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Paper

Evolutionary Dynamics of Bluetongue virus serotypes 3, 4, and 8 circulating in Italy, 2024–2025

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Abstract

Bluetongue virus (BTV) continues to pose a major threat to ruminant health in Europe, where repeated introductions, the co-circulation of multiple serotypes and frequent reassortment shape its genomic diversity. During 2024–2025, Italy experienced a marked resurgence of bluetongue, driven mainly by BTV-3 and BTV-8. In this study, we performed whole-genome sequencing of BTV-3, BTV-4 and BTV-8 strains collected in Italy during 2024–2025 and integrated these data with representative genomes from Italy and Tunisia generated over the previous decade. A total of 47 BTV whole-genome sequences were analysed. Multiple reassortant genomic constellations were identified among BTV-3 and BTV-4 strains, reflecting extensive segment exchange between North African and European lineages. In contrast, all Italian BTV-8 genomes showed near-complete nucleotide identity with the contemporary BTV-8 FRA 2023 lineage, indicating nationwide circulation of a single strain. Despite its widespread diffusion, BTV-8 did not acquire heterologous genome segments, whereas its internal genes were frequently incorporated into BTV-3 and BTV-4 genomic backgrounds. These findings highlight Italy as a key convergence point for BTV lineages in the Mediterranean basin and underscore the value of whole-genome surveillance for tracking viral introductions and reassortment dynamics.

Keywords

BTV-3, BTV-4, BTV-8, Bluetongue, Bluetongue virus, Genomic characterization, Reassortment, Italy

Introduction

Bluetongue virus (BTV), the causative agent of bluetongue (BT) disease, is a *Culicoides*-borne orbivirus that affects domestic and wild ruminants and remains one of the WOAH-listed major infectious diseases with substantial economic impact (Coetzee et al., 2012; Mellor et al., 2000). The virus, a member of the family*Sedoreoviridae*, genus *Orbivirus* (https://ictv.global/report/chapter/sedoreoviridae/sedoreoviridae/orbivirus), possesses a genome composed

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of ten double-stranded RNA segments (Seg-1 to Seg-10) that encode seven structural proteins (VP1–VP7) and five non-structural proteins (NS1, NS2, NS3/NS3a, NS4 and NS5) (Ratinier et al., 2011; Roy, 2017; Schwartz-Cornil et al., 2008). To date, 36 serotypes for BTV have been described. These include 24 classical BTV serotypes and 12 that are considered atypical (Ries et al., 2020; Ries et al., 2021). Only classical serotypes have the potential to produce the disease (Jimenez-Clavero, 2012) and largely fail to confer cross-protective immunity (Fay et al., 2021; Martinelle et al., 2018).

Although point mutations contribute to genetic diversification, BTV evolution is primarily driven by genomic reassortment, a process inherent to segmented RNA viruses and well documented in both experimental and field settings (Carpi et al., 2010; Nomikou et al., 2015).

Because BTV transmission depends on competent *Culicoides* vectors, its distribution is closely linked to climatic conditions and vector ecology (Maclachlan and Guthrie, 2010; Verwoerd and Erasmus, 2004). The Mediterranean basin, characterized by intense animal movement, suitable environmental conditions for vector proliferation, and strong climatic connectivity between continents, has historically functioned as a key interface for the introduction and northward spread of BTV into Europe (Calistri et al., 2004; Hammami, 2004). Repeated incursions of different serotypes—including BTV-1, BTV-2, BTV-3 and BTV-4, from North Africa into southern Europe have been documented, confirming the central Mediterranean as a persistent hot-spot of viral exchange between African and European ecosystems. Within this ecological corridor, Italy and Tunisia have played a historically important role in surveillance and molecular epidemiology, allowing reconstruction of evolutionary linkages between outbreaks on both sides of the basin, as previously demonstrated for BTV-1, BTV-2, BTV-3, BTV-4 and recently also for epizootic hemorrhagic disease virus serotype 8 (Cappai et al., 2019; Lorusso et al., 2013; Lorusso et al., 2014a; Lorusso et al., 2017; Lorusso et al., 2023; Martinez et al., 2025; Sghaier et al., 2022).

Over the past two years, Italy has undergone an intense re-emergence of bluetongue (BT), characterized by the simultaneous circulation of several serotypes, including BTV-3 and BTV-8 and with a lesser extent, BTV-4. Moreover, in September 2025, BTV-5 was detected in Sardinia, marking its first occurrence in Europe (Bollettino Epidemiologico Nazionale Veterinario, accessed December 10th, 2025). BTV-5 is not further addressed in the present study, as its emergence will be comprehensively described in a dedicated accompanying paper entitled "*Emergence of Bluetongue virus serotype 5 in Sardinia, Italy, 2025.*"

BTV-3 first entered Europe in 2017, when it was detected in Sicily and shown to be closely related to a Tunisian strain identified one year earlier in Cap Bon (Lorusso et al., 2018; Sghaier et al., 2017). Although BTV-3 circulated in Sardinia at low levels between 2018 and 2023, it did not spread extensively until the marked epidemic expansion of 2024 (Bollettino Epidemiologico Nazionale Veterinario, accessed December 10th, 2025). More recently, a genetically divergent BTV-3 strain emerged in the Netherlands in 2023 (Holwerda et al., 2024) and subsequently spread across several European countries (Barros et al., 2024; Larska et al., 2025; Newbrook et al., 2025; Van Leeuw et al., 2025), further increasing the heterogeneity of circulating BTV-3 lineages.

BTV-8—first identified in northern Europe in 2006 and responsible for the most severe BT epidemic ever recorded (Elbers et al., 2008; Maan et al., 2008)—continues to circulate in France. However, in 2023, two genetically distinct BTV-8 lineages co-circulated: the long-established enzootic strain present since 2015 and a novel lineage of unknown origin, which rapidly became predominant (Gondard et al., 2024).

Before 2014, BTV-4 circulation in the Mediterranean area was broadly partitioned into two well-defined phylogenetic clades. One lineage, associated with the Eastern Mediterranean, comprised viruses historically identified in Greece (e.g., 1979, 1999 and 2000). A second lineage was circulating in the Western Mediterranean and included strains detected from 2004 onwards in North Africa (Morocco, Tunisia, Algeria) and in several European countries such as France (Corsica), Spain and Italy; these incursions were largely attributed to wind-borne dispersal of infected *Culicoides* from North Africa. This scenario changed in 2014 when Greece reported the emergence of a BTV-4 strain that was genetically divergent to either of the two established Mediterranean lineages. This "Balkan" strain spread very rapidly throughout south-eastern Europe and caused substantial outbreaks across the Balkan Peninsula. In the following years, viruses belonging to this lineage were also identified further west, including in parts of Italy, Corsica and mainland France, and later re-appeared in the Balkans during 2020 (reviewed in Romero-Trancon et al., 2021, and references therein).

