13.9)		D/OK, D/660	Chiapponi, et al. (2019)
IT	2018–9	Po Valley	Passive	Lung fragments	7/250 (2.8)		D/660	Chiapponi et al. (2019)
IT	2018–9	Po Valley	Passive	BAL	0/22 (0)			Chiapponi et al. (2019)
IT	2020–2	Po Valley	Passive	NS	44/719 (6.1)	35/270 (13.5)	D/660	This study
IT	2020–2	Po Valley	Passive	Lung fragments	3/126 (2.3)	3/131 (2.2)	NA	This study
LU	2016–21		Passive	NS, lung fragments	1/25 (4.0)		D/OK	This study
IE	2014–6	Whole country	Passive	NS	18/320 (5.6)		D/OK, D/France-2012	Flynn et al. (2018)
UK	2017–8	Northern Ireland	Passive	NS/trachea, lung fragments	9/104 (8.7), 5/104 (4.8)		D/OK	Dane et al. (2019)
DK	2015		Passive	BAL			D/OK	Goecke et al. (2022)
DK	2018–20		Active	NS		12/100	D/OK (2019–20), D/660 (2020)	Goecke et al. (2022)
CH			Passive	NS	31/764 (4.1)			Studer et al. (2021)

Abbreviations: FR, France; IT, Italy; LU, Luxembourg; IE, Ireland; UK, United Kingdom; DK, Denmark; CH, Switzerland. NS, Nasal swab; BAL, Bronchoalveolar lavage.

comparison are listed in [Supplementary Table S2](#). For statistical analysis, an unpaired parametric t-test with a false-discovery rate approach was performed on GraphPad Prism v9.3.1 (GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>).

## Results

### Frequency of IDV infection in cattle and swine in Europe

An overview of IDV viro-prevalence in cattle and swine in different European countries is available in [Tables 1](#) and [2](#), respectively. These tables include newly generated results and results from previously published data. IDV is more frequently detected in cattle than swine: IDV was detected in almost every cattle cohort tested ([Table 1](#)), while it was only detected at low prevalence rates in 6/12 swine cohorts ([Table 2](#)). In cattle, IDV has been present in France since at least 2011 ([Ducatez, Pelletier, and Meyer 2015](#)).

Our molecular epidemiological data show that IDV has continued to circulate since then, being detected in the 2017–8 and 2018–9 winter seasons. In particular, high IDV shedding was detected in three veal calves' farms (named A, B, and C, respectively) in February and March 2018 in the Occitanie region. While in Farms A and C, the animals displayed no to limited respiratory signs with a positivity rate ranging from 13 per cent (Farm C) to 31 per cent (Farm A), IDV was isolated during a respiratory outbreak in Farm B. Animals displayed mild to severe respiratory signs. In the second cohort of nasal swabs collected from thirteen farms in the winter season of 2018–9 in the same region, the samples tested negative for IDV presence. IDV was also detected in the Normandie region and the Côte-d'Or department (sample CO-E2256.01), where the virus presence was never described before. In Italy, IDV was detected for the first time in 2014 ([Rosignoli et al. 2017](#)) and in almost every cohort tested since then, as well as in a recent cohort from 2020 to 2022. Despite previous serological results