Within this rapidly evolving context—defined by repeated viral introductions, the co-circulation of multiple serotypes and the increasing frequency of reassortment events—the systematic characterization of whole-genome constellations has become essential for understanding epidemic dynamics of newly emerging strains. Building on the analytical framework adopted in previous Italian–Tunisian comparative efforts, a comprehensive genomic assessment of recent

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strains is necessary to contextualize current outbreaks within the broader evolutionary landscape of the central Mediterranean.

This study therefore aimed to characterize the genomic constellations of BTV-3, BTV-4 and BTV-8 strains collected across several Italian regions in 2024–2025, integrating them with representative strains from Italy and Tunisia detected during the past decade. By providing an updated overview of the genetic diversity and reassortment patterns of the viruses currently circulating in Italy, this work contributes to a deeper understanding of the evolutionary forces shaping BTV dynamics at the Europe–North Africa interface.

Materials and methods

Specimen collection (2024–2025)

From July 2024 to December 2025, a total of 17,689 ruminant biological samples—mainly EDTA blood and spleen collected from cattle, sheep, and goats—were submitted to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM, Teramo, Italy) for confirmatory diagnosis of bluetongue virus (BTV), in accordance with current legislation (Nota DGSAF 17050 del 28/05/2024 - Febbre catarrale degli ovini (Bluetongue): orientamenti sulle misure di controllo e di gestione sul territorio nazionale e sulle attività di sorveglianza sierologica ed entomologica https://www.anmvioggi.it/images/CIRCOLARE_INDICAZIONI_OPERATIVE_BLUE_TONGUE.pdf).

BTV detection and genotyping by real time RT-qPCR

Total RNA was extracted from EDTA blood and spleen homogenates using the MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems, St. Austin, TX, USA) on a KingFisher™ Flex Purification System (Thermo Fisher Scientific, MA, USA), following the manufacturer's instructions. After denaturation, all specimens were screened using a real-time RT-qPCR assay simultaneously detecting both BTV and epizootic hemorrhagic disease virus (EHDV) RNA (pan-BTV/pan-EHDV; Portanti et al., 2025). Samples testing positive for BTV RNA were then serotyped using: i) the VetMAX™ European BTV Typing Kit (Applied Biosystems, St. Austin, TX, USA), able to detect serotypes 1, 2, 4, 6, 8, 9, 11 and 16 circulating or previously circulating in the Mediterranean basin; and ii) an in-house real-time RT-qPCR assay targeting Seg-2 of BTV-3 (Lorusso et al., 2018).

Whole Genome Sequencing and downstream analyses

As for Italy, sequencing candidates were selected based on geographical origin (region and province), serotype, and Ct value ≤ 25 obtained from the pan-BTV/pan-EHDV assays. Only biological samples positive for a single serotype were included. Additionally, BTV-3 and BTV-4 strains from Tunisia and Italy collected between 2019 and 2022 were incorporated into the analysis. A total of eight BTV-8 cell culture isolates were also included (Supplementary Table).

RNA purification, SISPA amplification, and library preparation

Total RNA was purified and DNase-treated using the RNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, USA) and subsequently subjected to Sequence-Independent Single-Primer Amplification (SISPA) following established protocols (Lorusso et al., 2023; Marcacci et al., 2016; Sghaier et al., 2022; Thabet et al., 2024). PCR products were purified with Expin™ PCR SV (GeneAll Biotechnology Co., Seoul, Korea) and quantified using the Qubit DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were generated using the Illumina DNA Prep Kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's protocol. Sequencing was performed on Illumina NextSeq1000 and NextSeq2000 platforms using the NextSeq 1000/2000 P2 Reagent Kit (300 cycles; 150-bp paired-end reads).

Bioinformatic analysis

Raw sequencing data were subjected to quality assessment using FastQC v0.11.5 and trimming with Trimmomatic v0.36 on the GENPAT platform (https://genpat.izs.it/cmdbuild/ui/#login). The resulting FASTQ files were uploaded to CZ-ID (https://czid.org/) to identify the closest matching BTV reference sequences for each genome segment.

The sequences showing the highest similarity were used as references for read mapping with iVar v1.3.1 (https://github.com/andersen-lab/ivar) to generate consensus genomes. Each consensus sequence was manually

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inspected and curated using Geneious Prime® v2025.1.1 (www.geneious.com).

Final genome sequences were deposited in GenBank and analyzed using BLAST. For each genome segment, BLAST comparisons were restricted to full-length nucleotide sequences available in public databases, excluding partial or incomplete sequences, in order to ensure accurate segment-level identity estimates. Nucleotide identity values were calculated over the entire length of each segment and used to infer the most likely parental origin. In this context, a nucleotide identity >99% with known reference strains was considered a proxy for shared segment ancestry and genome reassortment, when supported by the known circulation of putative parental strains.

Results

BTV-3 and 8 were responsible for the major BT outbreaks in Italy from July 2024 to December 2025

In 2024, Italy experienced intense circulation of BTV-3, which—six years after its first detection in Sardinia—caused a major epidemic with more than 2,330 outbreaks and 109,851 confirmed cases in that region, along with the involvement of three additional regions (Sicily, Tuscany, and Trentino-Alto Adige/South Tyrol). Concurrently, BTV-8, already present in Sardinia since the previous year, spread throughout the island and across much of mainland Italy, resulting in 3,847 outbreaks and 131,946 cases. In 2025, BTV-8 continued to expand into Italian regions not affected during the previous year's wave (2,449 outbreaks), while BTV-3 showed only limited circulation, with 409 confirmed outbreaks. As of December 2025, 102 outbreaks had been confirmed, largely concentrated in the southern part of the island. Residual circulation of serotypes BTV-4 and BTV-1 was also detected in several Italian regions.

Of the more than 9,000 outbreaks confirmed during the study period, approximately 9% involved multiple serotype detections. In most cases, co-infections included BTV-3 in combination with BTV-8, or these serotypes together with BTV-1 and BTV-4.

A total of 47 complete genomes were obtained and used for downstream analyses

A total of 47 BTV whole-genome sequences were obtained: 16 from Italian strains of 2021 (six for BTV-4, and one for BTV-3), 2022 (one for BTV-1, and five for BTV-3), and 2023 (two for BTV-4 and one for BTV-8); 7 from Tunisian strains from 2019 to 2022 (two for BTV-3 and five from BTV-4) (Figure 1); 24 from Italian strains of 2024-2025 (five for BTV-3, six for BTV-4, and thirteen for BTV-8) (Figure 2). All data related to the sequenced strains are reported in the Supplementary Table.

Seven BTV strains were selected as putative parental strains

To simplify the interpretation of genomic data, we operationally defined seven BTV strains circulating in Italy or in geographically adjacent areas as putative parental strains. For clarity, we did not assess whether these strains themselves resulted from reassortment events, reserving a more in-depth phylogenetic evaluation for future studies. The strains included BTV-1 SAD 2012 (KJ577094–KJ577103), BTV-2 modified live vaccine MLV (KP820911-KP821031-KP821153-KP821273-AM773705-KP821513-KP821635-KP821755-KP821996), BTV-3 TUN 2016 (KY432369–KY432378), BTV-4 TUN 2019 (PV974505–PV974514), BTV-4 MKD 2020 (MT879201–MT879210), BTV-3 NET 2023 (OR603992–OR604001), and BTV-8 FRA 2023 (PP199261–PP199270), as shown in Figure 3.

BTV-3 and BTV-4 strains of 2019-2023 from Italy and Tunisia show multiple genome constellations

A representation of the genomic constellations of BTV from 2019 to 2023 is presented in Figure 4 while Figure 5 shows the geographic distribution of these strains. For clarity, only reassortant genome constellations were assigned alphabetical labels (A, B, C, D, E, etc.) to aid interpretation of their geographical distribution, regardless of serotype.

As far as BTV-3 is concerned, the Tunisian strains from 2021 (BTV-3/TUN-312752.1.1 TUN2021) and 2022 (BTV-3/TUN-35502.1.2 TUN2022) were nearly identical to each other (99.89–100%). They also displayed a high

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nucleotide identity (99.69–99.88%) across all genomic segments, with the exception of Seg-5 and Seg-10, when compared with the Tunisian BTV-3 TUN 2016 strain (KY432369–KY432378) identified in 2016 (Sghaier et al., 2017). Seg-5 showed 99.61% identity with the BTV-2 vaccine strain (AM773705/), a finding already reported previously in a BTV-16 field strain of 2002 originated from reassortment between two modified live vaccine (MLV) strains including BTV-2, vaccine heavily used in Italy in 2002 (Batten et al., 2008). Seg-10 showed 99.74% identity with BTV-1 SAD 2012 strain detected in Sardinia in 2012 (KJ577094–KJ577103) (Lorusso et al., 2014b).These two segments showed lower identity to BTV-3 TUN2016 (94.85% for Seg-5 and 95.24% for Seg-10). This genome constellation has been identified as BTV-3A. The Sardinian BTV-3 strain of 2021 (BTV-3/SU-139639 ITA2021) shared 99.40–99.97% nucleotide identity with BTV-3 TUN 2016 and >99.85% identity with its Italian offspring, namely BTV-3 SAR2018 strain (MK348537–MK348546; Cappai et al., 2019). On the other hand, Sardinian BTV-3 strains collected in 2022 showed a reassortant genome constellation with BTV-4 (BTV-3B). Indeed, segments 2, 3, 6, and 7 shared >99.63% identity with BTV-3 TUN 2016, whereas Seg -1, - 4, -5, -8, -9, and -10 showed high identity (99.30–99.91%) with the Tunisian BTV-4 TUN 2019 strain.

Concerning BTV-4, BTV-4 TUN 2019 strain (PV974505–PV974514) was used as the main reference for downstream comparison. Three Tunisian strains from 2020 (BTV-4/TUN-320799.1.1, .15, .19 TUN2020) showed almost complete nucleotide identity with each other, and a high identity (99.50–100%) with BTV-4 TUN 2019. The Tunisian strain collected in 2021 (BTV-4/TUN-312752.1.21 TUN2021) shared >99.30% identity with BTV-4 TUN 2019. Italian BTV-4 strains collected in 2021 displayed two distinct genome constellations. One group—BTV-4/OR-301633 ITA2021, BTV-4/NU-301257 ITA2021, BTV-4/SU-311759 ITA2021, BTV-4/CA-311837 ITA2021, BTV-4/RM-312306 ITA2022—was strictly related to BTV-4 TUN 2019, showing 99.30–100% identity across all segments. The other constellation was represented by the Sicilian strain BTV-4/PA-310552.1.4 ITA2021, which showed higher nucleotide identity (99.26–99.82%) to the Balkan strain BTV-4 MKD 2020 (MT879201–MT879210) from North Macedonia. In 2023, both BTV-4 lineages were identified in Latium region (BTV-4/VT-23089 ITA2023) and in Sardinia (BTV-4/NU-18526 ITA 2023) being BTV-4 TUN-2019 and BTV-4 MKD 2020-like viruses, respectively.

Following the detection of BTV-1–derived genome segments in BTV-3 strains, we identified a BTV-1 strain collected in 2022 (BTV-1/TE-13617.1.9 ITA 2022) showing an extremely high nucleotide identity (99.81–100%) across all genome segments with the BTV-1 SAD 2012 strain detected in Sardinia in 2012, indicating the circulation of a virtually unchanged BTV-1 lineage over a ten-year period.

A BTV-8 strain detected in Sardinia in October 2023 (BTV-8/OR-23537 ITA 2023) showed near-complete nucleotide identity (99.89-100%) across all genome segments with French BTV-8 strains (PP199261–PP199270).

We detected at least four distinct genomic constellations of BTV-3 from samples collected in 2024 and 2025: three derived from multiple reassortment events involving both European and North African BTV strains, and one closely related to the BTV-3 lineage circulating in northern Europe.