**Table 2.** Overview over IDV viro-prevalence in swine in Europe.

Country	Year of sampling	Region/ department	Type of surveillance	Type of specimen	No. of positive samples/no. of collected samples (per cent)	No. of positive herds/total no. of sampled herds (per cent)	Clade detected	References
FR	2015–8	Whole country	Passive	NS	0/452 (0)	0/137 (0)		Gorin et al. (2019)
IT	2013	Po Valley	Passive	NS	0/32 (0)			Foniet al. (2017)
IT	2014	Po Valley	Passive	NS	0/22 (0)			Foni et al. (2017)
IT	2014–5	Po Valley	Passive	NS	1/150 (0.7)		D/OK	Chiapponi et al. (2016)
IT	2015–6	Po Valley	Passive	NS	14/350 (4.0)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2015–6	Po Valley	Passive	Lung fragments	3/361 (0.8)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2015–6	Po Valley	Passive	Oral fluids	4/134 (3.0)	9/448 (2)	D/OK	Foni et al. (2017)
IT	2017–22	Po Valley	Passive	NS, lung fragments	3/594 (0.5)	3/263 (1.1)	NA	This Study
SE	2014–5		Active	NS	0/330 (0)	0/22 (0)		This study
LU	2009		Active	NS	0/232 (0)	0/56 (0)		Snoeck et al. (2018)
LU	2014–5		Active	NS	3/427 (0.7)	2/36 (5.6)	D/OK	Snoeck et al. (2018)
LU	2018–21		Passive	Lung fragments	0/23 (0)			This study
Twelve countries	2015–7		Passive	NS, lung fragments, oral fluids, BAL	1/4033 (0.02)	1/707 (0.14)	D/OK	Henritzi et al. (2019)

Abbreviations: FR, France; IT, Italy; LU, Luxembourg; SE, Sweden; NS, Nasal swab.

indicating IDV circulation in Luxembourg cattle (Snoeck et al. 2018), IDV was not molecularly detected at high prevalence in this geographic region. In a cohort of twenty-five samples submitted for IDV diagnostic between 2016 and 2021, one was positive for IDV in 2018. The presence of IDV in swine has been investigated to a lower extent than in cattle. IDV was not molecularly detected in France so far but was detected in several swine cohorts in Italy. In Sweden, no positive samples were detected in a cohort of 330 swine samples collected in 2014–5. In Luxembourg, active surveillance previously detected IDV in swine in 2014–5, while none of the twenty-three samples screened for passive surveillance in 2018–21 were positive. Supplementary Fig. S1 shows the genetic distances between different European IDV sequences.

### Genetic diversity and phylogeny of IDV in Europe

In recent years, different provisional names have been used to refer to emerging circulating lineages of IDV. In this work, we therefore adopted different criteria to assign IDV strains to specific clades based on the complete coding sequence of the HEF segment. A summary of mean genetic distance within different clades is available in Fig. 1 and Supplementary Fig. S2.

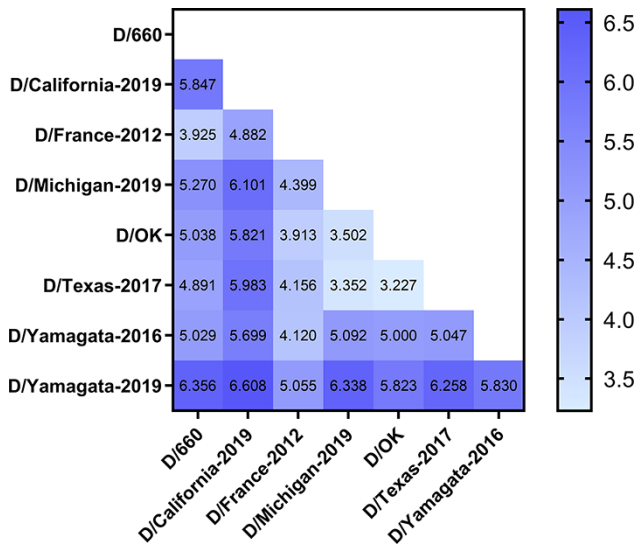
All IDV sequences obtained from 2011 to 2018 samples collected in France and Luxembourg belong to the D/OK clade, as shown in Fig. 2. Sequences belonging to the D/France-2012 clade were not detected in cohorts used in this study, suggesting a possibly minor circulating clade compared to the D/OK and D/660 clades. In Italy, ten new IDV sequences from nasal swabs collected from 2020 to 2022 were obtained. Starting in 2019, only clade D/660 sequences were detected, as previously

reported (Chiapponi et al. 2019). Similarly to Italy, an increase in D/660 group detection was also observed starting in 2020 in Denmark (Goecke et al. 2022). The topology inferred by the BEAST analysis confirmed the clade assignment inferred by the phylogenetic reconstruction carried out by the ML method (Supplementary Fig. S3).