The first constellation (BTV was represented by strains BTV-3/SA-30816 ITA2024 and BTV-3/SU-21113 ITA2024 (BTV-3C, Figure 6), which showed a genomic backbone (Seg-2, -5, -6, -7, -10; 99.61–99.91% nt id) characteristic of BTV-3/TUN-312752.1.1 TUN2021 and BTV-3/TUN-35502.1.2 TUN2022. Notably, Seg-1, -3, -4, and -8 were derived from BTV-4 TUN 2019 (99.13–99.91% nt id), while Seg-9 likely originated from BTV-1 strains circulating in 2012 (98.95% nt id). The second constellation (BTV-3D, Figure 6) was represented by a BTV-3 strain identified in Sicily, BTV-3/PA-30694 ITA2024, which showed a genomic backbone corresponding to Sardinian BTV-3 strains collected in 2022 (Seg-1, -2, -6, and -7; 99.62–100% nt id). Segments 3, 4, 5, 8, 9, and 10 instead showed >99.81% nucleotide identity with BTV-8 FRA2023 (strain 8645) (PP199261–PP199270). The third constellation (BTV-3F, Figure 7) was represented by a BTV-3 strain identified in Sicily in 2025 (BTV-3/CT-23193 ITA 2025). Its genome constellation included Seg -2, -6, and -7, which showed 99.63-100% nt id with BTV-3 TUN2016; Seg -1, -3, -4, -5, and -8 showed 99.82-99.91% nt id with BTV-8 FRA2023 (strain 8645), whereas Seg -9 and -10 had 98.95-99.61% nt id with BTV-1 strains circulating in 2012. The fourth genome constellation was represented by BTV-3/TN-2360 ITA2025 (Figure 7), a strain circulating in northern Italy (Trentino-Alto Adige/South Tyrol), which showed high nucleotide identity across all segments (99.62–100%) with BTV-3 NET 2023 (OR603992–OR604001), first detected in the Netherlands in 2023.

As far as BTV-4 is concerned, four different genome constellations were identified. The first, represented by BTV-4/TN-28765 ITA2024, BTV-4/RN-3037 ITA2024 (Figure 6), and BTV-4/BG-29678 ITA2025 (Figure 7), detected in central-northern Italy, showed >99.00% nucleotide identity with BTV-4 TUN-2019 across all gene segments.

The second, represented by BTV-4/TP-32693 ITA2024 (BTV-4 E, Figure 6), showed >99.45% identity with BTV-4/PA-310552.1.4 ITA2021 for Seg-2, -4, and -6 (99.45–99.51%). All remaining segments (Seg-1, -3, -5, -7, -8, -9, and -10) were shown to be derived from BTV-8 FRA2023 (strain 8645), with nucleotide identities ranging from

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99.76% to 100%. The third, represented by Sardinian strain BTV-4/NU-24558 ITA2025 (BTV-4 G, Figure 7), showed Seg -1, -3, -4, -7, -8, -9, and -10 with nt id ranging from 99.72 to 100% with BTV-8 FRA2023 (strain 8645); Seg -2 and -6 had high nt id, 99.62 and 99.33% respectively, with the Balkan strain BTV-4 MKD 2020; Seg -5 showed 99.21% nt id with the BTV-2 vaccine strain. The fourth constellation was represented by another Sicilian strain, BTV-4/TP-27405 ITA 2025 (BTV-4 H, Figure 7), that showed a reassortant constellation potentially related to five different parental strains: Seg -1, -3, and -8 had a nt id with BTV-4 TUN 2019 (97.82-99.70%); Seg -2, -6, -7, and -10 derived from BTV-4 MKD 2020 (97.87-99.43%); Seg -4 showed a nt id (99.79%) with BTV-8 FRA 2023; Seg -5 and Seg-9 instead showed a 99.20% nt id with the BTV-2 vaccine strain and BTV-1 strains circulating in 2012 (96.89%), respectively. However, for this strain, full-length genome quality was suboptimal, and therefore the robustness of this finding cannot be considered conclusive, warranting further analyses.

BTV-8 strains characterized in Italy is connected with the contemporary BTV-8 strain from France

Whole-genome sequencing revealed nearly complete nucleotide identity among all Italian BTV-8 strains analyzed (Figures 4, 6, and 7), indicating the circulation of a single viral strain in Italy (Figure 8). These genomes shared 99.63–100% nucleotide identity with BTV-8 FRA2023, which has been heavily circulating in France (Gondard et al., 2024). Notably, despite the extensive national circulation of BTV-8 FRA 2023-like viruses in Italy, no reassortment events involving the acquisition of heterologous genome segments by BTV-8 were detected, nor was BTV-8 VP2/Seg-2 identified in the genomic constellations of other serotypes, in contrast to BTV-3 and BTV-4, which showed multiple reassortment patterns with genomic segments of BTV-8.