The estimated mean within-group distance of European D/OK sequences is 0.84 per cent, whereas for European D/660 is calculated as 0.59 per cent, whereas the mean within-group genetic distance of all existing D/OK sequences at a global level is 1.26 per cent and 1.46 per cent for clade D/660. Intra-farm genetic diversity analyses were carried out on IDV sequences obtained for two veal farms in France (Farms A and B), where multiple genomes were obtained from the same herd. The genetic distance within the same farm was overall limited, ranging from 0.0 per cent to 0.31 per cent. A higher genetic distance was found between the two different farms, ranging from 0.11 per cent to 0.71 per cent (Supplementary Table S3). No within-clade reassortment events were detected in IDV sequences included in the data set. However, we detected reassortments within the two main clades (D/OK and D/660) for the Italian sequences for the Nucleoprotein segment, similar to what was previously described in the same country (Chiapponi et al. 2019). Phylogenetic trees obtained from the other six IDV genomic segments are available in Supplementary Fig. S4.

### IDV evolutionary rate

We used a molecular clock approach to calculate IDV evolutionary rate for the HEF gene. The rate of nucleotide substitution of all existing IDV sequences was estimated as an overall

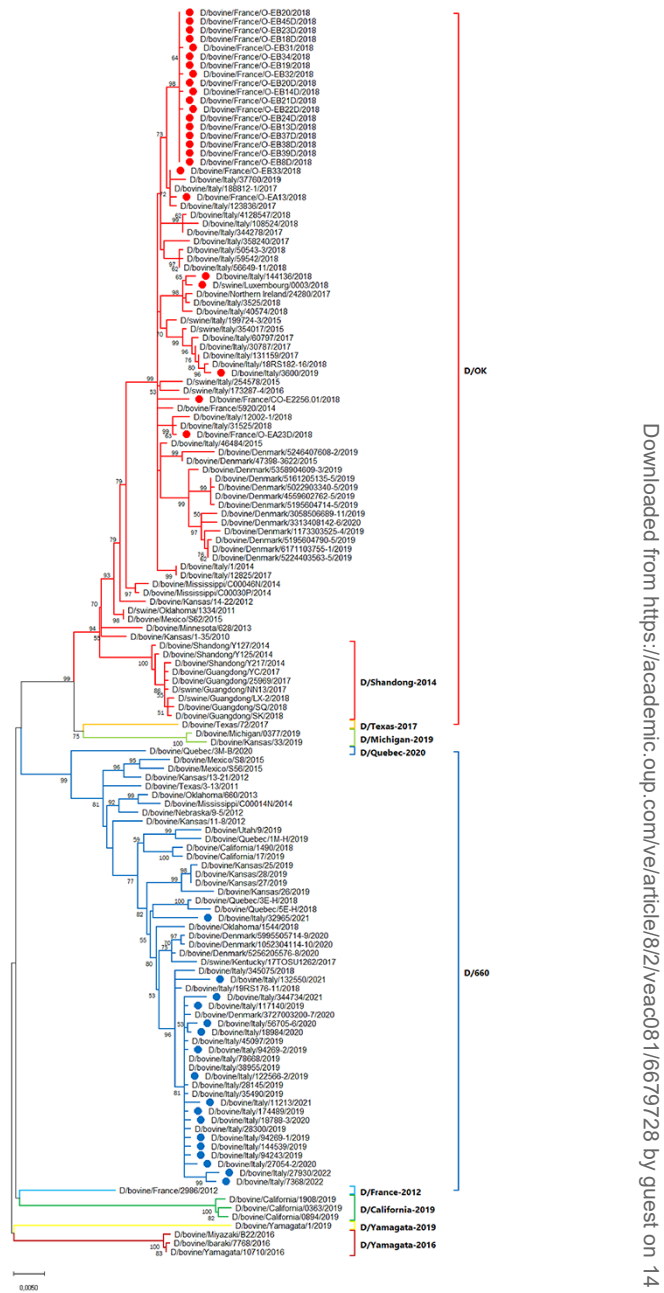


**Figure 1.** Matrix showing the mean distance between different IDV clades. As the mean genetic distance between D/OK and D/Shandong-2014 was  $\leq 2.5$  per cent, this latter was considered a D/OK subclade in phylogenetic analyses. Similarly, D/Quebec-2020 was considered a D/660 subclade. The mean distance between different genetic groups was calculated using the maximum composite likelihood method with gamma distribution rate (shape parameter = 1). In the matrix, the mean distance is expressed as a percentage. The colour code for the mean distance is in the function of increasing genetic distance (light to dark blue).

mean of  $1.403 \times 10^{-3}$  substitutions/site/year (95 per cent HPD interval:  $1.156 \times 10^{-3}$ – $1.633 \times 10^{-3}$ ). As reconstructed by BEAST analysis, an increasing pattern of diversification of IDV lineages was highlighted, especially between 2013 and 2018, when the majority of sequences were retrieved (Fig. 3A). The mean substitutions/site/year was similar for all IDV clades. However, it was higher for European D/OK (0.0017) compared to European D/660 (0.0014), probably due to the greater number of sequences available for European D/OK (Table 3). We then compared the IDV HEF rates of nucleotide substitution with those of HA of other influenza viruses reported in the literature (Fig. 3B; evolutionary rates of HA segments of IAV and IBV were included as a comparison to the evolutionary rates of ICV and IDV). The nucleotide substitution rate of IDV HEF glycoprotein was significantly higher than HEF of ICV ( $P < 0.0001$ ), and no significant differences were found with the HA of seasonal human H1N1 ( $P = 0.0792$ ), H3N2 ( $P = 0.0259$ ), and IBV ( $P = 0.0286$ ), probably due to the high variability of the estimated mutation rates among the studies.

**Dating the tMRCAs of IDV clades**

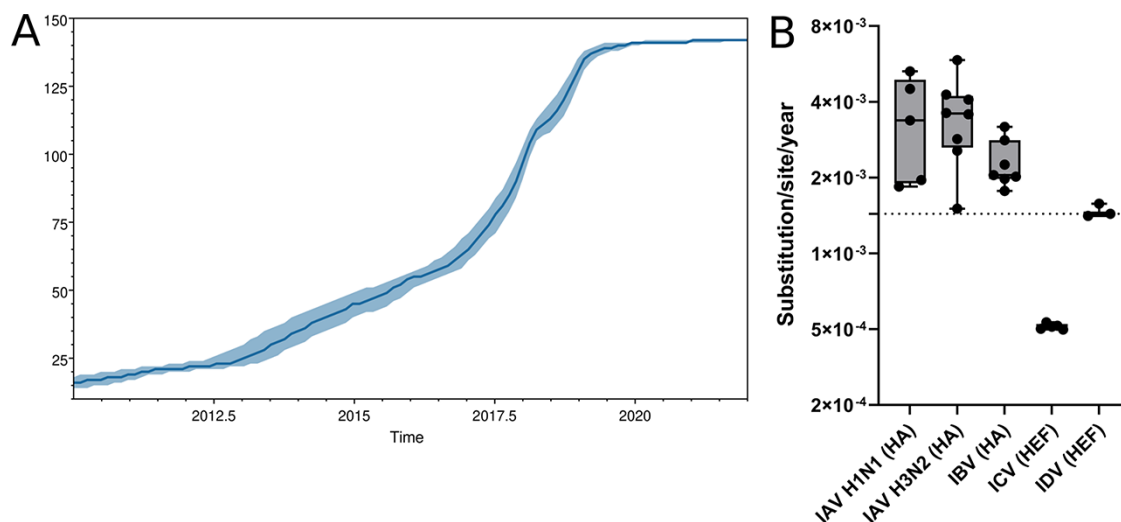
Based on HEF sequences, tMRCAs were inferred for different IDV clades and European clusters of both D/OK and D/660 clades by the Bayesian method using MCMC (Fig. 4; Supplementary Fig. S5 (equivalent to Te 4 but with sequences colouring based on their geographic origin); Table 3). The tMRCA of all existing IDV sequences was estimated as 1995 (95 per cent HPD interval: 1989–2000)—16 years before its discovery. The tMRCA was estimated as 2007 for both clades D/660 and D/OK. However, the mean tMRCA of European D/OK sequences was estimated to be January 2013 (95 per cent HPD interval from May 2012 to July 2013) and July 2014 for European D/660 (95 per cent HPD interval from July 2012 to May 2015). In addition, the tMRCA for D/France-2012 was estimated earlier, in 1998.