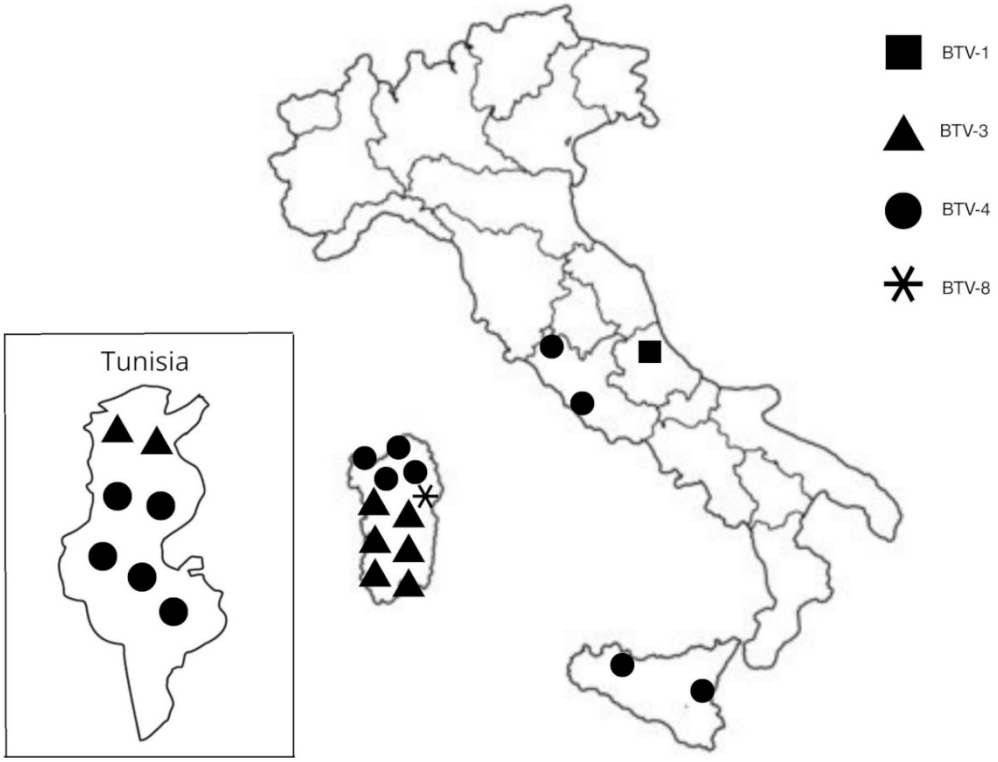

Figure 1. Sampling locations (2019–2023) and BTV serotypes. The map shows the geographical distribution of sampling sites in Italy (main panel) and Tunisia (inset) from which Bluetongue virus (BTV)-positive ruminant samples were obtained and sequenced for the analyses presented in this study. Samples were collected between 2019 and 2023. Symbols indicate BTV serotypes: squares represent BTV-1 (n=1), triangles represent BTV-3 (n=8), circles represent BTV-4 (n=13), and asterisks represent BTV-8 (n=1). While the geographic localization within Italy is as precise as possible, the same does not apply to Tunisia, for which the reported locations are only indicative.

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Figure 2. Sampling locations (2024–2025) and BTV serotypes. The map shows the geographical distribution of sampling sites in Italy from which BTV-positive ruminant samples sequenced and analyzed in this study were collected in 2024 and 2025. Symbols indicate BTV serotypes: triangles represent BTV-3 (n=5), circles represent BTV-4 (n=6), and asterisks represent BTV-8 (n=13).

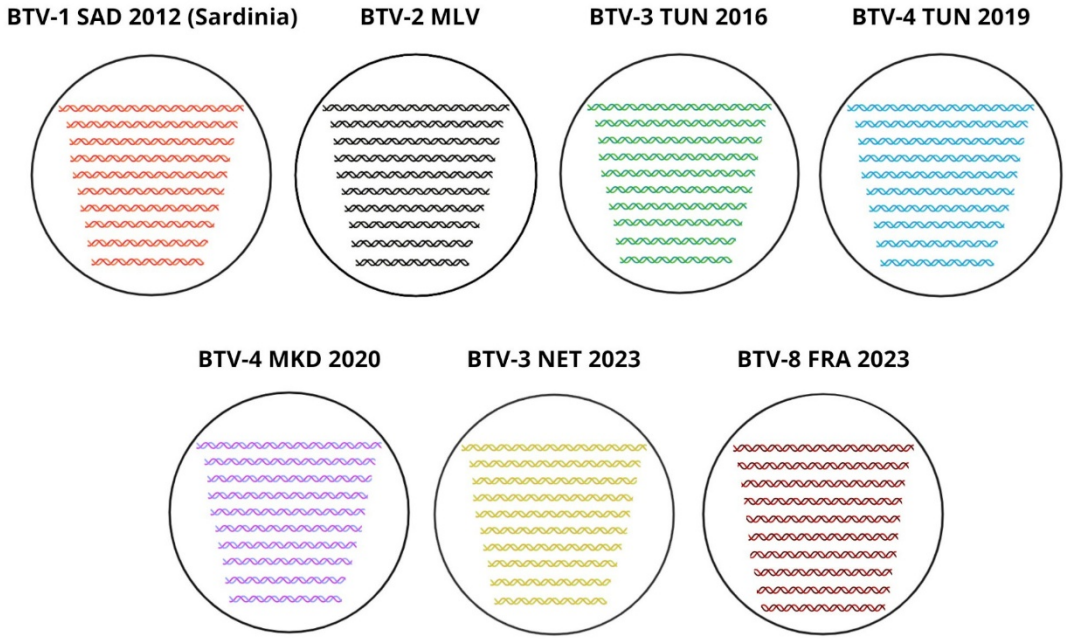

Figure 3. Putative parental BTV strains used for genome-constellation assignment. The figure provides a schematic representation of the putative parental BTV strains used as references for genome-constellation analysis. The genome constellation of each parental strain is represented using a single monochromatic colour to simplify interpretation, meaning that potential previous reassortment events were not considered. Colours correspond to the following reference strains: orange for Italian BTV-1 SAD 2012 (Sardinia), black for BTV-2 modified live vaccine (MLV), green for BTV-3 TUN 2016, light blue for BTV-4 TUN 2019, purple for Balkan BTV-4 MKD 2020, yellow for BTV-3 NET 2023 and dark red/brown for BTV-8 FRA 2023. Country abbreviations are as follows: SAD, Sardinia region in Italy; TUN, Tunisia; MKD, North Macedonia; NET, The Netherlands; FRA, France.

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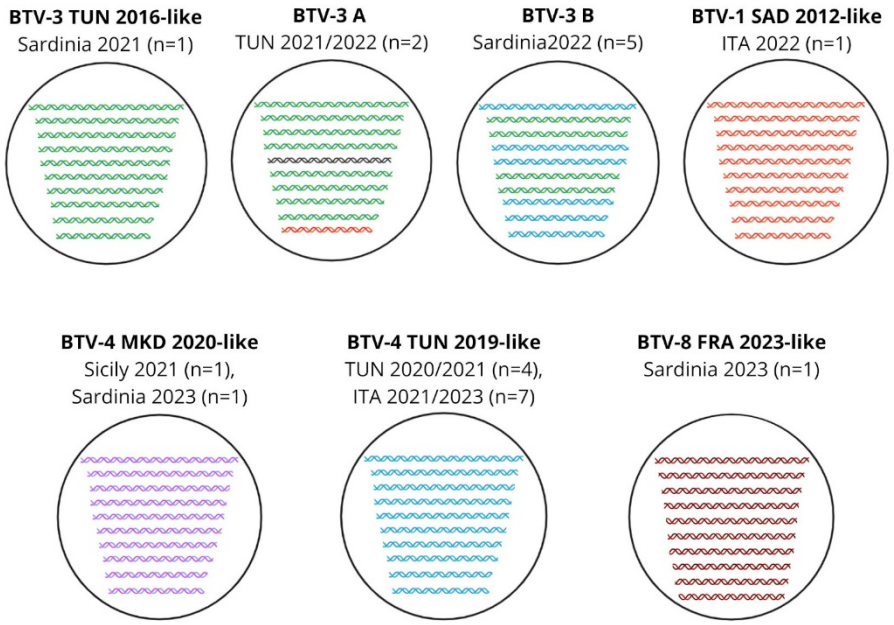