**Figure 2.** ML phylogenetic tree of complete HEF sequences showing the different clades and subclades of IDV. The new D/OK and D/660 European sequences generated in this work are displayed with a red and a blue dot. The tree was constructed using 1,000 bootstrap replicates. The scale bar represents the number of nucleotide substitutions/site/year.

**Discussion**

As the genetic diversity of IDV unfolds, the introduction of criteria for clade assignment becomes necessary. Therefore, in this work, we proposed standardized criteria that can be used to classify new strains based on their HEF sequence. Since IDV discovery, this emerging pathogen has been detected on almost all continents and in several animal species. Our virological screening confirmed previous serological results, where IDV circulation was described at a higher prevalence in cattle than in swine. Indeed, IDV was present in almost all bovine cohorts tested and all countries investigated in our study, whereas only a few swine samples tested



**Figure 3.** (A) Lineages-through-time plot as reconstructed by molecular dating analysis with BEAST and visualized in Tracer v1.7.2. The shading represents the 95 per cent HPD for the number of lineages (y-axis) distributed in time (x-axis). (B) Estimates of the nucleotide substitution rates/site/year for IDV HEF and the hemagglutinin of other influenza viruses (list of references available in [Supplementary Table S2](#)).

**Table 3.** tMRCA and mean evolutionary rates of the HEF gene for different IDV clades, as estimated by BEAST analysis. The estimated month is indicated after the year and the dot in the table.

Group	Mean tMRCA	95 per cent HPD	Node mean rate (s/s/y × 10 <sup>-3</sup> )
All IDV	1995.1	1989.1-2000.7	0.0014
D/Yamagata-2019	1997.1	1993.3-2001.7	0.0014
D/Yamagata-2016	2015.4	2015.1-2016.3	0.0014
D/France-2012	1998.1	1993.3-2002.2	0.0013
D/California-2019	2018.2	2017.8-2019.3	0.0015
D/Texas-2017	2006.8	2003.9-2010.3	0.0013
D/Michigan/2019	2006.8	2003.9-2010.3	0.0013
D/OK	2007.8	2006.5-2009.6	0.0014
European D/OK	2013.1	2012.5-2013.9	0.0017
D/660	2007.8	2006.5-2009.6	0.0015
European D/660	2014.7	2013.1-2015.8	0.0014

Abbreviation: s/n/y, substitutions/site/year.

positive for IDV. While the genetic diversity of IDV in swine seems to reflect the circulating cattle strains, IDV diversity in other hosts remains elusive, and future studies might also reveal the existence of distinctly evolving clades for IAV circulating in avian, swine, or humans. Therefore, the genetic thresholds proposed as part of those criteria will need to be monitored and possibly adapted in the future as a consequence of improved coverage of viral diversity at both geographic and host levels, as well as constant viral evolution.

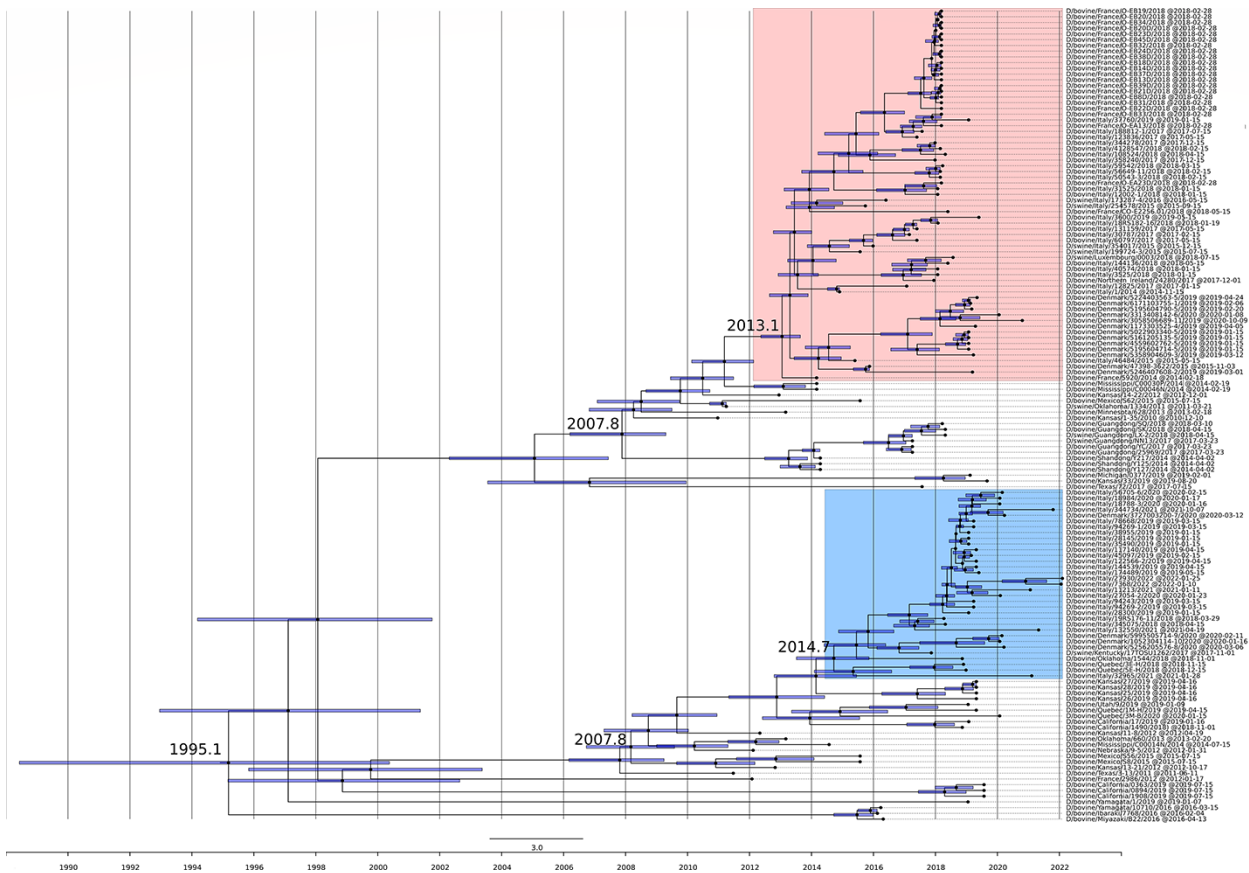
Our genetic analyses based on molecular screening and sequencing of recent cohorts showed an increase in IDV diversity in Europe throughout the years, resulting from a combination of genetic drift (mutations on the HEF glycoprotein), introduction of a new clade, and genetic shift (reassortment). In fact, the surveillance data collected from 2012 to 2019 only highlighted the presence of D/OK as the main circulating clade ([Ducatez, Pelletier, and Meyer 2015](#); [Chiapponi et al. 2016](#); [Foni et al. 2017](#); [Flynn et al. 2018](#)). A minor clade D/France-2012 was only detected in France in 2012 ([Ducatez, Pelletier, and Meyer 2015](#)) and Ireland in 2014 ([Flynn et al. 2018](#)), but the paucity of detections is probably due to