Figure 4. Genome constellations identified in BTV field strains sampled in 2019–2023. The figure shows the genome-constellation patterns identified among BTV field strains sequenced between 2019 and 2023. Each circle represents a genome constellation, and individual genome segments are coloured according to their closest putative parental origin, following the same colour code as in Figure 3. For BTV-3 a single BTV-3 TUN 2016-like strain was identified in Sardinia in 2021. For BTV-1, a single BTV-1 SAD 2012-like strain was identified in 2022 in mainland Italy. For BTV-4, two BTV-4 MKD 2020-like strains representing the BTV-4 Balkan lineage were identified in Sicily in 2021 and Sardinia in 2023; multiple BTV-4 TUN 2019-like strains (n=11) representing the Northern African lineage were identified in Tunisia in 2019 (n=1), 2020 (n=3) and 2021 (n=1), in mainland Italy in 2021 (n=1) and 2023 (n=1), in Sardinia in 2021 (n=4) as indicated in the figure. For BTV-8, a single BTV-8 FRA 2023-like strain was identified in Sardinia in 2023. The remaining genome constellations shown in the figure (BTV-3 A and BTV-3 B) are reassortant and follow the same colour code as in Figure 3.

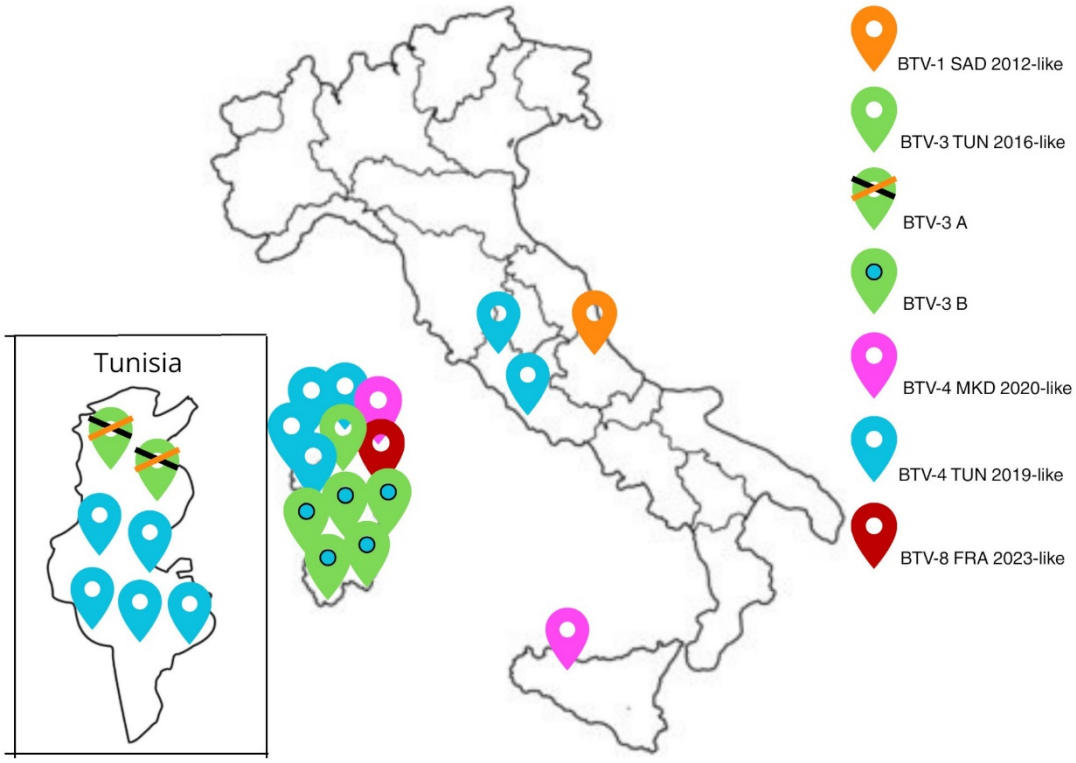

Figure 5. Distribution of BTV genomic constellations (2019-2023). The map shows the geographical distribution of sampling sites in Italy (main panel) and Tunisia (inset) for Bluetongue virus (BTV)-positive ruminant samples collected between 2019 and 2023. Colored symbols represent distinct genomic constellations, as indicated in the legend: orange for BTV-1 SAD 2012-like strain, green for BTV-3 TUN 2016-like strain, light blue for BTV-4 TUN 2019-like strains, purple for BTV-4 MKD 2020-like strains, and dark red for BTV-8 FRA 2023-like strain. BTV-3 A and BTV-3 B represent reassortant BTV strains, whose genomic constellations are shown in Figure 4. While the geographic localization within Italy is as precise as possible, the same does not apply to Tunisia, for which the reported locations are only indicative.

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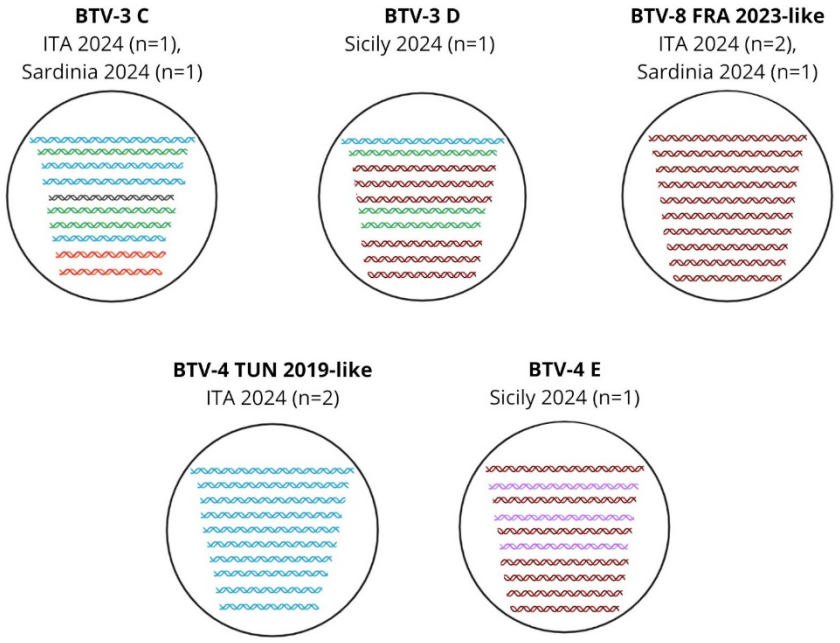

Figure 6. Genome constellations identified in BTV field strains sampled in 2024. The figure shows the genome-constellation patterns identified among BTV field strains sequenced in 2024. Each circle represents a genome constellation, with genome segments coloured according to their putative parental origin using the same colour code as in Figure 3. For BTV-8, multiple BTV-8 FRA 2023-like strains were identified in mainland Italy (n= 2) and Sardinia (n=1). For BTV-4, two BTV-4 TUN 2019-like strains were identified in mainland Italy. The remaining genome constellations, designated BTV-3 C, BTV-3 D, and BTV-4 E, represent reassortant profiles and follow the same colour code as in Figure 3.

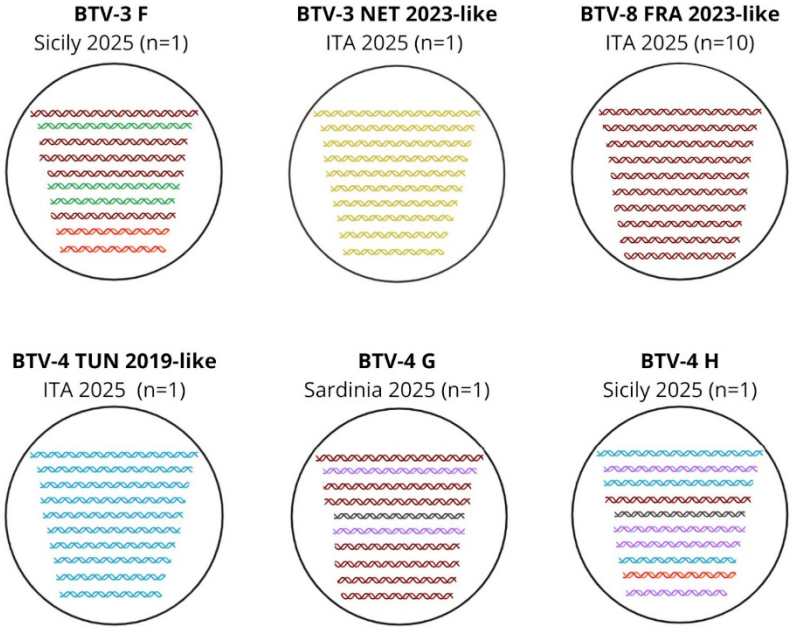

Figure 7. Genome constellations identified in BTV field strains sampled in 2025. The figure shows the genome-constellation patterns identified among BTV field strains sequenced in 2025. Each circle represents a genome constellation, with genome segments coloured according to their putative parental origin using the same colour code as in Figure 3. For BTV-3, a single BTV-3 NET 2023-like strain was identified in mainland Italy. For BTV-8, multiple BTV-8 FRA 2023-like strains (n=10) were identified in mainland Italy. For BTV-4, one BTV-4 TUN 2019-like strain was identified in mainland Italy. The remaining genome constellations, designated BTV-3 F, BTV-4 G, and BTV-4 H, display reassortant profiles and follow the same colour code as in Figure 3.

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Figure 8. Distribution of BTV genomic constellations (2024–2025). The map shows the geographical distribution of sampling sites in Italy for BTV-positive ruminant samples collected in 2024 and 2025. Coloured map markers indicate the detected BTV serotype, with green representing BTV-3, light blue BTV-4 and red BTV-8. BTV-3 C, BTV-3 D, BTV-3 F, BTV-4 E, BTV-4 G, and BTV-4 H represent reassortant BTV strains, whose genomic constellations are shown in Figure 6 and 7.

Novel genomic constellations of BTV-3 and BTV-4 strains were identified in 2024-2025

A representation of the genomic constellations of BTV from 2024 to 2025 is presented in Figures 6-7 whereas Figure 8 shows the geographic distribution of these strains. For clarity, only reassortant genome constellations were assigned alphabetical labels (A, B, C, D, E, etc.) to aid interpretation of their geographical distribution, regardless of serotype.

Discussion

To reconstruct the genomic trajectories of bluetongue virus (BTV) strains circulating in Italy during 2024–2025, it was necessary to extend the analysis beyond the immediate study period and include reference sequences from previous years, particularly those originating from Tunisia. Tunisia represents a long-standing and highly active partner in the molecular epidemiology of orbiviruses in North Africa, and its inclusion allowed the newly generated Italian genomes to be placed within a broader evolutionary and geographic framework. This retrospective integration clarified the relationships among Italian, North African, Balkan, and northern European BTV lineages.

In 2023, two distinct BTV-8 strains were reported to circulate simultaneously in France: an endemic lineage persisting since 2015 and a genetically distinct strain of unknown origin (Gondard et al., 2024). Whole-genome sequencing unequivocally demonstrates that the BTV-8 detected throughout mainland Italy in recent years corresponds to the latter strain. Its introduction into Italy is most plausibly explained by the movement of viremic animals, consistent with long-recognized mechanisms of BTV dissemination across Europe.

Collectively, the genomic data presented here depict a highly dynamic evolutionary scenario in which the Italian BTV

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landscape is shaped by repeated viral introductions, co-circulation of multiple serotypes, and frequent reassortment events involving segments from different lineages. In this context, Italy emerges as a point of genetic and geographic convergence for BTV strains circulating across the Mediterranean basin.

Our analyses identified four distinct BTV-3 genomic constellations circulating during 2024–2025. Several of these constellations contained genomic backbones derived from Tunisian BTV-3 strains (TUN2016, TUN2021, and TUN2022), combined with genome segments originating from BTV-4 TUN 2019 and, in specific cases, from BTV-1 strains that began circulating in Italy in 2012. Notably, BTV-4 TUN 2019 and its derived constellations were confirmed to carry Seg-3 and Seg-5 of BTV-1 origin, supporting previous observations that repeated viral introductions into North Africa facilitate extensive segment mixing across lineages (Lorusso et al., 2025 personal communication). In addition to the well-documented Balkan BTV-4 lineage—still detected in Sardinia in 2023—our data further indicate that a BTV-4 TUN2019-like strain was circulating concurrently in Italy during the same year and was subsequently identified in the Trentino–Alto Adige/South Tyrol region.