limited surveillance. The presence of the D/660 clade on the continent was observed for the first time in 2019 in Italy ([Chiapponi et al. 2019](#)) and then in Denmark in 2020 ([Goetze et al. 2022](#)). Since then, the relative frequency of D/OK versus D/660 detections shifted, and no new sequence belonging to D/OK was reported over the last 2 years in Europe. This suggests that D/660 could currently be the major circulating strain on the continent compared to the last decade. However, a systematic IDV surveillance in the majority of European countries is missing and could provide additional insight into the real genetic diversity on the continent. Reassortant viruses combining segments from the D/OK and D/660 clades were also observed for the first time in Europe and Italy in 2019. In North America, multiple reassortment patterns between different clades have been detected since 2015 ([Collin et al. 2015](#); [Saegerman et al. 2022](#)). This apparent difference in the time frame and frequency of reassortant detection could be due to (1) longer circulation of IDV on the American continent, (2) the greater size of cattle farms, (3) higher intensity of mixing animals from different farms, and/or (4) the higher number of exchanges between distant locations in Northern America, providing more opportunities for co-circulation of genetically different viruses in a single setting.

The mechanisms suspected to drive IDV evolution are already well described for other influenza viruses ([Guarnaccia et al. 2013](#); [Barbezange et al. 2018](#); [Kim 2018](#); [Linster et al. 2019](#)) and are associated with an increase in viral fitness and antibody escape ([Ma et al. 2015](#); [Rajão et al. 2015](#); [Pulit-Penalzo et al. 2018](#); [Gao et al. 2019](#)). However, the consequences of reassortment on viral fitness remain unknown for IDV. Our estimated evolutionary rates for the HEF gene are similar to what was previously described ([Su et al. 2017](#); [He et al. 2021](#)). Despite the genomic similarity between IDV and ICV, IDV seems to evolve faster than ICV. This could indicate that the novel IDV is still not fully adapted to the cattle population, from which the majority of sequences used in this study are derived. Sequences from animal species other than swine are currently missing and could provide additional insight into IDV evolution in other hosts.

To better understand the introduction dynamics of IDV in Europe, the tMRCA of European clusters was estimated through molecular clock analysis with the Bayesian method. The tMRCA of D/France-2012 was estimated as 1998, indicating that it was





**Figure 4.** tMRCAs of IDVs. The tree was generated using BEAST under a relaxed clock model and a constant coalescent tree prior. The nodes correspond to the mean tMRCAs, and the 95 per cent HPD interval is represented with blue boxes. The D/OK European strains are highlighted in pink, whereas the D/660 Italian strains are highlighted in blue. The scale bar represents the number of nucleotide substitutions/site/year.

probably the first clade present on the continent. However, it is difficult to draw a precise phylodynamic pattern for this clade, given the lack of related sequences. The tMRCAs of the two major circulating clades were different, being estimated as January 2013 for D/OK and July 2014 for D/660 viruses. This could indicate a first introduction of D/OK in Europe followed by a more recent independent introduction of clade D/660, which is also supported by virological results. The high nucleotide identity (99.55 per cent) between IDV detected in swine in the USA in 2017 (D/swine/Kentucky/17TOSU1262/2017) (Thielen et al. 2019) and in Denmark in 2020 (D/bovine/Denmark/5256205576-8/2020) (Goetze et al. 2022) suggests that the introduction of D/660 had potentially taken place from North America. Likewise, 99.9 per cent of nucleotide identity was found between the first isolated IDV (D/swine/Oklahoma/1334/2011) in the USA and some 2014–8 D/OK European sequences. This is also supported by the fact that the sister branches of both European clades are located in America. The lack of geographic segregation in these first years of the spread of clades D/OK and D/660 suggests several recent intercontinental spillovers. However, the mode of transmission from one continent to another remains undefined. Exchanges of livestock such as cattle or swine from one continent to another are virtually absent, suggesting the implication of another unknown host. IDV was shown to bind to the epithelial surface of other domestic and wild animals (Nemanichvili et al. 2022). Additional IDV permissive species are horses, shown to seroconvert and replicate the virus (Collin et al. 2015; Sreenivasan et al. 2022), and camelids (Salem et al. 2017; Murakami et al. 2019). While exchanges of