Interestingly, the expected reciprocal reassortment patterns involving BTV-8 were not observed. Despite the extensive national circulation of BTV-8 FRA 2023–like viruses, neither the acquisition of heterologous genome segments by BTV-8 nor the presence of BTV-8 VP2/Seg-2 in the genomic backgrounds of other serotypes was detected. Instead, we observed the opposite pattern: internal genome segments derived from BTV-8 were incorporated into BTV-3 and BTV-4 genomic constellations carrying VP2/Seg-2 typical of serotypes 3 or 4. This pattern mirrors what has been described for other segmented viruses in which surface proteins define antigenic identity, most famously influenza A virus, where HA and NA determine subtype while internal genes reassort more freely to optimize viral fitness (McDonald et al., 2016).

Our findings also reinforce a well-established principle in bluetongue virus evolution: genome segments 2 and 6, and frequently segment 7, tend to segregate together, reflecting functional constraints that stabilize the VP2–VP5–VP7 module during reassortment. This phenomenon has been consistently reported across multiple studies (Carpenter et al., 2024; Jacquot et al., 2019; Nomikou et al., 2015; Pullinger et al., 2016; White et al., 2019). Such constraints likely explain the apparent stability of VP2-based serotype identity in BTV-8, even though internal BTV-8 genes can be successfully accommodated within heterologous genomic backgrounds.

The marked increase in BTV-3 cases observed in 2022 coincided with the emergence of a reassortant form of this lineage, a pattern that has recurred in subsequent years. Importantly, the original BTV-3 TUN2016-like genomic constellation—previously detected in Sardinian strains from 2018 and 2021—has effectively disappeared from the recent genomic landscape. This suggests that it may have been outcompeted by reassortant variants displaying greater epidemiological fitness. This observation should not be interpreted as evidence that reassortment intrinsically increases virulence; rather, it underscores the multifactorial nature of viral success, which likely depends on a combination of viral fitness, vector competence, host immunity, extrinsic incubation period, and replication efficiency.

Given the high number of reassortant constellations identified and the apparent ease with which reassortment events were detected, it is reasonable to question whether some of these patterns might reflect mixed infections involving multiple serotypes. However, several lines of evidence support the robustness of our conclusions. First, real-time RT-PCR consistently identified a single serotype in each analyzed sample, a finding that was subsequently confirmed by whole-genome sequencing. Second, nucleotide identity values between reassorted segments and their putative parental strains were sufficiently high to allow confident assignment of segment origin. Together, these observations argue against widespread undetected co-infections as the primary explanation for the reassortment patterns observed.

Despite these insights, several limitations must be acknowledged. Many genomes sequenced during 2024–2025 were partial and are not shown here, preventing full reconstruction of all reassortment events and leaving open the possibility that additional genomic constellations remain undetected. Moreover, the apparent absence of heterologous segments within BTV-8 genomes should be interpreted cautiously. It is possible that the number of BTV-8 strains sequenced was limited or that sampling occurred predominantly in areas with low levels of serotype co-circulation. In addition, several BTV-8 genomes were obtained from geographically close locations, introducing a potential spatial sampling bias that may have further reduced the likelihood of detecting reassortment involving BTV-8. Phylogenetic analyses addressing these issues are currently ongoing and will provide additional resolution.

This study also does not estimate the prevalence of individual genomic constellations, quantify their temporal dynamics, or assess potential competitive interactions among them. Additionally, although sporadic detections of BTV-1, BTV-2, and BTV-16 were recorded during the study period, sample numbers were too limited to allow meaningful genomic characterization. Their presence nevertheless warrants attention, as even low-level circulation

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may provide genetic material for future reassortment events with potential epidemiological relevance. Finally, not all African sequences currently available in our repositories were included in this analysis; a more comprehensive comparative study is ongoing in collaboration with international partners.

For these reasons, the present work should be regarded as a snapshot of the current genomic landscape rather than an exhaustive reconstruction. Nevertheless, it provides a timely update on recent evolutionary trends and highlights the critical value of whole-genome sequencing for tracking viral introductions and reassortment. Future efforts should aim to integrate genomic constellation data with spatial and ecological variables—such as animal density, vector distribution, and livestock movement patterns—to better understand the drivers of viral spread and evolution.

In conclusion, our findings highlight the evolving and increasingly complex genomic landscape of bluetongue virus in Italy. Continuous genomic surveillance, strengthened international collaboration, and systematic analysis of archived African datasets will be essential to elucidate the evolutionary trajectories of BTV lineages and to better anticipate their potential impact on animal health and the livestock sector.

At the time this manuscript was prepared, BTV-5 (PX460302-PX460311) was detected in Sardinia. Seg-2 and Seg-6 were clearly linked to a BTV-5 strain isolated in Nigeria in the 1980s (AJ585182 and AJ586702, respectively) whereas the internal genome segments appeared to be derived from a shared Mediterranean gene pool encompassing multiple circulating serotypes, including those analyzed here.

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Ethical approval

No ethical authorization was required. All included samples were collected for routine testing and surveillance programmes for BTV.

Conflict of interest

The authors declare no conflicts of interest. Authors declare that no competing interests exist. Mention of trade names or commercial products in this article is solely for providing specific information and does not imply recommendation or endorsement by the IZSAM. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Author Contributions

Alessio Lorusso: Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing, Supervision, Validation, Data curation; Gloria Plebani: Methodology, Investigation, Data curation; Andrea Palombieri: Methodology, Investigation, Formal analysis, Validation; Soufien Sghaier: Investigation, sample collection and initial processing in Tunisia; Gardenia Gatta: Investigation, Data curation; Thameur Ben Hassine: Field Investigation in Tunisia; Valentina Curini: Investigation, Data curation; Marta Cresci: Investigation, Data curation; Sarah Thabet: Processing of samples in Tunisia; Francesca Parolini: Investigation, Data curation; Salah Hammami: Supervision of studies conducted in Tunisia and review; Massimo Ancora: Investigation, Data curation; Massimo Spedicato: Validation, Data curation, Conceptualization; Daria Di Sabatino: Conceptualization, Data curation, Formal analysis; Maurilia Marcacci: Software, Formal analysis, Data curation, Validation, Conceptualization. Stacey L. P. Scroggs: Conceptualization, Writing- review & editing. All authors have read and agreed to the published version of the manuscript.

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