live horses between America and Europe take place (FAOstat), exchanges of camelids or horses between America and Africa, where IDV also circulates (Salem et al. 2017; Murakami et al. 2019; Sanogo et al. 2021), are not described (FAOstat). Given the extent and rapidity of IDV spread, the role of humans should also be considered. IDV was detected in urban environments such as a hospital emergency room bioaerosol (Choi et al. 2018), in an airport bioaerosol (Bailey et al. 2018), but also in human samples such as a nasal swab of a farmer working on a pig farm in Malaysia (Borkenhagen et al. 2018). Serologic evidence for IDV in humans remains of more difficult interpretation, as antibody cross-reaction with ICV was highlighted (Eckard 2016). However, the presence of anti-IDV antibodies was highlighted by hemagglutination inhibition assay and virus neutralization assay in a cohort of randomly selected human sera ( $n=1,281$ ) from two different geographic Italian regions from 2005 to 2017 (Trombetta et al. 2019). Interestingly, few sera were already positive in 2005 (5.1 per cent) with a constant increase in time (9.8 per cent in 2007, 24.1 per cent in 2010, 39.0 per cent in 2013, and 42.0 per cent in 2014) and a constant decrease starting from 2015 (21.8 per cent in 2015 and 7.9 per cent in 2017). The frequency of anti-ICV antibodies is generally high in the adult population (Sederdahl and Williams 2020). Therefore, an increase in the time frame as previously described is not likely attributable to an increase in anti-ICV antibodies in the general Italian adult population. In addition, anti-IDV antibodies were highlighted in four veterinarian sera collected in 2004 (4.9 per cent 4/82) in Italy (Trombetta et al. 2022), as well as at a higher prevalence in cattle-exposed workers (97 per cent) versus people

without cattle exposure (18 per cent) in Florida (White et al. 2016). While the presence of IDV in the human population has been highlighted, the precise role of humans remains yet unknown. The first description of IDV in the cattle population was assessed in 2003 in the USA by a serological study conducted on a cohort of sera collected from 1977 to 2010 (Eckard 2016). In addition, starting from 2005, an increase in IDV human seropositivity was highlighted (Trombetta et al. 2019). It is therefore possible that humans could have been exposed as accidental hosts following IDV circulation at high prevalence in cattle (zoonotic transmission), rather than a conversely species jump in the opposite direction (anthroponotic transmission) with a consequent adaptation in cattle. However, it is also possible that IDV circulation in humans started in other geographical areas before cattle and remained undetected for several years. IDV circulation in cattle before 2003 in other geographic areas is currently unknown, and serology studies using cohorts of human and cattle sera collected from the same time frame of the putative IDV spread, as well as serology studies on other animal species that were not considered so far, could help to provide a better understanding of the role of humans in the virus origin and transmission.

## Conclusion

Together, these global genomic data provide new insight into the different evolutionary dynamics exhibited by IDVs. Our molecular surveillance data confirm that the IDV is actively circulating in Europe, with an increased genetic diversity due to genetic drift, the recent introduction of the D/660 clade, and inter-clade reassortments. Extending surveillance geographically is required to understand the real prevalence of the virus in Europe and globally and obtain a better overview of its genetic diversity. Surveillance in cattle and other animal species could provide additional insight into IDV origins, evolution, and interspecies transmission. In particular, surveillance in human cohorts is warranted to assess their susceptibility to infection and their importance in IDV transmission.

## Supplementary data

Supplementary data are available at *Virus Evolution Journal* online.

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**Conflict of interest.** The authors declare no competing interests.

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